Antimicrobial Actions of Reactive Oxygen Species

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Everything should be as simple as it can be, but not simpler.—Attributed to Albert Einstein (1)

ABSTRACT **Reactive oxygen species (ROS) are produced by host phagocytes and exert antimicrobial actions against a broad range of pathogens. The observable antimicrobial actions of ROS are highly dependent on experimental conditions. This perspective reviews recent controversies regarding ROS in *Salmonella*-phagocyte interactions and attempts to reconcile conflicting observations from different laboratories.**

IMPORTANCE OF ROS IN HOST DEFENSE

In 1932, the uptake of *Micrococcus* by canine leukocytes was found to result in a burst of oxygen consumption (2). This phenomenon was later rediscovered (3, 4) and linked to the formation of hydrogen peroxide (5), suggesting a possible role in microbial killing by phagocytes (6, 7), as hydrogen peroxide was known to exhibit antimicrobial activity. The NADPH-dependent NOX2 phagocyte oxidase complex responsible for the generation of reactive oxygen species (ROS) is now well characterized (8). The enhanced susceptibility to infection of individuals with inherited deficiencies of specific NOX2 components, a condition known as chronic granulomatous disease (CGD), has unequivocally demonstrated the importance of ROS production in host defense (9). Important opportunistic pathogens in CGD include *Salmonella enterica*, *Staphylococcus aureus*, *Serratia marcescens*, and *Aspergillus* spp. (10, 11). Mouse models with targeted disruption of NOX2 exhibit impaired host resistance comparable to that of humans with CGD (12). However, the mechanisms by which phagocyte-derived ROS kill microbes and by which pathogens resist ROS-dependent antimicrobial actions remain controversial. This perspective will provide a brief overview of ROS-dependent antimicrobial actions, critically assess selected recent publications concerning ROS and *Salmonella*, and attempt to reconcile conflicting observations.

INTERCONVERSION OF ROS

The product of NOX2 is superoxide radical ($O_2\cdot^-$), which can undergo spontaneous or enzymatic dismutation to hydrogen peroxide (H_2O_2). The cytotoxic potential of H_2O_2 results to a large extent from its ability to oxidize ferrous iron (II), in what is referred to as Fenton chemistry (13), to form highly reactive hydroxyl radicals (OH·). $O_2\cdot^-$ and H_2O_2 exhibit synergistic cytotoxicities, suggested by Haber and Weiss to result from the reduction of ferric iron (III) by $O_2\cdot^-$ (14), but studies of *Escherichia coli* have demonstrated an alternative mechanism, the mobilization of iron from iron-sulfur clusters by $O_2\cdot^-$ (15, 16), thereby increasing the availability of free iron to participate in Fenton-mediated damage. H_2O_2 itself can also mobilize iron from iron-sulfur clusters (17). In neutrophils, myeloperoxidase (MPO) catalyzes the formation of hypochlorous acid (HOCl) from H_2O_2 and chloride ion. Although HOCl dramatically enhances the microbicidal activity of H_2O_2, MPO appears to be nonessential for host defense, as MPO-deficient individuals do not have a high frequency of infections, with the exception of an increased susceptibility to *Candida* spp. (18, 19).

PHAGOSOMAL ROS CONCENTRATIONS

During the respiratory burst, professional phagocytes can convert 3 to 4 nmol of oxygen to ROS per 10^6 cells each minute (20). However, much of the generated H_2O_2 is released from the cell (21), as H_2O_2 diffuses freely across membranes. Attempts to model steady-state ROS concentrations within the neutrophil phagosome have estimated concentrations of $O_2\cdot^-$ to be 25 μM, with H_2O_2 concentrations in the low micromolar range, but levels rise to $>$100 μM $O_2\cdot^-$ and 30 μM H_2O_2 if MPO is absent (22). These values are somewhat lower than the extracellular concentrations of H_2O_2 required for observable antimicrobial actions *in vitro*, although intracellular concentrations as low as 1 μM are toxic for *E. coli* (17). The higher H_2O_2 concentrations required in order to demonstrate antimicrobial actions in experimental systems are largely an artifact of the rapid degradation of H_2O_2 by concentrated cell suspensions, which does not occur when a single bacterium is situated within a phagosome. Moreover, it is likely that bacteria located close to the source of ROS generation experience considerable oxidative stress out of proportion to that caused by steady-state H_2O_2 concentrations. Recent studies of *Moraxella catarrhalis* indicate that high levels of flux through a truncated denitrification pathway result in nitric oxide (NO·)-dependent protein modification and substantial cytotoxicity even though steady-state NO· levels remain so low that they are undetectable with a sensitive electrode (23). By analogy, exposure to a constant ROS flux generated in close proximity should not be considered equivalent to treatment with a bolus administration of H_2O_2 in a test tube.

