Myeloperoxidase: friend and foe

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Abstract: Neutrophilic polymorphonuclear leukocytes (neutrophils) are highly specialized for their primary function, the phagocytosis and destruction of microorganisms. When coated with opsonins (generally complement and/or antibody), microorganisms bind to specific receptors on the surface of the phagocyte and invagination of the cell membrane occurs with the incorporation of the microorganism into an intracellular phagosome. There follows a burst of oxygen consumption, and much, if not all, of the extra oxygen consumed is converted to highly reactive oxygen species. In addition, the cytoplasmic granules discharge their contents into the phagosome, and death of the ingested microorganism soon follows. Among the antimicrobial systems formed in the phagosome is one consisting of myeloperoxidase (MPO), released into the phagosome during the degranulation process, hydrogen peroxide (H_2O_2), formed by the respiratory burst and a halide, particularly chloride. The initial product of the MPO-H_2O_2-chloride system is hypochlorous acid, and subsequent formation of chlorine, chloramines, hydroxyl radicals, singlet oxygen, and ozone has been proposed. These same toxic agents can be released to the outside of the cell, where they may attack normal tissue and thus contribute to the pathogenesis of disease. This review will consider the potential sources of H_2O_2 for the MPO-H_2O_2-halide system; the toxic products of the MPO system; the evidence for MPO involvement in the microbicidal activity of neutrophils; the involvement of MPO-independent antimicrobial systems; and the role of the MPO system in tissue injury. It is concluded that the MPO system plays an important role in the microbicidal activity of phagocytes. *J. Leukoc. Biol.* **77: 598–625; 2005.**

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I have had a love affair with myeloperoxidase (MPO) for over 40 years, with particular regard to its role in the microbicidal activity of phagocytes. I would like to describe to you how this affair began, indicate some of its pleasures and disappointments, and consider some unanswered questions regarding the role of this enzyme.

In February 1957, I began a post-doctoral fellowship at The Rockefeller University (New York, NY), working in the laboratory of Reginald Archibald. This was an endocrine laboratory, and my interest at that time was in the thyroid gland, specifically in the biosynthesis of the thyroid hormones and in their mechanism of action. Thyroid hormone synthesis involves the iodination of tyrosine residues of thyroglobulin to form mono- and diiodotyrosine, which then couple to form the thyronine derivative, thyroxine or triiodothyronine. Both of these steps in thyroid hormone synthesis are catalyzed by a thyroid peroxidase. In regard to mechanism of action, it was found that phenolic hormones, namely the thyroid hormones and estrogens, had a marked stimulatory effect on some, but not all, reactions catalyzed by peroxidase [1, 2]. Thus, the oxidation of epinephrine, uric acid, and ferrocytochrome C by peroxidase and hydrogen peroxide (H_2O_2) was strongly stimulated by the phenolic hormones, whereas the oxidation of guaiacol was not. The stimulatory activity of thyroxine and estradiol on peroxidatic reactions was lost on the blocking of the phenolic hydroxyl group of the hormones by the formation of the methyl ether, suggesting that the phenolic hormones acted as oxidation-reduction catalysts, first being oxidized by peroxidase and H_2O_2 to a form, probably the phenoxy radical, which was then reduced back to its original form by reaction with an electron donor, whose oxidation was thus accelerated (**Fig. 1**). In the absence of an appropriate electron donor, the phenolic hormones were further oxidized and inactivated.

With the excitement of youth, the question was posed: can the stimulation of peroxidases account in part for the mechanism of action of these hormones? Early studies were with horseradish peroxidase, and mammalian peroxidases were sought, whose function might be stimulated by the phenolic hormones. A number of distinct peroxidases exist in mammalian tissues, which differ in their primary structure, in their heme prosthetic group, and to some degree, in the reactions they catalyze but have in common the ability to increase the rate of H_2O_2-dependent reactions many orders of magnitude. Two mammalian peroxidases had, at that time, been purified, lactoperoxidase (LPO) [3] and MPO [4], although their function was unknown. Both peroxidases were isolated, and it was found that reactions catalyzed by these peroxidases also were strongly stimulated by phenolic hormones. Tyrosine was iodinated by a beef thyroid preparation [5] as well as by LPO and MPO, but
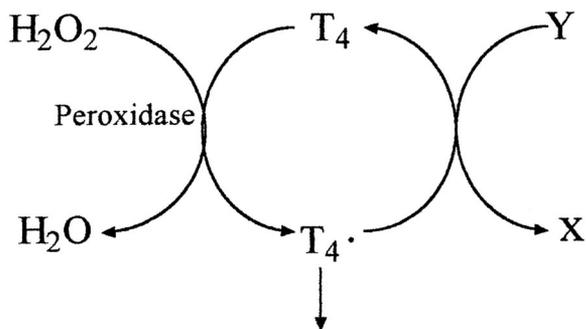
Fig. 1. Stimulation of peroxidase-catalyzed reactions by thyroxine.

a stimulation of this reaction by the phenolic hormones was not observed. It was decided to concentrate on MPO, to search for a function for this enzyme and if found, to see whether this function could be influenced by the phenolic hormones.

Agner [6], who had prepared MPO in a highly purified form in the early 1940s, had reported its presence in neutrophils at concentrations no less than 1–2% of the dry weight of the cells, and others [7] reported its presence at concentrations greater than 5%. Agner initially called the enzyme verdoperoxidase, as a result of its intense green color, but the name was subsequently changed to myeloperoxidase. It is what gives pus its green color. Cytochemical studies dating back to the early 1900s suggested the presence of a peroxidase in the cytoplasmic granules of mature granulocytes, and subsequent studies indicated its presence entirely in the azurophil (primary) granules of these cells. MPO synthesis is initiated in the promyelocyte stage of neutrophil development and terminates at the beginning of the myelocyte stage, at which time the MPO-containing azurophil granules are distributed to daughter cells, where they intermingle with the newly formed peroxidase-negative, specific (secondary) granules. The MPO-containing granules in the mature human neutrophil are heterogeneous by density [8] and by morphology [9]. Human monocytes also contain MPO-positive granules, although they are fewer in number than in neutrophils [10]. The MPO-containing granules are formed in bone marrow promonocytes, are readily apparent in mature monocytes, and are generally lost when monocytes mature into macrophages in tissues (see Atherosclerosis and Multiple sclerosis below for evidence for the presence of MPO in tissue macrophages under certain conditions). A peroxidase is also present in the cytoplasmic granules of eosinophils [11]; however, it differs from MPO structurally and in its function [12].

MPO is the product of a single gene, which has been cloned in a number of laboratories [13–19]. The gene is ~11 kb in size, composed of 11 introns and 12 exons [20], and located in the long arm of chromosome 17 in segment q12–24 [17, 21–23]. Its initial translation product is an ~80-kD protein [24], which following proteolytic removal of the 41 amino acid signal peptide, undergoes N-linked glycosylation with the incorporation of mannose-rich side-chains [25, 26] to generate an 89- to 90-kD enzymatically inactive apoproMPO [20], which forms a complex in the endoplasmic reticulum with the calcium-binding proteins calreticulin and calnexin, which act as molecular chaperones [27, 28]. With the insertion of a heme, apoproMPO is converted to the enzymatically active proMPO [29–31]. The removal of the N-terminal 125 amino acid pro-region by proteolytic cleavage results in the production of a 72- to 75-kD protein, which undergoes a second proteolytic cleavage to generate the 467 amino acid heavy (α) subunit (57 kD) and the 112 amino acid light (β) subunit (12 kD) of MPO, which associate as a heavy-light protomer. Mature MPO has a molecular mass of ~150 kD and consists of a pair of heavy-light protomers [32–34] whose heavy subunits are linked by a disulfide bond along their long axis [32]. The mannose-rich carbohydrate and the two hemes are covalently bound to the heavy subunit [33–35]. Reduction and alkylation result in the cleavage of MPO into hemi-MPO, which consists of a single α and β protomer. It retains enzymatic and bactericidal activity [32, 36]. The two heavy (α) subunits appear to be structurally different [37, 38]. The X-ray crystallographic structure of canine [39–42] and human [43] MPO has been described.

In 1920, Graham [44] first reported the release of peroxidase from neutrophil cytoplasmic granules during phagocytosis as follows: "Very marked changes in the granules may readily be determined by the study of leucocytes engaged in phagocytosis, as for example, in opsonic preparations or in smears of pus. Smears from an active case of gonorrhoea are very satisfactory. When such preparations are stained with a 'peroxidase' reagent, interesting examples of the more or less complete disappearance of the granules from the individual cells may be obtained. While exceptions may occur, it may be stated in general that the granules disappear from the leucocytes progressively as the number of bacterial inclusions in the cell increases." In 1960, Hirsch and Cohn [45] described in detail the degranulation process in phagocytes, in which the membrane of the cytoplasmic granules fused with that of the developing phagosome, the common membrane then ruptured, and the contents of the granules were discharged with great force into the phagosome. Cohn and Hirsch [46] then isolated the granules of rabbit granulocytes and demonstrated the presence in them of a variety of hydrolases as well as an antimicrobial protein, which in 1956, Skarnes and Watson [47] had called leukin, and Hirsch [48] had called phagocytin. The concept that granule components released into the phagosome may be toxic to ingested organisms was thus born. Subsequent studies by myself [49] and others indicated that MPO was among the granule enzymes discharged into the phagosome during the degranulation process. MPO also can be released to the outside of the cell by leakage before complete closure of the developing phagosome or in response to stimulation by an antibody/complement-coated surface too large to be ingested. MPO is a strongly basic protein with an isoelectric point >10 [6] and thus binds avidly to negatively charged surfaces. It can be seen coating ingested microorganisms and when released to the outside of the cell, to biological membranes. MPO, when released, can be inactivated by products of the respiratory burst [50, 51] or be cleared from the extracellular fluid by uptake by macrophages [52, 53] through reaction with the mannose receptor [53]. Further, the uptake of microorganisms coated with extracellularly released MPO [54] or eosinophil peroxidase [55–57] may arm the macrophages, resulting in an associated increased destruction of the ingested organisms.

It was clear at that time (1959–60) that the process of phagocytosis and degranulation by phagocytes was associated with increased metabolic activity [58, 59]. Of particular interest was the burst of oxygen consumption, which was first reported by Baldridge and Gerard [60] in 1933. However, it was not until 1961, when Iyer, Islam, and Quastel [61] reported that the respiratory burst of phagocytes was associated with the formation of H_2O_2, that a product of the respiratory burst was implicated in antimicrobial activity. They stated, "In the consideration of the various factors that may operate in bringing about bactericidal action during phagocytosis, the possibility that hydrogen peroxide is formed during this process must be taken into account. It is unlikely that hydrogen peroxide is wholly responsible for the non-specific bactericidal effects of phagocytosis, but it will surely be one of the responsible agents."

As the primary function of neutrophils is the phagocytosis and destruction of microorganisms, the question was posed: Is the function of MPO in neutrophils the destruction of ingested microorganisms? With a vial of green MPO in hand, I proposed to Jim Hirsch that we look at the antimicrobial properties of this enzyme. Our first studies in this regard in 1961–62 were disappointing. We mixed viable microorganisms with MPO and sublethal levels of H_2O_2 and found no fall in the viable cell count. It was theoretically possible that MPO, rather than being directly toxic to bacteria in the presence (or absence) of H_2O_2, could act indirectly by catalyzing the conversion of a nontoxic agent to a toxic one. Thus, Kojima [62] reported that peroxidase (source unspecified) and H_2O_2 can increase the germicidal effect of a number of phenols by conversion to the corresponding quinone. Iodide seemed ideal for this purpose, as it is nontoxic but is oxidized by peroxidase and H_2O_2 to iodine, a well-known germicidal agent. When iodide was added to the MPO-H_2O_2 system, the solution turned yellow as iodine was formed, and the microorganisms were killed. The concentration of iodide required, however, was considerably greater than physiologic. At this point, I moved from The Rockefeller University to the University of Washington (Seattle), and this line of study was put to one side.

My interest in the antimicrobial properties of peroxidases resurfaced a few years later in saliva. In 1934, an antilactobacillus system was described in saliva, which differed from the then-known antimicrobial systems. Its nature remained a mystery until 1959 when Zeldow reported [63] that this system could be divided into a heat-stable, dialyzable component and a heat-labile, nondialyzable component, each of which was ineffective alone but which when combined, was toxic to the lactobacilli. In 1962, Dogon, Kerr, and Amdur [64] reported that the heat-stable, dialyzable component was the thiocyanate ion, and in 1965, Luebke and I reported [65] that the heat-labile, nondialyzable component was the salivary peroxidase (LPO). H_2O_2 was an additional requirement for this system [65, 66]. It was fortuitous that lactobacillus was the target organism used in the early studies, as this organism can generate H_2O_2 in large amounts and thus, was able to generate the H_2O_2 required for its own destruction when combined with LPO and thiocyanate ions. When H_2O_2 or a H_2O_2-generating system was added, toxicity was extended to microorganisms which did not

generate H_2O_2 themselves. A similar antimicrobial system was detected in milk [67].

The observation at that time that MPO could replace LPO in the peroxidase-thiocyanate-H_2O_2 antimicrobial system [65, 66] renewed interest in the possibility that MPO served an antimicrobial function in the neutrophil. In 1967, I reported that MPO, H_2O_2, and iodide, bromide, chloride, or thiocyanate ions formed a powerful antimicrobial system in neutrophils [68–70] (**Fig. 2**). Of particular interest was the observation that chloride, at physiologic concentrations, could meet the halide requirement of the MPO-dependent antimicrobial system, although it was ineffective in the LPO-H_2O_2 system. In 1967, McRipley and Sbarra [72] reported that a guinea pig leukocyte extract was bactericidal when combined with H_2O_2, and they subsequently confirmed the requirement for a halide [73].

MECHANISMS OF H_2O_2 FORMATION FOR THE MPO SYSTEM

The H_2O_2 required for the MPO-mediated antimicrobial system may come from a variety of sources.

Phagocyte NADPH oxidase

Rossi and Zatti [74] first proposed in 1964 that a NADPH oxidase was responsible for the respiratory burst of phagocytes. In 1973, Babior, Kipnes, and Curnutte [75] reported that the initial product of the respiratory burst oxidase was the O_2^- as follows:

$$NADPH + O_2 \rightarrow O_2^- + NADP^+ + H^+$$
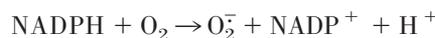
and there followed 30 years of effort worldwide designed to elucidate the nature of the O_2^--generating NADPH oxidase system of phagocytes (for review, see refs. [76–79]). A major advance was the demonstration that the NADPH oxidase could be activated in cell-free leukocyte preparations by an anionic
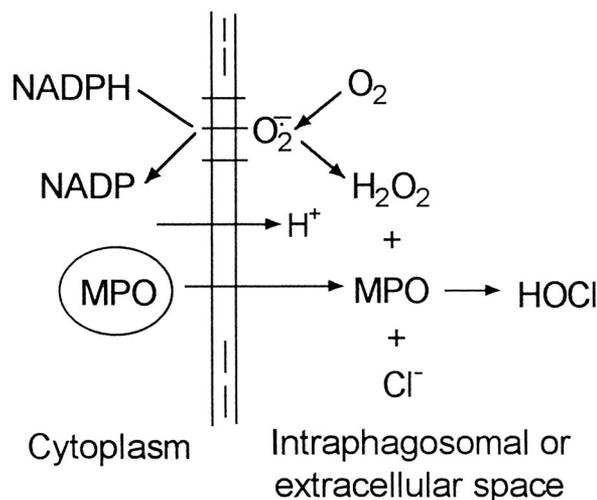

Fig. 2. The MPO-H_2O_2-chloride antimicrobial system (taken from ref. [71]). NADPH, Reduced nicotinamide adenine dinucleotide phosphate; O_2^-, superoxide anion; HOCl, hypochlorous acid.

detergent such as arachidonic acid, sodium dodecyl sulfate, or other cis-unsaturated fatty acids [80–82]. The NADPH oxidase activated in this way could be separated by centrifugation into a particulate (membrane) fraction and a soluble (cytosolic) fraction, both of which were required for NADPH oxidase activity. In 1978, Segal and Jones [83] reported the presence of a novel b-cytochrome in high concentrations in normal neutrophils as well as in monocytes, macrophages, and cosinophils [84]. It is a heterodimer consisting of a 91-kD glycoprotein (β subunit), designated gp91^{phox}, and a 22-kD polypeptide (α subunit), designated p22^{phox}. The b-cytochrome has a characteristic absorption peak at 558 nm and a low mid-point potential (–245 mV), which allows it, by oxidation-reduction, to directly reduce O_2 to O_2^-. The b-cytochrome also contains a flavin (flavin adenine dinucleotide) as part of the electron transport chain and thus, has been termed flavocytochrome b$_{558}$. The gene for the heavy subunit of the b-cytochrome (gp91^{phox}) was first identified and cloned by Orkin and his colleagues [85, 86] by "reverse genetics" in which the gene was initially located on the X chromosome by linkage analysis and its protein product subsequently determined from the base sequence. In the resting cell, the b cytochrome is present largely in the membranes of secondary (specific) granules and secretory vesicles in the cytoplasm and is transported to the cell (or phagosomal) membrane when the leukocyte is stimulated. It is the particulate (membrane) component of the cell-free NADPH oxidase system. The soluble (cytoplasmic) components of the oxidase consist of 67 kD, 47 kD, and 40 kD proteins, designated p67^{phox}, p47^{phox}, and p40^{phox} and the low molecular weight guanosine 5′-triphosphate (GTP)-binding protein rac 1 [87] or rac 2 [88]. Rac 2, which is a member of the Rho family of small GTPases, appears to be the predominant GTP-binding protein of human neutrophils [89]. The development of a polyclonal antiserum, which recognized p47^{phox} and p67^{phox} [90], led to the cloning and structural characterization of the cytoplasmic components of the oxidase [91, 92]. The NADPH oxidase is activated by the migration of the cytoplasmic components to the cell membrane to form a complex with the membrane component flavocytochrome b$_{558}$. This is facilitated by the phosphorylation, largely by protein kinase C, of p47^{phox}, which binds to the 22-kD α subunit of the b-cytochrome. The dissociation of Rac from a cytoplasmic complex with an inhibitor molecule (guanosine diphosphate dissociation inhibitor) occurs, with the conversion of Rac from its guanosine 5′-diphosphate-bound inactive form to its GTP-bound active form (for a review of NADPH oxidase regulation by Rac, see ref. [93]). The activated NADPH oxidase transports electrons from NADPH on the cytoplasmic side of the membrane to oxygen in the extracellular fluid or when the membrane is invaginated in the intraphagosomal space to form O_2^-. The NADPH-binding site, flavin and heme, required for this electron transport, appears to be present in the gp91^{phox} component of the NADPH oxidase, with the cytosolic components serving an activating function.

In 1967 Quie et al. [94] described a microbicidal defect in the neutrophils of patients with chronic granulomatous disease (CGD) of childhood, a condition that had been described clinically 10 years earlier by Berendes, Bridges, and Good [95]. CGD is characterized by severe, repeated, and widespread infections, generally involving staphylococci, certain Gram-negative bacteria, and fungi and affecting a variety of tissues. Infections generally appear in infancy and often result in early death. The lesions are often characterized by granulomata with characteristic lipid-filled histiocytes and thus the name chronic granulomatous disease. In 1967, Holmes, Page, and Good [96] reported that the neutrophil microbicidal defect in CGD was associated with the absence of respiratory burst activity, suggesting an important role for the respiratory burst in the microbial activity of phagocytes. The basis for the respiratory burst defect in CGD is mutations in components of the NADPH oxidase, namely gp9l^{phox}, p47^{phox}, p67^{phox}, and p22^{phox} (for review, see refs. [78, 79, 97, 98]). The gp91^{phox} gene is located on the X-chromosome, and thus, CGD due to mutations in that gene (~60% of cases), affects males and is generally [99] but not always [100] associated with the absence of flavocytochrome b$_{558}$. The remaining cases of CGD are autosomal in nature and occur with equal frequency in males and females. Approximately 30% of patients with CGD have a mutation in p47^{phox}, 5% in p67^{phox}, and 5% in p22^{phox} [90, 97, 98, 101]. Recently, an infant with decreased Rac2 levels (41% of normal in neutrophil lysate, 31% of normal in neutrophil cytosol), a dominant-negative Rac2 mutation, multiple neutrophil functional abnormalities, and delayed wound-healing, complicated by infection, has been described [102–104].

The initial product of the respiratory burst oxidase, O_2^-, exists in equilibrium with its protonated form, the perhydroxyl radical (HO$_2$·) The pK_a of the dissociation is 4.8 so that the radical exists almost entirely as O_2^- at neutral or alkaline pH. O_2^- reacts predominantly as a reductant, where it gives up an electron and is converted back to oxygen. It can, however, also act as an oxidant, where it accepts an electron and is converted to H_2O_2. When two molecules interact, one is oxidized, and the other is reduced in a dismutation reaction with the formation of O_2 and H_2O_2. This reaction can occur spontaneously or be catalyzed by superoxide dismutase (SOD). Spontaneous dismutation occurs optimally at pH 4.8, where O_2^- and HO$_2$· are present in equal concentrations. At this pH, the rate constant for spontaneous dismutation approaches that of SOD-catalyzed dismutation. As the pH rises, and O_2^- predominates, the rate of spontaneous dismutation falls sharply, and catalysis by SOD becomes more significant. There is no evidence that leukocytic SOD is released into the phagosome; SOD, however, can be introduced there as a component of the ingested organism.

Vascular NAD(P)H oxidase

A NAD(P)H oxidase, similar but not identical to the leukocyte enzyme, is present in vascular cells [105–110] and may serve as a source of reactive oxygen species (ROS) in or adjacent to the vessel wall. It is of interest in this regard that in a rodent model of acute inflammation in which rats are treated with lipopolysaccharide, MPO, presumably released by stimulated phagocytes in the bloodstream, can be detected on the endothelial surface, within the endothelial cells, and in the subendothelial space, where it may react with H_2O_2 formed by the vascular NAD(P)H oxidase to modulate nitric oxide (NO·)-dependent signaling [111].

NOX/DUOX enzymes

The NADPH oxidase of phagocytes is the most reactive of a family of oxidases designated NOX for NADPH oxidase or DUOX for dual oxidase [112, 113], which contain a region with homology to the gp91^{phox} of the phagocyte NADPH oxidase. The DUOX enzymes also contained a peroxidase domain. A NOX homologous to the phagocyte gp91^{phox} was first described in colon epithelial cells by Lambeth and his colleagues in 1999 [114] and was designated MOX1 (currently NOX1). In subsequent studies, this group and others [115–119] have identified NOX1 as well as homologues, designated NOX3, NOX4, and NOX5, in a variety of other tissues [113]. The phagocyte NADPH oxidase was designated NOX2. In general, ROS production by the nonphagocyte NOX enzymes is considerably less than that produced by the phagocyte NOX (NOX2). However, recently, the proteins NOX organizer 1 and NOX activator 1, with homology to the NOX2 p47^{phox} and p67^{phox}, respectively, have been cloned from colon epithelial cells [120–123], and their inclusion with NOX1 greatly increases ROS production. Elevated calcium levels can also increase superoxide production by some NOX enzymes, e.g., NOX5, which contains four EF-hands [118, 124].

DUOX1 and 2 were first described in the thyroid gland [116, 117, 125, 126] and were designated THOX1 and -2 [117]. The THOX proteins are colocalized with the thyroid peroxidase at the apical membrane of human thyroid cells [125], and these two enzymes thus may combine to catalyze thyroid hormone synthesis. The DUOX (THOX) enzymes by virtue of having a superoxide-generating and a peroxidase center also may serve to generate and use H_2O_2 for thyroid hormone synthesis, although it is not clear that the DUOX enzymes have appreciable peroxidase activity. Mutations in DUOX2 (THOX2) are associated with a loss of thyroid hormone synthesis and can lead to congenital hypothyroidism [126]. The DUOX1 of *Caenorhabditis elegans* uses its dual functionality to facilitate cuticle formation by cross-linking of tyrosine residues in extracellular proteins [121]. DUOX1 and DUOX2 were found in salivary gland, rectum, trachea, and bronchium, and it was proposed that these enzymes serve as the source of H_2O_2 for a LPO-mediated antimicrobial system at mucosal surfaces [127].

Soluble H_2O_2-generating enzymes

Certain soluble enzyme systems can form H_2O_2, some via a O_2^- intermediate and others, without the formation of detectable O_2^-. Thus, xanthine oxidase and amine oxidase form O_2^- and H_2O_2, whereas glucose oxidase forms H_2O_2 without an apparent O_2^- intermediate. Xanthine oxidase has been implicated as a source of ROS in ischemia-reperfusion injury [128].

Mitochondrial metabolism

Mitochondrial electron transport systems in a variety of cell types generate small amounts of O_2^- and H_2O_2; however, it is not clear whether the H_2O_2 formed in this way can be released to the outside of the cell in sufficient amounts to be damaging to adjacent tissue.

Microbial metabolism

Certain microorganisms, namely those designated as lactic acid bacteria, generate H_2O_2 [129]. These microorganisms, which include strains of streptococci, pneumococci, and lactobacilli, lack heme and thus do not use the cytochrome system (which converts oxygen to water) for terminal oxidations. Rather, flavoproteins are used, which convert oxygen to H_2O_2. These organisms also lack a heme catalase and thus do not efficiently degrade H_2O_2, which accumulates in the medium. The finding that lactobacilli can serve as a source of H_2O_2 for the peroxidase-mediated antimicrobial system is pertinent to the female genital tract, as the predominant organism in the normal human vagina is the lactobacillus. In one study [130], 96% of normal women harbored H_2O_2-generating lactobacilli, whereas 4% harbored lactobacilli, which did not generate H_2O_2. Bacterial vaginosis typically occurs in sexually active women who complain of vaginal discharge, irritation, and odor. There is an associated overgrowth with Gram-negative coccobacilli, *Gardnerella vaginalis*, and certain anaerobes in the vagina associated with a decrease in H_2O_2-generating lactobacilli. In our series, only 6% of women with bacterial vaginosis harbored H_2O_2-producing lactobacilli, and 36% harbored lactobacilli which did not generate H_2O_2 [130]. Similarly, the absence of H_2O_2-generating lactobacilli predisposes women to vaginal *Escherichia coli* colonization and urinary tract infection [131]. H_2O_2-producing lactobacilli, particularly in the presence of peroxidase and a halide, are bactericidal to *G. vaginalis* [132] and *E. coli* [133] and are viricidal to human immunodeficiency virus type 1 (HIV-l) [134]. Lactobacilli alone at low concentrations also are toxic to certain anaerobes through the formation of H_2O_2 [132]. Peroxidase activity has been detected in vaginal fluid specimens from most women in amounts sufficient to induce a microbicidal effect in vitro [132], and chloride is also present in human cervical mucus in excess [135]. These findings raise the possibility that production of H_2O_2 by lactobacilli may represent a nonspecific host defense mechanism in the normal vagina in the presence or absence of peroxidase of leukocytic or uterine origin.

Nonoxynol-9 is a nonionic detergent with spermicidal activity that is widely used as the active ingredient in a number of vaginal contraceptive preparations. In addition, nonoxynol-9 is toxic to a variety of microorganisms, suggesting that nonoxynol-9-containing contraceptive preparations also may provide protection against certain genital infections. Epidemiological studies have provided support for this protective effect [136]. Paradoxically, women who use spermicides have increased vaginal colonization with *E. coli* [131, 137] and an increased incidence of bacteriuria with this organism. *E. coli* are resistant to the direct toxic effect of nonoxynol-9 [138], whereas lactobacilli are highly sensitive. This raises the possibility that suppression of the growth of lactobacilli in the vagina by nonoxynol-9 may favor the survival of *E. coli* by preventing its destruction by lactobacilli-derived H_2O_2 in the presence or absence of peroxidase and a halide. Nonionic detergents including nonoxynol-9 form peroxides when exposed to oxygen for a prolonged period, and the peroxides so formed can be toxic to *E. coli* when combined with MPO and chloride [133].
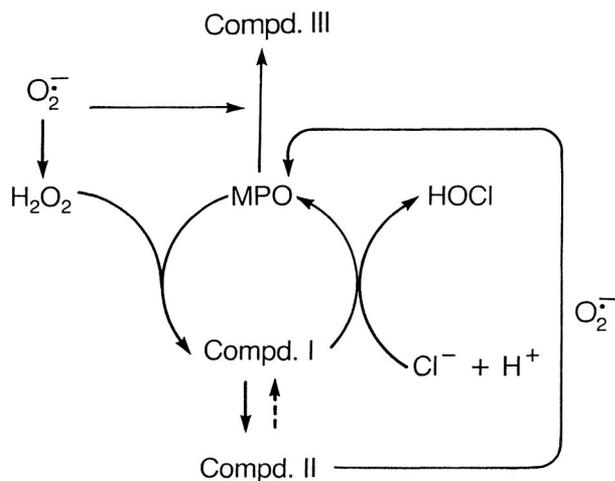
Fig. 3. MPO complex formation (taken from ref. [71]).

PRODUCTS OF THE MPO-MEDIATED ANTIMICROBIAL SYSTEM

HOCl, chlorine, and chloramines

It is the prevailing view that the initial product formed by the oxidation of chloride by MPO and H_2O_2 is HOCl [139–141]. MPO forms three different complexes on reaction with products of the respiratory burst of phagocytes, compounds I, II, and III (**Fig. 3**), with distinct spectral properties. H_2O_2 at stoichiometric concentrations [142] (or more efficiently, at a 20-fold excess of H_2O_2 [143]), reacts rapidly with the iron of MPO (which is normally in the ferric form) to form a complex compound I [142], which has an oxygen bound by a double bond to the heme iron. Compound I can also be formed by the reaction of MPO with HOCl [144]. Compound I, the primary catalytic complex of MPO, reacts with a halide in a two-electron reduction to form the corresponding hypohalous acid and regenerating the native Fe^{3+}-MPO. The reaction of compound I with excess H_2O_2 results in the formation of compound II, which is inactive with respect to the oxidation of chloride [145]. Compound II can be reduced to the active, native enzyme by O_2^- [146–148], as well as by a number of other reducing agents [146, 149, 150] with restoration of the ability to oxidize chloride to form HOCl. Kettle and Winterbourn [146, 148, 151] have proposed that one of the functions of O_2^- may be to maintain MPO in an active form in the presence of excess H_2O_2. O_2^- may thus potentiate oxidant damage at inflammatory sites by optimizing the MPO-dependent production of HOCl, and the anti-inflammatory effect of SOD may be in part a result of the inhibition of this reaction. However, the conversion of compound II to native MPO by O_2^- is considerably slower than the comparable conversion by certain other reducing agents, e.g., ascorbic acid [143, 152], raising a question about the physiologic role of the O_2^--dependent activation of MPO compound II. O_2^- can also react directly with native MPO to form compound III [142, 153–156], an oxyperoxidase, which like oxyhemoglobin, has oxygen attached to the heme iron [157, 158]. Compound III is unstable, decaying to native MPO with a half-decay time of several minutes at room temperature [157,

159]. Compound III also can be converted to the native enzyme by reducing agents such as ascorbic acid with the return of compound I-dependent HOCl production [152]. Compound III, which has been detected in intact, stimulated neutrophils [154], can react with a number of compounds, both electron donors [157, 160–162] and electron acceptors [162], raising the possibility that it is a catalytically active form of MPO in neutrophils. The reaction of MPO with O_2^- to form compound III, however, is an order of magnitude slower than the reaction of native MPO with H_2O_2 [156].

As HOCl has a pK_a of 7.53, it exists as a mixture of the undissociated acid and the hypochlorite ion at physiologic pH levels (**Fig. 4**). When the pH is lowered, as may occur in the phagosome, HOCl predominates, and it can react with excess chloride to form molecular chlorine (Cl_2) [163–165]. These products, which are the reactive components of standard household bleach, are highly reactive and thus, short-lived, oxidizing agents that can attack the microorganisms at a variety of chemical sites [166]. Essentially, any oxidizable group on the organism, e.g., sulfhydryl groups, iron-sulfur centers, sulfur-ether groups, heme groups, unsaturated fatty acids, can be oxidized, and as a consequence, there may be a loss of microbial membrane transport [167], an interruption of the membrane electron transport chain [168], dissipation of adenylate energy reserves [169], and suppression of DNA synthesis with associated disruption of the interaction of the microbial cell membrane with the chromosomal origin of replication [170]. In addition, HOCl reacts with nitrogen-containing compounds to form nitrogen-chlorine derivatives such as monochloramines and dichloramines, which can degrade to the corresponding aldehyde [171, 172]. Some of these compounds retain oxidizing activity. Taurine, which is present at a high concentration in neutrophil cytoplasm [173, 174], reacts with HOCl to form taurine chloramine, which is less toxic than HOCl, and this reaction has thus been implicated as a mechanism by which neutrophils are protected from HOCl released into the cytoplasm. Taurine chloramine, however, retains some biologic activity [175], as does histamine chloramine [176]. The chloramines are long-lived, thus providing a mechanism for the prolongation of the oxidant activity of the peroxidase system and for the penetration of MPO-derived oxidants into complex biological fluids to be toxic at a distance under conditions in
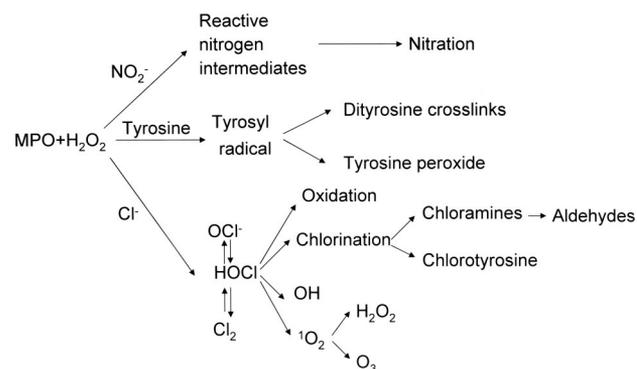

Fig. 4. Products of the MPO-mediated amtimicrobial system (modified from ref. [71]). ·OH, Hydroxyl radical.

which the more reactive products are readily scavenged. Tyrosine or tyrosine residues of protein also can be chlorinated to form 3-chlorotyrosine or 3,5-dichlorotyrosine [163, 177]. Although it is not known whether tyrosine chlorination is itself damaging to microorganisms or tissues, it serves as a specific marker of MPO activity (see ref. [166]). MPO and H_2O_2 also can oxidize tyrosine directly, i.e., in the absence of chloride, to form the tyrosyl radical, which can cross-link to form free and protein-dityrosine linkages [178–181] or react with superoxide to form tyrosine peroxide [182].

Hydroxyl radical (·OH)

In 1894, Fenton [183] described the strong oxidizing activity of a mixture of ferrous sulfate and H_2O_2, and in 1934, Haber and Weiss [184] reported that ·OH was the powerful oxidant formed as follows:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + \cdot OH$$

When the free iron concentration is limiting, as is the case in biological fluids, the reduction of the ferric iron formed is required for the complete conversion of H_2O_2 to ·OH. This can be accomplished by O_2^- as follows:

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$
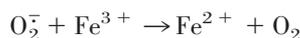
with the overall reaction being the iron-catalyzed interaction of H_2O_2 and O_2^- to form ·OH (Haber-Weiss reaction, superoxide-driven Fenton reaction) as follows:

$$H_2O_2 + O_2^- \rightarrow O_2 + OH^- + \cdot OH$$
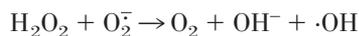
As H_2O_2 and O_2^- are formed by stimulated phagocytes, their interaction to form ·OH might be expected. Trace metal (e.g., Fe) catalysis of the Haber-Weiss reaction is required, as H_2O_2 and O_2^- do not interact directly at an appreciable rate. The free iron concentration in biological fluids is extremely low and would be expected to limit the formation of ·OH by the Haber-Weiss reaction. The bulk of the body's iron is bound to protein for storage and transport or to form a catalytic center. The ability of protein-bound iron to catalyze the Haber-Weiss reaction under physiological conditions has not been clearly demonstrated.

·OH appear to be formed by the MPO-H_2O_2-Cl$^-$ system in neutrophils, at least in small amounts (for review, see ref. [12]). One of the methods for the detection of highly labile free radicals is to form a relatively stable radical adduct with a spin trap, which can be detected by electron spin resonance spectroscopy. A new spin trap procedure for the detection of ·OH, in which ·OH reacts with ethanol to form the α-hydroxyethyl radical, which forms a measurable adduct with the spin trap α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN), was applied by Ramos et al. [185] to neutrophils. This procedure was an order of magnitude more sensitive than previously used methods, and with it, ·OH formation by stimulated neutrophils could be detected. Further, evidence was presented suggesting that the formation of ·OH by stimulated neutrophils was by a MPO-dependent mechanism. They proposed that MPO catalyses the H_2O_2-dependent formation of HOCl, which reacts with O_2^- to form ·OH as follows:

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$$

$$HOCl + O_2^- \rightarrow \cdot OH + O_2 + Cl^-$$
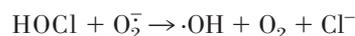
The evidence was as follows: ·OH formation was inhibited by SOD implicating O_2^-, by catalase implicating H_2O_2, and by azide implicating MPO. Further, the reaction of purified MPO with the xanthine oxidase system, which generates O_2^- and H_2O_2, resulted in the formation of ·OH in a reaction that was dependent on chloride and was inhibited by SOD, catalase, and azide. However, less than 1% of neutrophil O_2^- and H_2O_2 production could be accounted for by the formation of ·OH by this mechanism, raising a question about its physiological significance. ·OH is an extremely reactive radical, which will react with essentially the first molecule it meets. Thus, it would need to be formed in the immediate vicinity of the crucial target on the bacterial surface [186]. The HCO_3· radical, formed by the reaction of ·OH with CO_2, may be an effective microbicide under the conditions present in the phagosome [186].

Singlet oxygen ($^1O_2$)

In early studies, formation of $^1O_2$ by neutrophils could not be detected using the conversion of cholesterol to 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide as a specific marker of $^1O_2$ formation [187, 188] or by the use of instrumentation, which could detect the emission of delta $^1O_2$ decay at 1270 nm [189, 190], leading to the suggestion that $^1O_2$ is, at best, a minor product of the respiratory burst. However, Steinbeck et al. [191] have provided evidence supporting the formation of $^1O_2$ by the MPO system in neutrophils, using the conversion of 9,10 diphenylanthracene (DPA) to the DPA-endoperoxide as a specific and sensitive measure of $^1O_2$ formation. When neutrophils ingested beads coated with DPA, up to 19% of the oxygen consumed could be accounted for by the formation of $^1O_2$. $^1O_2$ was also detected with this technique as a product of the MPO-H_2O_2-chloride system, and the mechanism for its formation was the reaction of H_2O_2 with HOCl/OCl$^-$ (or Cl_2), a classical mechanism for the formation of $^1O_2$ as follows:

$$H_2O_2 + Cl^- \xrightarrow{MPO} H_2O + HOCl \leftrightarrow OCl^- + H^+$$

$$H_2O_2 + OCl^- \rightarrow {}^1O_2 + H_2O + Cl^-$$
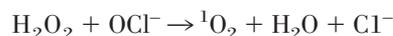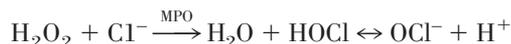
Arisawa et al. [192], using a chemiluminescence method for the detection of $^1O_2$, reported the formation of $^1O_2$ by the MPO-H_2O_2-chloride system, which peaked at pH 7. Further, using a highly sensitive detection system for light emission at 1270 nm, Kiryu et al. [193] detected $^1O_2$ formation by the MPO-H_2O_2-chloride system under physiological conditions, i.e., at pH 7.4 and without the use of deuterium oxide. However, the formation of $^1O_2$, in appreciable amounts by stimulated phagocytes, remains in question.

Ozone (O$_3$)

Recently, it has been proposed that antibodies, regardless of antigen specificity, can catalyze the oxidation of water by $^1O_2$ to produce H_2O_2 and O_3 [194–197]. O_3 has also been proposed as a product of the respiratory burst of neutrophils, using $^1O_2$

formed by the MPO system for its formation [198]. O_3 is bactericidal, and a combination of H_2O_2 and O_3 is more toxic to microorganisms than either alone. This thus may be an additional mechanism for the destruction of microorganisms by the MPO system of phagocytes.

The formation of O_3 by stimulated phagocytes was based largely on the conversion of indigo carmine to isatic sulfonic acid, a reaction that can be induced by O_3. However, O_2^- is also capable of this reaction, and the involvement of O_2^- in the conversion of indigo carmine to isatic sulfonic acid by stimulated phagocytes is suggested by the inhibitory effect of SOD and the absence of inhibition by catalase, azide, and methionine [199].

EVIDENCE FOR MPO INVOLVEMENT IN NEUTROPHIL MICROBICIDAL ACTIVITY

A number of lines of evidence support an important role for MPO in the microbicidal activity of neutrophils (for reviews, see refs. [12, 200, 201]).

MPO, H_2O_2, and a halide form a powerful antimicrobial system

Initial studies indicated that the halide requirement could be met by iodide, bromide, or chloride [68–70] or by the pseudohalide, thiocyanate [65, 66]. More recently, nitrite was shown to substitute for the halide in the MPO-mediated antimicrobial system in vitro [202, 203]. Nitrite was bactericidal at concentrations down to 10^{-3}M when combined with MPO and a source of H_2O_2 [202]. Nitrite can be formed in phagocytes as a product of the metabolism of NO·; however, it is not clear that nitrite is formed in sufficient amounts to contribute significantly to the microbicidal activity of the MPO system.

It is the prevailing view that chloride is the physiological halide, as it is present in biological fluids at concentrations considerably higher than that required. Iodide is the most effective halide on a molar basis; however, the concentration of free iodide in biological fluids is very low (<1 mg%). The iodinated hormone, thyroxine, can substitute for iodide in the cell-free MPO system [69], presumably, at least in part, as a result of its deiodination by the peroxidase system [204, 205]. Bromide is intermediate between iodide and chloride in effectiveness and concentration; however, at levels present in plasma, chloride is preferentially used by the MPO system [206]. The presence of brominated compounds (e.g., 3-bromotyrosine) as well as chlorinated compounds (e.g., 3-chlorotyrosine) in the peritoneal fluid of wild-type mice with sepsis at concentrations considerably higher than those seen in peritoneal fluid of septic MPO-deficient mice supports a contribution by bromide to the MPO-mediated antimicrobial system in vivo [207]. Thiocyanate is readily oxidized by MPO and H_2O_2 [208, 209] even in the presence of physiologic concentrations of chloride [208], suggesting that the product of thiocyanate oxidation, hypothiocyanous acid, may contribute to the antimicrobial activity of the MPO system.

MPO and H_2O_2 are formed or released by neutrophils at a time and place appropriate to the microbicidal act

MPO [49] and H_2O_2 [210, 211] can be detected in the phagosome by electronmicroscopic cytochemical techniques. The oxidase responsible for O_2^- [212] and H_2O_2 [211] production was detected in low amounts on the plasma membrane of resting neutrophils and following phagocytosis, was found in considerably increased amounts in the phagosome. These findings indicate that the oxidase is membrane-associated and is internalized and activated during phagocytosis with the generation of H_2O_2 within the phagosome.

The formation of HOCl accounts for a high proportion of the oxygen consumed in the respiratory burst

Values of at least 28% [213] and 72% [214] with different stimuli have been proposed. Similarly, in one study, 40% of the H_2O_2 generated by zymosan-stimulated neutrophils was used for the formation of HOCl [215]. These are minimum values, as they do not take into account the presence of proteins and other scavengers that compete for HOCl in the assay system [213]. Jiang et al. [216] concluded from their studies that "the neutrophil is capable of intraphagosomal generation of HOCl in sufficient quantities to kill entrapped bacteria on a time scale that is associated with bacterial death", and Hampton et al. [200] concluded from their studies that "enough HOCl is generated in the phagosome for it to be responsible for killing."

MPO, H_2O_2, and a halide interact in the phagosome adjacent to the ingested organism

The production of ROS and their reactions occur in the microenvironment of the phagosome, which is complex and constantly changing [186]. Initially, when opsonized bacteria are ingested by phagocytes, microbe-associated ligands, generally antibody and/or complement, bind to membrane receptors in a continuous and circumferential manner, as the cell membrane invaginates to form a tight phagosome, with little or no space between the microbe and phagosome membrane (zipper phenomenon [217]). This process is associated with the activation of the NADPH oxidase and with degranulation, which releases ROS and the granule components, including MPO, into the phagosome. The latter process creates a space between the microbe and the membrane of the phagosome, which contains a variety of granule components through which the secreted ROS would need to travel to reach the ingested microbe. Some ROS, e.g., ·OH, are highly reactive and would be expected to be scavenged quickly prior to reaching the microbe. Other ROS, e.g., H_2O_2, are less reactive and thus have longer diffusion distances. That H_2O_2 can reach the microbe and react with it is suggested by the OxyR-mediated transcriptional response in *E. coli*, which is elicited by reagent H_2O_2 and when complement-opsonized *E. coli* are ingested by intact phagocytes. This suggests that H_2O_2 formed in the phagosome can reach the microbe at concentrations adequate to initiate an OxyR response [218]. MPO, being present in human neutrophils at concentrations no less than 1–2% of the dry weight of the cells [6], would be expected to be present in the phagosome in very high concentrations. Being a highly cationic protein with an isoelectric point greater than 10 [6], it can bind to the negatively charged surface of the microorganism and react there with H_2O_2 to initiate MPO-dependent oxidant formation in close proximity to the ingested microbe.

When iodide is the halide, iodination is detected [69]. The fixed iodide can be localized in part in the phagosome by autoradiographic techniques [49] and by the detection of radioiodide in the isolated phagosome [219]. The bulk of the iodination appears to involve other neutrophil constituents [49, 220, 221] and extracellular proteins [222, 223]; however, autoradiographic studies demonstrated the presence of silver grains lining the surface of the ingested organism [49], indicating bacterial iodination as well. Chlorination and bromination also occur. Thus, free 3-chlorotyrosine and 3-bromotyrosine levels, used as markers of the reaction of MPO with H_2O_2 and chloride or bromide, rise in the peritoneal fluid of wild-type mice with experimentally induced sepsis but not (or to a considerably lesser degree) in septic mice deficient in MPO [207]. The bromination observed in the absence of MPO may be a result of the presence of eosinophil peroxidase [224]. 3-Chlorotyrosine has also been detected in tracheal aspirates from preterm infants, particularly those with respiratory distress [225], in human atherosclerotic lesions [226], in sputum specimens of patients with cystic fibrosis [227], and in bronchoalveolar lavage fluid proteins of patients with acute respiratory distress syndrome, the latter in association with increased nitrotyrosine levels and MPO [228]. Fluorescein-conjugated beads react with HOCl to form mono- and dichlorofluorescein [216]. When human neutrophils were exposed to opsonized fluorescein-conjugated beads, ~20 beads per cell became cell-associated, of which approximately two thirds were incorporated into sealed phagosomes. Near stoichiometric chlorination of the phagocytosed beads occurred [216]. Of particular interest is the demonstration that bacteria sequestered in the phagosome are chlorinated by a MPO-dependent mechanism, as indicated by the presence of 3-chlorotyrosine and 3,5-dichlorotyrosine in bacterial proteins [229, 230]. In contrast, neutrophils failed to nitrate bacterial proteins in the phagosome under conditions in which chlorination was readily observed [230]. Although it seems clear that HOCl is formed in adequate amounts to kill the ingested microorganisms, it would be expected to be scavenged to some degree if formed some distance from the microbe, as it travels through a fluid rich in scavengers, thus decreasing the amount of HOCl available for reaction with the ingested bacteria [229]. However, chlorination of tyrosine residues of bacterial proteins does occur [229, 230], and this may be only the tip of the iceberg, as kinetically more favored (and likely more toxic) reactions of HOCl with, among others, sulfhydryl groups, iron-sulfur centers, and sulfur-ether groups would be expected to precede chlorination. Thus, although not all of the HOCl formed by leukocytes is used to attack the ingested organism, some of it is, which raises the question: how much is enough? The microbicidal power of HOCl is compatible with the need for only a portion of the HOCl formed.

H_2O_2 generation by neutrophils is required for optimum microbicidal activity

Enzymes that scavenge H_2O_2, such as catalase, protect certain organisms, e.g., coagulase-positive staphylococci, from the toxic effect of reagent H_2O_2 [231, 232] and neutrophils [233] in vitro, and staphylococcal strains rich in catalase are more virulent in vivo [233]. Furthermore, neutrophils from patients

with CGD lack a respiratory burst, and the microbicidal defect in these cells can be reversed in part by the introduction of H_2O_2 into the cell [234–237]. Glucose oxidase, which forms H_2O_2 without an apparent O_2^- intermediate, can be used for this purpose, indicating that H_2O_2 is effective even when the cells remain deficient in O_2^-. Finally, certain microorganisms, e.g., strains of streptococci, pneumococci, and lactobacilli, generate H_2O_2, and these organisms are killed well by CGD leukocytes [238–240] and are rarely found in the lesions of infected patients. Presumably, they provide the H_2O_2 required for their own destruction by leukocytes that lack a cellular H_2O_2-generating system. This is supported by the finding that mutant strains of *Streptococcus faecalis* [241] and *Streptococcus pneumoniae* [242, 243] with diminished H_2O_2 production are killed less well by CGD leukocytes than are the wild-type strains. These organisms also lack a heme catalase; however, the studies with mutant strains suggest that the level of H_2O_2 production may be more important than the catalase content in their susceptibility to killing by CGD leukocytes.

MPO is required for optimum microbicidal activity

Peroxidase inhibitors such as azide [244, 245], cyanide [244, 245], and sulfonamides [246] decrease the microbicidal activity of normal neutrophils and have little or no effect on the microbicidal activity of MPO-deficient neutrophils, suggesting that they exert their effect on normal neutrophils largely by the inhibition of MPO. Neutrophil cytoplasts (neutroplasts) lack nuclei and are greatly depleted of cytoplasmic granules (and thus MPO) but have an intact respiratory burst [247, 248]. Neutroplasts phagocytose but do not kill *Staphylococcus aureus* unless the bacteria are coated with MPO [249], suggesting that the respiratory burst is not sufficient for the killing of the organisms but that the additional presence of MPO is required. Similarly, stimulated neutroplasts do not lyse erythrocytes [250] or inactivate α^1-proteinase inhibitor [251] unless MPO is added. Finally, human neutrophils, which lack MPO, have a microbicidal defect in vitro, although the defect is not as severe as in CGD. Thus, in 1969, Lehrer, Cline, and Hanifin [252, 253] described a diabetic patient with MPO deficiency and systemic candidiasis whose neutrophils had a prolonged candidacidal defect in vitro, whereas their staphylocidal activity was characterized by a lag period following which the organisms were killed. At this time, MPO deficiency was considered to be a rare event. However, the introduction of automated white blood cell counting techniques, which used the peroxidase stain, indicated that hereditary MPO deficiency was not uncommon in Europe and America (one in 2000–4000 [254, 255]), although it was less common in Japan (complete deficiency, one in 57,135 [256]). Although some of these patients had clinical infections, most were well despite the demonstration of a neutrophil microbicidal defect in vitro [254, 255, 257, 258]. In one study comparing 100 patients with total or subtotal MPO deficiency to 118 with normal MPO levels, there was a statistically significant, higher incidence of severe infection and chronic inflammatory processes in the deficient patients [259].

Does the microbicidal activity of human MPO-deficient leukocytes accurately reflect the contribution of MPO to the

microbicidal activity of normal cells? Evidence has been provided suggesting that it may not [244]. **Figure 5** demonstrates the effect of the peroxidase inhibitor azide on the microbicidal activity of normal and MPO-deficient neutrophils on three organisms, *Lactobacillus acidophilus*, coagulase-negative staphylococci, and *Candida tropicalis*. As reported earlier [252, 253], the organisms were killed less well by MPO-deficient than by normal neutrophils. Azide markedly decreased the microbicidal activity of normal neutrophils and had no effect on the microbicidal activity of MPO-deficient neutrophils, suggesting that it exerts its effect on normal cells by the inhibition of MPO. Of particular interest is the observation that the microbicidal activity of MPO-deficient leukocytes is greater than that of azide-treated, normal cells [244]. This suggests that the contribution of MPO to the microbicidal activity of normal neutrophils may be greater than that suggested by studies with MPO-deficient neutrophils, as the latter cells appear to have adapted to the long-term absence of MPO with an increase in the activity of the MPO-independent (azide-insensitive) antimicrobial systems. Thus, the microbicidal activity of MPO-deficient cells appears to underestimate the contribution of MPO to the killing by normal cells.

Mice that lack MPO have an increased susceptibility to infection under some experimental conditions but not others. Thus, MPO-knockout mice have an increased susceptibility to *Candida albicans* infection, whereas the clearance of *S. aureas* is normal [260]. MPO-deficient mice are also considerably more susceptible to the development of pulmonary infection following the intranasal instillation of *C. albicans*, *C. tropicalis*, *Trichosporon asahii*, and *Pseudomonas aeruginosa*, whereas susceptibility to infections with *Aspergillus fumigatus* and *Klebsiella pneumoniae* is increased to a lesser degree, and susceptibility to *Candida glabrata*, *Cryptococcus neoformans*,

S. aureus, and *S. pneumoniae* is comparable to that of the wild-type strain [261]. Further, mice lacking MPO are more susceptible to intra-abdominal infection and sepsis following cecal ligation and puncture than are wild-type mice [207]. In a study comparing the susceptibility to intraperitoneal *C. albicans* infection of wild-type, MPO-deficient, and CGD mice, it was concluded that when the fungal load is low, ROS formed by the NADPH oxidase of neutrophils are adequate to control infection in the absence of MPO, but that at high fungal load, respiratory burst products and MPO are needed [262]. Similarly, CGD and MPO-deficient mice are more susceptible to pulmonary infection with *C. albicans* or *A. fumigatus* than are normal mice, and the infection of CGD mice is more severe than that of MPO-deficient mice [263]. It should be emphasized, however, that mice are not humans and that the findings with the mouse model do not necessarily translate to humans. For example, the MPO level of mouse neutrophils is approximately 10% of that of human cells. Further, the inducible NO· synthase system with its associated microbicidal activity appears to be much more highly developed in rodent than in human phagocytes (see below, Reactive nitrogen intermediates) and is thus more likely to substitute for the MPO system in mice when MPO is absent. A comparison of the microbicidal activity of normal and MPO knockout mice when NO· synthase is also absent would be of interest in this regard.

It can be concluded from these studies that MPO is involved in the microbicidal activity of normal neutrophils, particularly in the early post-phagocytic period or when the microbial challenge is high, but that MPO-independent antimicrobial systems develop more slowly but are ultimately effective in MPO-deficient leukocytes, particularly when the microbial challenge is low. It should be emphasized that organisms differ in their susceptibility to oxygen-dependent antimicrobial systems. Thus *E. coli* are killed well by CGD and MPO-deficient
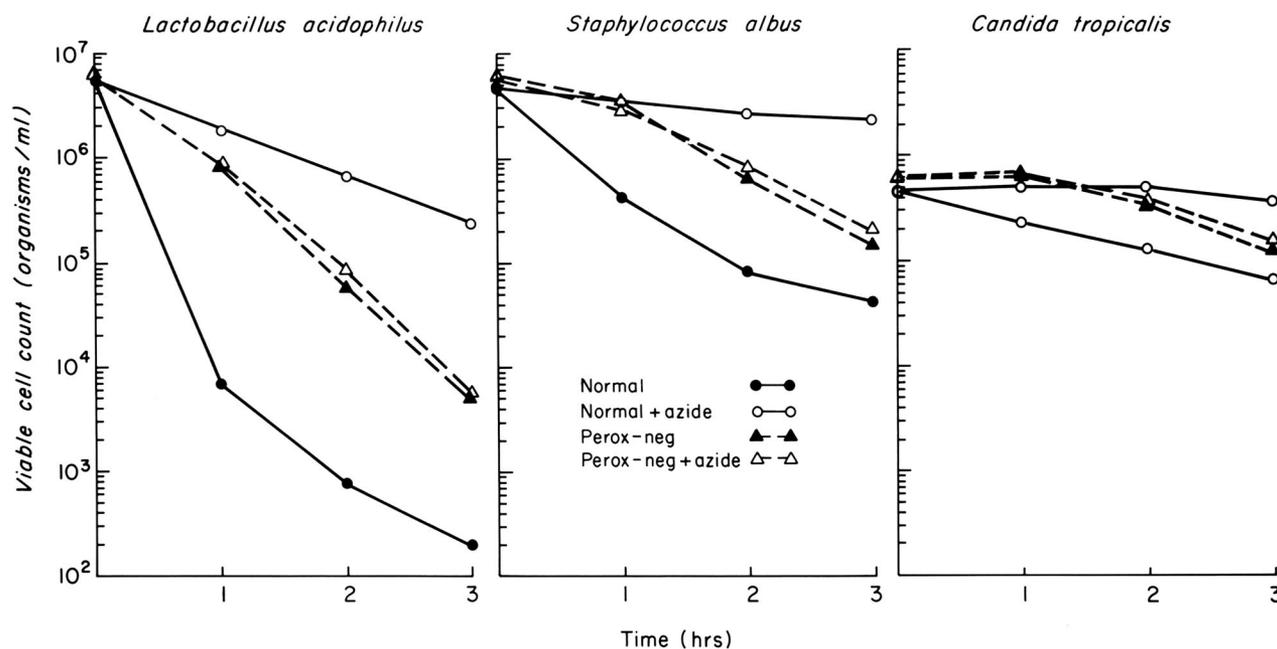

Fig. 5. Comparison of the bactericidal activity of MPO-deficient and azide-treated neutrophils (taken from ref. [244]).

leukocytes [264], fungi are particularly dependent on MPO for killing [252], and staphylococci are intermediate [253]. However, even if the MPO system is not *required* for the killing of a particular organism, it may normally do so in the early post-phagocytic period as a result of its rapid mobilization and power.

MPO-INDEPENDENT ANTIMICOBIAL SYSTEMS

What is the nature of the azide-insensitive (i.e., MPO-independent) antimicrobial systems of phagocytes? These may include ROS operating in the absence of MPO, reactive nitrogen intermediates, intravacuolar acidity, cationic peptides and proteins, and proteases. These systems may contribute to the microbicidal activity of phagocytes in the presence as well as in the absence of MPO.

ROS operating in the absence of MPO

The staphylocidal activity of MPO-deficient neutrophils is inhibited by anaerobiosis, indicating that the microbicidal activity is at least in part dependent on oxygen ([222]; see p. 437 in ref. [265]). The respiratory burst of murine [260] and human [222, 266–274] MPO-deficient leukocytes has been shown in a number of studies to be greater than normal, which may be a result of at least two mechanisms. First, MPO may be required for the termination of the respiratory burst [275], and thus, its absence would lead to increased production of toxic oxygen metabolites. Second, as H_2O_2 is degraded in part by the MPO system in neutrophils, the absence of MPO would be expected to lead to a build-up of H_2O_2 as well as that of other oxidants dependent on H_2O_2 for their formation. These oxidants may eventually kill or at least contribute to the killing of the ingested organisms in the absence of MPO.

O_2^- and H_2O_2 are recognized products of the respiratory burst of phagocytes, which do not require MPO for their formation. O_2^- could theoretically be directly toxic to ingested organisms. However, many biologically important compounds react rather sluggishly with O_2^-, leading to the suggestion that O_2^- does not have the necessary reactivity to be directly toxic to ingested organisms. However, following are a few words of caution. The chemical reactivity of O_2^- is increased considerably in a nonpolar environment, as exists in the hydrophobic region of a membrane, where the reactions of O_2^- are not in competition with the proton-requiring dismutation reaction. Under these conditions, O_2^- is a powerful base with considerable nucleophilicity and reducing activity. Further, the protonated form $HO_2 \cdot$ is a considerably stronger oxidant than is O_2^-, raising the possibility that a local fall in pH, as might occur at a membrane surface or within a phagosome, may cause a shift in the $HO_2 \cdot \leftrightarrow O_2^-$ equilibrium toward the more potent, protonated form, with localized damage to a membrane or ingested organism. Further, the low, steady-state concentration of O_2^- would limit its dissipation by spontaneous dismutation, and this together with its relatively low reactivity allow it to diffuse over significant distances as through the ion channels of some cell membranes [276], where it may be toxic at a distance through the formation of more reactive oxidants. H_2O_2 alone has antimicrobial properties at concentrations higher than those needed to generate toxic amounts of HOCl by the MPO system and thus, may contribute to the microbicidal activity of MPO-deficient phagocytes.

Reactive nitrogen intermediates (RNI)

Nitric oxide (NO·) is a recognized product of a cytokine-inducible NO· synthase in murine phagocytes where it contributes to microbicidal activity [277]. It acts synergistically with ROS under some conditions [278]. The production of NO· by human phagocytes, however, has been more difficult to demonstrate. Thus, in early studies, NO· formation by human phagocytes could not be detected under conditions in which NO· formation by murine phagocytes was readily apparent [279–281]. However, human neutrophils appropriately stimulated have been shown to contain an inducible NO· synthase (iNOS) [282–284], as do tissue macrophages from infected humans [285–288]. It has been concluded that although human mononuclear phagocytes can produce iNOS when appropriately stimulated, NO· production by these cells is very low as compared with mouse phagocytes [289, 290].

NO· reacts with O_2^- to form peroxynitrite (ONO_2^-) [291–293] (**Fig. 6**), which can oxidize nonprotein and protein sulfdryl groups [294]. ONO_2^-, at acid pH, is protonated to form peroxynitrous acid (ONO_2H), which decomposes to form a strong oxidant with the properties of ·OH [295, 296]. ONO_2^-/ONO_2H has bactericidal properties [297–299] and thus may contribute to the MPO-independent antimicrobial activity of phagocytes. However, the contribution of ONO_2^-/ONO_2H would be expected to be greater in rodent than in human phagocytes. Carbon dioxide (CO_2) reacts with ONO_2^- to form the ·$ONO_2CO_2^-$ adduct with a corresponding loss of bactericidal activity [299, 300].

Nitration of tyrosine and tyrosine residues of proteins has been used as a marker for the production of RNI. Initially, the mechanism proposed for the induction of nitration by phagocytes was the reaction of NO· with O_2^- to form ONO_2^-/ONO_2H, which was the nitrating species. Recently, doubt has been cast on the essential role of peroxynitrite in nitration by phagocytes [301] and evidence presented supporting a role for peroxidase in the nitration process [203, 302, 303] (for commentary, see ref. [304]). Nitrite is converted to a nitrating species, and tyrosine is oxidized to the tyrosyl radical by MPO and H_2O_2
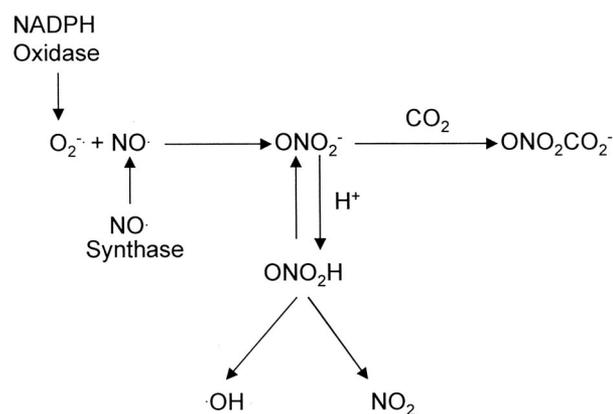

Fig. 6. Reactive nitrogen intermediates.

[305–307]. Further, phagocytes activated in medium containing nitrite can generate nitrating species through a MPO-dependent mechanism [306, 308], and murine macrophages activated by immunological stimuli can generate the nitrite required for catalysis of the nitration reaction by peroxidase and H_2O_2 [302]. Nitration of free or protein-associated tyrosine has been detected in areas of inflammation [309]. However, the absence of nitration in the phagosome, even at nitrite concentrations up to 0.1 M [310], would suggest that the oxidation of nitrite is not a major source of oxidants in the phagosome, although the oxidation of nitrite in the extracellular fluid by MPO and H_2O_2 released from phagocytes may occur in vivo [311]. The formation of nitrating and chlorinating species, possibly nitryl chloride (Cl–NO_2) or chlorine nitrite (Cl–ONO), by reaction of nitrite with HOCl with their involvement in tissue injury, has been proposed [312]. Paradoxically, the toxicity of the MPO-H_2O_2-chloride system or its product HOCl is inhibited by nitrite [202, 313–316] as a result of an interaction between nitrite and HOCl, which results in the stoichiometric removal of both [202]. Further, nitrite or a product of its oxidation can bind to the heme prosthetic group of MPO and thus modulate its activity [202, 307, 317–319]. Whether nitrite influences the MPO-mediated antimicrobial system in the phagosome positively or negatively remains to be established.

Intraphagosomal acidity

Studies of intraphagosomal pH have yielded conflicting results. In early studies, a fall in intraphagosomal pH was observed (for review, see pp. 447 and 448 in ref. [265]), although the extent of the fall varied. Thus, Metchnikoff [320] first reported that "the staining of the ingested elements indicates a feebly acid reaction inside the phagocytes." Further, he stated that "while the phagocyte is still living the acid juice which fills the vacuoles or permeates the ingested organisms does not mix with the protoplasm which is always alkaline." Subsequently, Rous [321, 322] reported that the "intragranular" pH of peritoneal exudate cells in mice or rats was 3.0 or below, Sprick [323] found the intraphagosomal pH surrounding mycobacteria ingested by mouse or guinea pig intraperitoneal cells to be 4.7–5.5, Pavlov and Solov'ev [324] found the pH surrounding bacteria ingested by mouse peritoneal cells to be 4.7–5.2, and Jensen and Bainton [325] reported that the intraphagosomal pH in rat peritoneal polymorphonuclear leukocytes (PMNs) fell to 6.5 in 3 min and to 4.0 in 7–15 min following the ingestion of yeast particles. In these studies, microorganisms coated with indicator dyes were used in vivo. Mandell [326], using human PMNs ingesting indicator dye-stained candida in vitro, found a fall in intraphagosomal pH to 6.0–6.5. Kakinuma [327], using the 5,5-dimethyl-2,4-oxazolidinedione method for determining intracellular pH, estimated the intraphagosomal pH of guinea pig PMNs to be 5.5–6.0. These findings led to the suggestion that intraphagosomal acidity may limit the growth of certain ingested microorganisms.

More recent studies, however, have suggested an initial rise in pH followed by a fall. Thus, Segal et al. [328] reported an initial rise in intraphagosomal pH to 7.75 within the first 2 min of phagocytosis by human neutrophils, followed by a slower fall in pH to 6.0–6.5 in 2 h. Similarly, Geisow et al. [329] described an initial rise in intraphagosomal pH in mouse peritoneal macrophages to 7.75 in 1.5–2 min, followed by a fall to levels below pH 6 at 7 min. Cech and Lehrer [330], using human neutrophils, reported an initial increase in pH to 7.8 in 5 min followed by a fall in pH to 6.35 in 30 min and 5.68 in 60 min. Also, Jiang et al. [216] reported a slight rise in pH to 7.5–7.7 over the first 15 min following phagocytosis by human neutrophils, followed by a slow decline to about pH 7.0 in 60 min. These findings would suggest that a fall in pH is unlikely to contribute to antimicrobial activity during the immediate post-phagocytic period but may do so later. It should be emphasized, however, that the intraphagosomal chlorination of the probes used to measure pH may alter their spectral properties, thus making their use for the measurement of pH equivocal [331]. Under conditions in which alteration in the properties of the fluorescent pH probe by chlorination was minimized by the inhibition of MPO, initial alkalinization of the phagosome was not detected, and the pH remained near neutral for at least 20 min [332]. When the NADPH oxidase was inhibited by diphenylene iodinium, a rapid acidification of the phagosome occurred, and the pH reached 5.1 in 2–8 min [332]. It is of interest in this regard that in CGD leukocytes (in which the respiratory burst and thus the MPO system are not operative), there is not an initial alkalinization but rather an immediate and rapid fall in pH to 6.7 in 2 min, with the final intraphagosomal pH being 5.5 [328]. Thus, measurements of intraphagosomal pH varied strikingly under different experimental conditions and may, to some degree, be artifactual due to the altered properties of chlorinated probes.

The gp9l ^{phox} membrane component of the NADPH oxidase of neutrophils has been reported to act as an H^+ channel, conducting protons across the membrane into the extracellular fluid and/or phagosome [333, 334]. However, other studies have suggested that the H^+ channel is separate from the NADPH oxidase [335–337]. Extracellular proton release, which is associated with the respiratory burst of normal neutrophils, was not seen when CGD neutrophils were used [338], despite the rapid and immediate fall in intraphagosomal pH [328].

Cationic peptides and proteins

A variety of cationic peptides and proteins present in leukocyte granules has been implicated in the microbicidal activity of phagocytes. These include defensins, seprocidins, bactericidal/permeability-increasing protein, lysozyme, cathelicidins, phospholipase A_2, and lactoferrin [339–342]. **Figure 7** compares the staphylocidal activity of a human neutrophil granule cationic protein preparation provided by Olsson and Venge [343] to that of the MPO system (see p. 459 in ref. [265]). The cationic proteins at a concentration of 50 µg/ml had a small but significant staphylocidal effect after 2 h of incubation, whereas the MPO system, with MPO at 2 µg/ml, was strongly bactericidal at the earliest time period used (7.5 min). These findings do not necessarily reflect the relative roles of these systems under other experimental conditions, with other peptide or protein preparations, against other microorganisms or in the intact cell where oxygen may be limiting and the cationic protein concentration high. However, it does emphasize the greater microbicidal potential of the MPO system against some organisms.
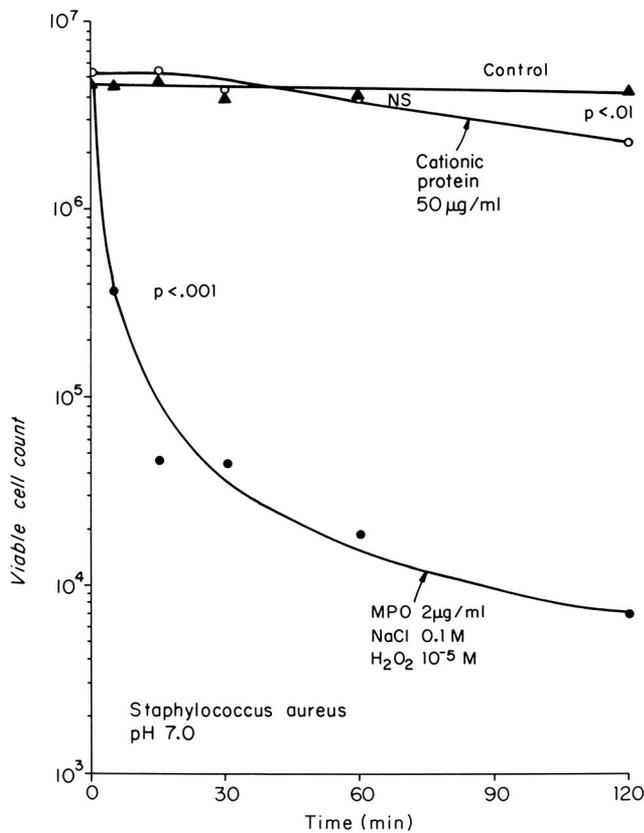
Fig. 7. Comparison of the staphylocidal activity of phagocyte-derived cationic proteins and the MPO-mediated antimicrobial system (taken from page 459 in ref. [265]). NS, Not significant.

Proteases

In a recent study [344], mice deficient in neutrophil granule elastase were shown to have an increased susceptibility to *S. aureus* but not to *C. albicans* infection, and mice deficient in cathepsin G had an increased susceptibility to *C. albicans* but not to *S. aureus* infection in vivo (for comments, see refs. [345–347]). Neutrophils from these animals had a corresponding killing defect in vitro. Mice deficient in neutrophil elastase and/or cathepsin G also had increased susceptibility to *A. fumigatus* infection [348]. Other studies suggested that mice deficient in neutrophil elastase were more susceptible to infection with *K. pneumoniae* and *E. coli* infection but not to infection with *S. aureus* [349, 350]. Further, cathepsin G-deficient mice have been reported to kill *S. aureus, K. pneumoniae*, and *E. coli* normally [351]. Thus, the susceptibility of microorganisms to proteases appears to vary under different experimental conditions. Neutrophil elastase has been reported to exert its toxic effect on *E. coli* by the degradation of the organism's outer membrane protein A (OmpA) [352], and neutrophil elastase, cathepsin G, and proteinase 3 can cleave the flagellin of Gram-negative organisms and thus influence the inflammatory response [353].

It has been proposed that the influx of O_2^- into the phagosome and its dismutation there consume protons leading to a small rise in intraphagosomal pH, which would facilitate antimicrobial activity of granule proteins with alkaline pH optima [328].

Further, an influx of high concentrations of K^+ through a Ca^{2+}-activated K^+ channel occurs in an attempt to maintain charge neutrality [344, 354] (for discussion of the role of K^+, see refs. [355, 356]). The resultant alkalinity and hypertonicity may result in the release of cationic proteins and neutral proteases with antimicrobial activity from intraphagosomal complexes with proteoglycans. It is of interest in this regard that microbial killing and digestion were abolished when the K^+ channel was blocked, despite normal NADPH oxidase activity, phagocytosis, and iodination [354]. It has been recently proposed that ion movement and ROS contribute to microbial killing by phagocytes, with the ion movement being most significant at low O_2^- production [357].

POTENTIAL TARGETS OF THE MPO SYSTEM

The MPO-H_2O_2-halide system, like the household bleach Clorox, is broadly toxic in vitro. It is toxic to a variety of microorganisms (bacteria, fungi, viruses, protozoa, amoebae, helminths), bacterial toxins, intact mammalian cells (tumor cells, granulocytes, lymphocytes, erythrocytes, spermatozoa), and low molecular weight mediators (chemotactic factors, α^1-proteinase inhibitor, leukotrienes). The MPO system, although generally inhibitory in its actions, also can be stimulatory at low levels, as for example, in the stimulation of platelet and mast cell secretion and the activation of certain proteases (e.g., collagenase, gelatinase; for review, see refs. [12, 358]). Other proteases, e.g., matrix metalloproteinase-7 [359], are inhibited by the MPO system. HIV-1 is given here as an example of a potential target of the MPO system and the approach used to detect an effect of the MPO system on survival. In this instance, ROS, formed by phagocytes, may have opposing effects, i.e., a viricidal effect as a result of the MPO system and the stimulation of viral replication as a result of activation of the HIV-1 long-terminal repeat (LTR) by H_2O_2.

HIV-1: viricidal effect

The MPO-H_2O_2-halide system is strongly toxic to HIV-1, as measured by the inability of the virus to replicate in the lymphocyte cell line CEM [134]. The MPO could be replaced by eosinophil peroxidase (EPO) [360], and the H_2O_2 could be replaced by a H_2O_2-generating enzyme system, such as amine oxidase [361]. Chloride, bromide, iodide, and thiocyanate ions could meet the halide requirement of the MPO system [134], and bromide, iodide, and thiocyanate could be used by the EPO system [360]. H_2O_2-generating lactobacilli are viricidal alone at high concentration to HIV-1, in part as a result of the formation of H_2O_2, and when the lactobacilli are decreased to a level where they are no longer viricidal alone, the further addition of MPO and a halide restores viricidal activity [134]. Peroxidase [132] and H_2O_2-generating lactobacilli [130] are present in the vaginal fluid of most women, raising the possibility that they may influence the heterosexual transmission of HIV-1 through their toxic effect on the virus. Neutrophils [362] and monocytes [363], when appropriately stimulated, were also toxic to HIV-1, an effect that was inhibited by catalase, implicating H_2O_2, and by the peroxidase inhibitor azide, implicating

MPO. Activity was also lost when chloride was replaced by sulfate in the medium. Stimulated cells from CGD patients were not viricidal unless H_2O_2 was added, and the viricidal activity of the H_2O_2-supplemented cells was inhibited by azide implicating endogenous MPO. Further, stimulated neutrophils or monocytes from patients with hereditary MPO deficiency were not viricidal unless MPO was added, and the viricidal effect of the MPO-supplemented cells was inhibited by catalase, implicating endogenously formed H_2O_2. When monocytes are allowed to differentiate in culture to macrophages, MPO is lost, and the respiratory burst and thus, H_2O_2 formation is greatly decreased. The viricidal effect on HIV-1 of 3- to 9-day monocyte-derived macrophages was lost unless MPO was added, whereas when 12-day monocyte-derived macrophages were used, MPO did not restore activity unless the cells were pretreated with interferon-γ, which increases the respiratory burst of macrophages [364]. Stimulated human eosinophils also are viricidal to HIV-1 through the action of a peroxidase (EPO)-H_2O_2 system [360].

HIV-1: activation of the LTR

H_2O_2 can activate the HIV-l LTR and increase HIV-1 production by latently infected cell lines [365–367], at least in part by activation of the nuclear transcription factor NF-κB [366]. H_2O_2-induced activation of the HIV-1 LTR and stimulation of virus production are greatly enhanced by vanadate, presumably as a result of the reaction of H_2O_2 with vanadate to form peroxides of vanadate, which have potent biological properties [367]. Activation of the HIV-1 LTR and viral replication by H_2O_2 are also strongly stimulated by polar, aprotic solvents such as dimethylsulfoxide, dimethylacetamide, and dimethylformamide [368]. H_2O_2-generating lactobacilli can activate the HIV-l LTR in Jurkat T lymphocytes, an effect that is enhanced by vanadate and inhibited by catalase, implicating H_2O_2 [369]. Thus, H_2O_2 generated by vaginal lactobacilli may influence viral replication in two opposing ways, by its viricidal effect, particularly when supplemented with peroxidase and a halide, and by the stimulation of viral replication by activation of the LTR.

Human neutrophils stimulated by phorbal myristate acetate (PMA) strongly activated the HIV-1 LTR in Jurkat T lymphocytes [370]. Activation was inhibited by catalase, was potentiated by vanadate, and was not observed when normal neutrophils were replaced by neutrophils that lack a respiratory burst, i.e., from patients with CGD, implicating H_2O_2. In contrast to its inhibitory effect on the viricidal effect of stimulated neutrophils [362], azide increased LTR activation by PMA- and opsonized zymosan-stimulated neutrophils, presumably by inhibiting the degradation of H_2O_2 [370]. Thus, products of the respiratory burst of neutrophils can have a dual effect on HIV-1, a viricidal effect through the release of components of the MPO-H_2O_2-halide system and a viral-promoting effect through activation of the LTR.

OTHER BENEFICIAL FUNCTIONS OF MPO

The studies described above strongly suggest that the primary physiologic function of MPO is to kill microorganisms in neutrophils and monocytes, and to do this, it forms highly reactive halide (particularly chloride)-derived oxidants in the confines of the phagosome. Does MPO have other "normal" functions beneficial to the host? Agner [371, 372] proposed that MPO played a protective role in infectious diseases by the detoxification of microbial toxins such as diphtheria or tetanus toxin. The MPO system also can induce platelet [373, 374] and mast cell secretion [375–377] and can activate certain proteases in vitro [12]. Lanza et al. [378–381] have reported a high incidence of malignancy in patients with complete MPO deficiency and have raised the possibility that MPO-dependent tumoricidal activity may be required to prevent the progression of some tumors. Kutter et al. [259], however, could not detect an increase in malignancies in their group of MPO-deficient patients. Persons who inherit two copies of an allele with a single-base substitution in the promoter region of the MPO gene (which markedly reduces expression of this gene) have been reported to have a decreased risk of lung cancer [382]. To my knowledge, a change in susceptibility to tumors has not been detected in MPO-deficient mice.

My initial goal was to find a biologic role for peroxidases in the mode of action of the thyroid hormones and estrogens. Although thyroxine can substitute for the halide in the MPO-H_2O_2-halide antimicrobial system [69], throxine is deiodinated under these conditions [204, 205], raising the possibility that the released iodide may be the required microbicidal component. The thyroid hormones have, however, been reported to stimulate the generation of chlorinating oxidants by the MPO-H_2O_2-chloride system [383, 384]. Impaired thyroid hormone or estrogen response has not been noted in patients with MPO deficiency, suggesting that the phenolic hormones do not require MPO for their action. Thyroxine is synthesized first by the iodination of tyrosine residues in thyroglobulin and then by the coupling of two diiodotyrosine residues to form thyroxine. The iodination and coupling reactions are catalyzed by a thyroid peroxidase. MPO is also capable of this synthesis [385, 386]; however, there is no evidence that it does so in vivo. On the contrary, stimulated phagocytes can inactivate thyroxine through a MPO-dependent deiodination reaction [204, 205]. The MPO system also inactivates estrogens [387], and the estrogens are covalently bound to cellular constituents when incubated with phagocytes during phagocytosis [388]. These findings raise the possibility that inactivation of thyroxine and estrogens by the MPO system may occur at sites of inflammation. In summary, a physiologic role for MPO, distinct from its contribution to the phagocyte antimicrobial armamentarium, remains to be established.

TISSUE INJURY

MPO can be released to the outside of the cell, as can H_2O_2, raising the potential for damage to an extracellular target. There are two types of extracellular targets: those to which the PMN is adherent and those to which it is not. When the PMN is adherent to a target too large to be ingested, as for example, to a multicellular organism or a cell membrane coated with antibody and/or complement, the PMN flattens on the surface of the target and is stimulated with the release of MPO into a

walled-off pocket of space between the cell and the target to which it is adherent. H_2O_2 formed by the stimulated PMN is released into the pocket, and toxicity is induced in a manner similar to that occurring in an intracellular phagosome containing an ingested microorganism.

Toxicity to a target to which the PMN is not adherent can occur when MPO is released into the extracellular fluid by leakage during phagocytosis, by cell lysis, or when the PMN is exposed to a variety of soluble stimuli. A major deterrent to the production of toxicity at a distance by ROS produced by phagocytes is the presence of scavengers in the intervening fluid. Thus, proteins such as serum albumin, as well as a number of low molecular weight-reducing agents, react rapidly with the highly reactive products of the MPO system and prevent them from reaching a sensitive target of biological importance. We are thus protected from indiscriminant damage by phagocyte-produced oxidants. By definition, the more reactive the product of the MPO system, the more likely it will be scavenged. Under conditions in which HOCl or chlorine is readily scavenged, less-reactive PMN products, such as certain chloramines or H_2O_2, may penetrate the extracellular fluid to be toxic at a distance. MPO and EPO are strongly basic proteins and thus bind avidly to negatively charged surfaces such as cell membranes. H_2O_2, reaching that site from a distance, can react with the peroxidase located there to induce damage. MPO, when bound to albumin, may be transported across the endothelium by reaction with albumin-binding proteins located in caveolae [389].

Can the MPO system damage normal tissue and thus contribute to disease? There is considerable evidence to suggest that it has this potential [12, 258].

Carcinogenesis

A number of studies have implicated MPO in the development of malignancies. A polymorphic site is located 463 bp upstream of the MPO gene (−463 G/A) in the Alu hormone-responsive element of the promoter region. The G allele acts as a strong SP1 transcription factor-binding site, which reacts with SP1 to increase MPO expression [390]. This allele is enhanced in patients with acute myeloid leukemia in association with high levels of MPO mRNA and expression [391]. The G-to-A nucleotide base shift, which is associated with decreased SP1 binding and thus lower MPO gene expression, has been associated with an overall decreased risk for lung cancer by some investigators [382, 392–401] (for commentary, see ref. [402]) but not by others [403–405], and with a decreased risk of larynx cancer [392], bladder cancer [406], and hepatoblastoma [407]. Although G/A polymorphism alters transcription rates, there are few studies indicating corresponding changes in MPO protein or enzyme activity. The MPO system, in cell-free form or in intact, stimulated neutrophils, can catalyze the conversion of certain procarcinogens to their carcinogenic form [408–411] and in this way, may contribute to the development of malignancies. Further, the formation of 5-chlorouracil and 5-bromouracil by the MPO (or EPO) system and their incorporation into nuclear DNA can be mutagenic [412, 413]. The MPO −463 AA/AG genotypes are associated with reduced benzo[a]pyrene diol epoxy DNA adduct formation in skin of tar-treated patients and thus decreased carcinogenesis [414].

Renal injury

MPO has been implicated in the pathogenesis of renal disease (for review, see ref. [415]). There is considerable evidence that neutrophils mediate glomerular injury in certain experimental models of nephritis [416]. This is best documented in antiglomerular basement membrane nephritis in which antibody to glomerular basement membrane forms an antigen-antibody complex on the basement membrane, which activates complement to form chemotactic factors, which attract neutrophils to the region. Glomerular injury is evidenced by proteinuria, and the involvement of neutrophils in the damage is suggested by the prevention of injury by depletion of neutrophils with antineutrophil serum or cytotoxic agents and the reinstitution of injury by the intravenous (i.v.) infusion of neutrophils to a leukocyte-depleted animal. The mechanism by which neutrophils induce glomerular injury may include the release of lysosomal hydrolases, cationic peptides, and ROS, particularly H_2O_2, operating in the absence or presence of MPO. What is the evidence for the involvement of the MPO system?

MPO, when infused into the renal artery of the rat, binds to the glomerular basement membrane, as indicated by light and electron microscopic examination of sections stained for peroxidase by the diaminobenzidine method [417]. MPO was detected 1 min following perfusion throughout the basement membrane, with concentration in the subepithelial space along the base of the epithelial cell-foot processes. MPO is a highly cationic protein and may bind to the glomerular basement membrane through ionic bonds with negatively charged groups on sialoglycoproteins and heparin sulfate proteoglycans. When the infusion of MPO into the renal artery is followed by an infusion of H_2O_2, renal damage was indicated by an increased 24-h urinary protein excretion, which was not observed following the infusion of MPO or H_2O_2 alone. Morphologically, light microscopy at 4 h revealed a reduction in nuclear staining of resident glomerular cells, cell swelling, and a wrinking of the glomerular basement membrane [417, 418]. At 24 h, these changes were more prominent with the occlusion of many capillary loops with a weakly eosinophilic granular material. Electron microscopy at 4 h indicated the presence of endothelial cell swelling with occasional denudation. Platelets were frequently present in the capillary lumen, whereas infiltrating leukocytes were not common. Focal epithelial cell foot process effacement was present, but no discontinuities in the glomerular basement membrane were evident.

These studies suggest that MPO bound to the glomerular basement membrane can react with the infused H_2O_2 in a chloride-containing medium to induce glomerular injury. This injury was associated with in vivo iodination of glomeruli when radioiodide was added to the last perfusate, and electron microscopic autoradiography revealed the presence of silver grains along the glomerular basement membrane [417]. These findings indicate that MPO can react with H_2O_2 to oxidize iodide to a form that binds in covalent linkage to adjacent glomerular basement membrane components.

In the studies described above, glomerular injury was induced by infusion of MPO and H_2O_2 into the renal artery of normal rats. Does this system contribute to renal damage in a neutrophil-dependent model of glomerulonephritis? The model

used involved the infusion of concanavalin A (Con A) into the renal artery, which bound to the glomerular basement membrane, serving as a planted antigen [419]. When this infusion was followed by an infusion of rabbit anticon A antibody, an antigen-antibody complex formed on the basement membrane, which attracted neutrophils to the region. Proteinuria occurred, which was not seen when the anticon A was replaced by normal rabbit immunoglobulin G (IgG) and was significantly reduced but not abolished when the animals were neutrophil-depleted with antineutrophil antibody. Iodination of the glomeruli was evident when radioiodide was administered i.v. after the Con A-anticon A infusion and was not seen in control rats given Con A and normal rabbit IgG or when the rats were neutrophil-depleted with antineutrophil antibody. These findings are compatible with a contribution by the MPO-H_2O_2-halide system to the renal damage in experimental neutrophil-dependent glomerulonephritis.

A role for MPO in the glomerular injury observed in rapidly progressive glomerulonephritis in humans was suggested by the presence of MPO in the glomeruli of most patients with this condition in association with an increase in the titer of MPO-specific, antineutrophil cytoplasmic antibody (MPO-ANCA) [420]. Further evidence of the involvement of MPO-ANCA in a mouse model of vasculitis and glomerulonephritis using MPO knockout mice immunized with mouse MPO has been presented [421] (for commentary, see ref. [422]). The activation of tumor necrosis factor α-primed neutrophils by MPO-ANCA has an absolute requirement for cellular MPO [423]. The GG genotype of the −463 G/A MPO promoter polymorphism was found to be associated with an increased risk of MPO-ANCA-associated vasculitis in females but not males, and the A allele was associated with an increased incidence of relapses and an earlier age of diagnosis [424]. The G-to-A conversion at position −463 is also associated with a lower prevalence of cardiovascular complications in end-stage renal disease [425]. An interaction between MPO-ANCA and glomerulus-associated MPO may produce changes similar to those observed in the Con A-anticon A model described above. Similarly, recent studies have proposed a role for MPO and MPO-ANCA in the injury observed in a mouse model of coronary artery vasculitis [426, 427]. MPO and HOCl-modified proteins have been demonstrated in diseased human renal tissue, often in association [428], and 3- chlorotyrosine, a specific marker of the MPO-H_2O_2-halide system, was detected in the plasma proteins of chronic hemodialysis patients [429].

Lung injury

MPO has been implicated in the induction of lung injury. In vivo studies, in which the intratracheal infusion of glucose oxidase (as a source of H_2O_2) and peroxidase (LPO, MPO) into rats produced severe acute lung injury, which progressed to interstitial fibrosis under conditions in which infusion of glucose oxidase or peroxidase alone produced little damage [430]. The alveolar epithelial lining fluid of most patients with idiopathic pulmonary fibrosis contains increased levels of MPO and inflammatory cells, which spontaneously released increased amounts of H_2O_2. This is compatible with the MPO system playing a role in the epithelial cell injury found in this disorder in humans [431]. Further, protein carbonyl [432] and

3-chlorotyrosine [225] levels, which are markers of protein damage by the MPO system, were elevated in tracheal asperates of premature infants. 3-Chlorotyrosine levels were high in infants with low birth rates or who developed chronic lung disease and correlated strongly with MPO levels [225].

Atherosclerosis

Of particular recent interest is the proposed involvement of MPO in the development of atherosclerosis [433–442]. MPO binds to low-density lipoproteins (LDL) [443], and under appropriate conditions, the LDL modified by MPO-catalyzed reactions is taken up by macrophages in increased amounts, converting them into the lipid-laden foam cells characteristic of the early atherosclerotic lesion [164, 444, 445]. The oxidation of chloride [226, 446–448], bromide [449], thiocyanate [450], nitrite [451, 452], or tyrosine [179, 453] by MPO and H_2O_2 can form intermediates capable of the oxidative modification of LDL (or high-density lipoproteins [454, 455]), with the chloride-dependent formation of HOCl being likely the most important. The active tyrosine-derived oxidant can be the tyrosyl radical [179, 456, 457] or the product of tyrosine oxidative deamination, p-hydroxyphenylacetaldehyde [453, 458]. The detection of MPO [459–461] and specific markers of MPO-catalyzed halogenation [226, 460, 462–466] in human atherosclerotic lesions, often colocalized [460], is compatible with the involvement of MPO in the development of atherosclerosis in vivo. Nitration of LDL [467] in human atherosclerotic lesions [467, 468] also occurs, and although nitration reactions can be catalyzed by MPO in the presence of nitrite [469], other mechanisms for the generation of nitrating species exist. Serum/plasma protein-associated nitrotyrosine [470] and plasma LDL 3-chlorotyrosine [455] levels are elevated in patients with coronary artery disease, raising the possibility that these changes may serve as a marker of this condition. Nitrotyrosine elevation was modulated by statin therapy [470]. Chlorination of LDL or its associated protein, apolipoprotein A-1 (APOA-1), impairs the removal of cholesterol from cultured cells by adenosine 5′-triphosphate-binding cassette transporter A1(ABCA-1)-dependent cholesterol transport [455], suggesting a mechanism for the promotion of atherogenesis by MPO-catalyzed reactions.

It should be emphasized that the presence of MPO in or adjacent to pathologic lesions does not necessarily implicate it in the pathologic process. The detection of chemical markers of MPO-catalyzed reactions in the lesion suggests that at some point, the MPO was active. But did that activity contribute to the disease? Is there more definitive evidence for MPO involvement in atherosclerosis in vivo? Recent evidence using gp91^{phox} and MPO knockout mice has suggested that MPO-dependent reactions are not required for the initiation of atherosclerosis in mice. Gp91^{phox} is the component of the NADPH oxidase of phagocytes most commonly defective in CGD, and mice deficient in this protein have phagocytes that lack a respiratory burst and thus, H_2O_2 production. These animals develop atherosclerosis normally on a high-fat diet. APO-E-deficient mice develop atherosclerosis on a normal diet. Atherosclerotic plaque development was similar in APO-E-deficient mice to that seen in CGD mice crossed with APO-E-deficient mice [471]. These studies do not support the

involvement of the products of the respiratory burst of phagocytes in the development of atherosclerosis in mice. Other sources of H_2O_2 for use by MPO, e.g., vascular NAD(P)H oxidase, may be available. Neutrophils from MPO-knockout mice, when stimulated by PMA, can oxidize LDL, although they are not as effective as neutrophils from wild-type mice in this regard [472]. This suggests that MPO is not essential for the oxidation of LDL by neutrophils in this species. The oxidation by cells from wild-type and MPO-deficient mice was completely inhibited by SOD, suggesting that it is a result of O_2^- rather than H_2O_2 generation by phagocytes. Brennan et al. [473] (see ref. [474] for commentary) used a model of atherosclerosis in which mice deficient in the LDL receptor were lethally irradiated, their bone marrow repopulated with wild-type or MPO-deficient cells, and the development of atherosclerosis on feeding the animals a high-fat, high-cholesterol diet monitored. No decrease in atherosclerosis was detected in the MPO-deficient mice; indeed the lesions were ~50% larger than in the control animals. Further, in contrast to human atherosclerotic lesions, MPO and the marker of MPO reactivity, 3-chlorotyrosine, were not detected in greater amounts in the lesions of wild-type as compared with MPO-deficient mice.

These studies with MPO-deficient mice indicate that MPO is not required for the development of lesions in an experimental mouse model of atherosclerosis. However, this does not exclude the possibility of MPO involvement in human atherogenesis. Neutrophil MPO levels were significantly higher in patients with coronary heart disease than in normal controls [475]. Variations in the MPO content of peripheral blood monocytes in coronary heart disease were not determined in this study. Further, increased plasma [476] or serum [477] MPO levels in patients with coronary artery disease may serve to identify patients with an increased risk for further cardiovascular events (see ref. [478] for comment). However, Hiramatsu et al. [479] have reported that monocytes from a MPO-deficient patient induced lipid peroxidation of LDL to the same degree as that induced by normal cells. A long-term epidemiological study of the susceptibility of MPO-deficient patients to atherosclerosis would be helpful but difficult to do. However, autopsies of individual MPO-deficient patients may answer the following question: Do MPO-deficient patients in general have cleaner blood vessels than age-controlled, normal individuals?

The presence of MPO in human atherosclerotic lesions raises an additional issue. What is the source of the MPO, and when did it arrive? Is the MPO in atherosclerotic lesions a relic of enzyme deposited there by earlier recruitment of monocytes, is it evidence of the more recent presence of neutrophils or monocytes in the lesion, or is it an indication that mature macrophages can be induced to begin again to synthesize MPO under the conditions prevailing in the atherosclerotic lesion? Progression of atherosclerotic plaques is associated with robust migration of monocytes/macrophages into the lesion and their reduced clearance, leading to the build-up of these cells [480]. There has been increasing interest in the possibility that atherosclerosis is in part an infectious process as a result of the detection of evidence for *Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus, or herpes simplex virus 2 infection in atherosclerotic lesions [481, 482]. Is MPO brought to the lesion by phagocytes responding to an infectious agent? It is the

prevailing view that MPO is synthesized and packaged in the promyelocyte stage of neutrophil development and in the promonocyte in cells of macrophage lineage and that synthesis ceases with further maturation of the cells. Circulating monocytes contain MPO, but as the monocytes mature into macrophages in tissues, this peroxidase is lost. MPO is a strongly cationic protein, which binds to negatively charged particles and cell surfaces and may be taken up by macrophages by pinocytosis or phagocytosis. Thus, the presence of MPO in macrophages doesn't necessarily indicate its synthesis there. However, the reinstitution of MPO synthesis in mature macrophages in atherosclerotic lesions, possibly under the control of granulocyte macrophage-colony stimulating factor [461], is an intriguing possibility. Other evidence for the reinstitution of MPO synthesis in mature cells of macrophage lineage has come from studies of brain tissue (see below).

Multiple sclerosis

MPO has been detected in microglia/macrophages in and around the lesions in multiple sclerosis, as measured by the immunocytochemical detection of MPO protein and by the detection of MPO mRNA sequences in brain microglia in this condition, which was not detected in normal brain tissue [483]. Further, the G allele of the −463G/A MPO polymorphism, which is associated with increased MPO production, is overrepresented in early-onset multiple sclerosis in females [483]. Peripheral blood leukocyte MPO levels have been reported to be decreased in patients with multiple sclerosis [484]. Experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis, developed in 90% of MPO-knockout mice as compared with 33% of wild-type mice, suggesting that MPO is protective to the animal [485].

Alzheimer's disease

MPO protein also has been detected by immunohistochemical techniques in microglia adjacent to senile plaques in the cerebral cortex of patients with Alzheimer's disease [486]. The MPO colocalized with amyloid β, and its gene expression could be induced by amyloid β in cultured rodent microglial cells. APO-E, which colocalizes with amyloid β in senile plaques of patients with Alzheimer's disease, is highly susceptible to oxidation by the MPO system [487, 488]. MPO has also been detected in increased amounts in certain Alzheimer brain neurons [489]. Studies relating MPO −463G/A polymorphism to the risk of Alzheimer's disease have been contradictory. Reynolds et al. [486] reported that the G allele was overrepresented in females, whereas the A allele was overrepresented in males with Alzheimer's disease. In a subsequent study with a Finnish cohort, the MPO A and the APO-E ε4 alleles were found to synergize to significantly increase the risk of Alzheimer's disease in men but not in women [490]. Crawford et al. [491] detected an association between the MPO G/G genotype and Alzheimer's disease in a Caucasian but not in a Hispanic population, with no gender association or relationship to the APO-E gene. Leininger-Muller et al. [492] concluded that the −463G/A MPO polymorphism was statistically associated with Alzheimer's disease in females but not males, although the low *P* value led to their conclusion that there was

no causal relationship between the MPO genotype and Alzheimer's disease. In this regard, Combarros et al. [493] and Styczynska et al. [494] could not detect an association between the MPO −463G/A genotype and Alzheimer's disease.

Brain infarction

The A allele of the G −463A polymorphism is associated with a poorer short-term, functional outcome of brain infarcts, and carriers of the A allele of the G −129A polymorphism had significantly larger infarcts. There was no significant relationship between these polymorphisms and the risk of developing a brain infarct [495].

Parkinson's disease

Parkinson's disease is associated with the loss of dopaminergic neurons from the substantia nigra pars compacta area of the brain. NADPH oxidase levels (as measured by the up-regulation of gp91^{phox} and p67^{phox}) are increased in this area of the brain in Parkinson's disease and in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a model for Parkinson's disease, and mice deficient in NADPH oxidase have less neuronal loss following MPTP administration than do wild-type mice [496]. These findings are compatible with a role for NADPH oxidase-derived ROS in the development of the lesions in Parkinson's disease. The tyrosyl radical generated by MPO and reactive nitrogen species have also been implicated in the brain lesions in mice treated with MPTP [497]. The presence of MPO in the lesions in certain neurodegenerative diseases and its absence from equivalent normal nervous tissue raise the question of the possible role of MPO in the pathogenesis of these diseases. Studies along the lines of those discussed above in relation to atherosclerosis are needed to resolve this issue.

CONCLUSIONS

The evidence, I believe, strongly suggests that the primary function of MPO is to kill microorganisms in neutrophils and monocytes, and to do this, it forms highly reactive halide (particularly chloride)-derived oxidants in the confines of the phagosome. Chlorine disinfection is of course not new. In 1827, Thomas Alcock in an "Essay on the use of Chlorets of Oxide of Sodium and Lime" recommended the use of what is now called sodium and calcium hypochlorite as disinfectants. Its most famous early proponent was Ignaz Philipp Semmelweis, who noted that death from puerperal fever was considerably higher among women giving birth in a ward in which medical students and doctors attended births as compared with a second ward staffed by midwives. He concluded that the medical students and doctors, who also participated in autopsies, carried "cadaveric material" on their hands to the bedside. In May 1847, he posted a sign on the clinic door, which indicated that any doctor or student coming from the postmortem room must wash his hands thoroughly in a basin of chlorinated water before entering the maternity ward. Shortly thereafter, the death rate from puerperal fever fell to that observed in the midwife-run ward. In 1881, Robert Koch

demonstrated the microbicidal effect of hypochlorites on pure cultures of bacteria. Chlorine-based disinfectants subsequently became widely used in the treatment of open and infected wounds until the introduction of the less-toxic antibiotics. Chlorine derivatives, however, are still widely used in water and sewage treatment and in the disinfection of laundry, instruments, and swimming pools. By 1990, 98.6% of disinfective practices in the United States used chlorine-based disinfectants. The body, thus, has found a way to generate chlorine-based disinfectants in the confines of the phagosome and in this way, to kill ingested microbes and control infection.

In conclusion, is MPO a friend or foe? The evidence suggests that MPO is a friend that can, to some degree, be replaced and a foe that has the potential to produce damage and contribute to disease.

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