INTERACTION OF ROS WITH OTHER HOST DEFENSES

The challenge of analyzing ROS-dependent antimicrobial actions in tissue culture or animal models is increased by potential ROS interactions with other mediators. The reaction of $O_2\cdot^-$ and NO· can generate the cytotoxic peroxynitrite ($OONO^-$) anion (24), and NO· can also potentiate the antimicrobial actions of H_2O_2 (25, 26). ROS appear to interact synergistically with certain neutrophil proteases (27), although a paper providing some of the evidence

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underpinning this claim has recently been retracted due to an inability to reproduce the original findings (28). The involvement of ROS and NOX2 in signal transduction, phagocyte activation, and the regulation of autophagy must also be considered (29, 30).

MICROBIAL ROS TARGETS

One of the most important cellular targets of ROS is DNA (31). Base oxidation, particularly guanine, may be mutagenic (17), and blocking lesions or strand breaks may be lethal unless they are repaired (32, 33). As previously mentioned, iron-sulfur cluster-containing proteins are also vulnerable to ROS damage (34) and may substantially restrict metabolic pathways even if the damage is not microbicidal. The presence of SOD in the periplasm has suggested the existence of extracytoplasmic $O_2\cdot^-$ targets (17), although these are as yet unidentified.

MICROBIAL ROS DEFENSES

A number of enzymes can transform ROS into less toxic products. Among the most important of these are catalases, peroxiredoxins, and superoxide dismutases (SODs). *Salmonella enterica* carries three catalases (KatE, KatG, KatN), three peroxiredoxins (AhpC, TsaA, Tpx), and four SODs (SodA, SodB, SodCI, SodCII) (35–41). Catalases and peroxiredoxins are scavengers of H_2O_2, and superoxide dismutases are scavengers of $O_2\cdot^-$. Although SODs create 0.5 mol of H_2O_2 per mol of $O_2\cdot^-$, SODs may actually reduce overall H_2O_2 levels by preventing the reaction of $O_2\cdot^-$ with other reductants (42); SOD may also prevent cytotoxic interactions of $O_2\cdot^-$ and NO· (43). The redundancy of antioxidant enzymes is more apparent than real. Several of these enzymes differ with regard to cofactors, regulation, stability, or cellular compartmentalization, and some mutants lacking individual antioxidant enzymes exhibit enhanced ROS susceptibility. As intracellular free iron is limiting for Fenton chemistry, mechanisms to sequester iron or control its uptake are important determinants of ROS susceptibility (44). The importance of DNA as a microbicidal target is underscored by the existence of a protein called Dps, which simultaneously sequesters iron to prevent its interaction with H_2O_2 and physically protects DNA (44–46). Dps-deficient mutant bacteria are highly susceptible to killing by H_2O_2 and attenuated for virulence in macrophages and mice (44, 45). In addition, a plethora of repair enzymes can reverse oxidative DNA lesions (17).

A unique mechanism of ROS evasion has been described in *Salmonella*. The type III secretory system (T3SS) encoded by *Salmonella* pathogenicity island 2 (SPI2) is expressed within the phagosome, translocates effector proteins into the host cell cytosol, and interferes with the localization of a functional NOX2 complex in *Salmonella*-containing vacuoles (47–49). Moreover, the SPI2-encoded T3SS reduces the colocalization of intracellular *Salmonella* and H_2O_2, detected as cerium perhydroxide precipitate by transmission electron microscopy (49), and enhances *Salmonella* survival in activated primary peritoneal macrophages from C57BL/6 mice but not in their NOX2-deficient counterparts (49) or in macrophages deficient in the tumor necrosis factor p55 or SLAMF1 receptors required for the recruitment of active NOX2 to the phagosome (48, 50). The colocalization of intraphagosomal *Salmonella* and nitrotyrosine, indicative of peroxynitrite formation from $O_2\cdot^-$ and NO·, has also been reported to be abrogated by SPI2 (51). Casbon et al. observed NOX2 within Rab11-positive recycling endosomes (52), and it has been suggested by those authors that Rab11 may participate in SPI2-dependent depletion of NOX2 from the *Salmonella*-containing vacuole, as described for CD44 (53).

VARIABLES AFFECTING ROS SUSCEPTIBILITY

A number of experimental variables have a significant impact on *in vitro* ROS-dependent antimicrobial actions; these include ROS concentration, bacterial cell density, growth phase, metabolic activity, and the mode of ROS generation. H_2O_2 exhibits bacteriostatic actions at low concentrations and bactericidal actions at higher concentrations (54). DNA damage plays an important role in *E. coli* at micromolar concentrations, with additional targets involved in killing by higher H_2O_2 concentrations (55). ROS concentration must be evaluated in concert with cell density. At high cell densities and high H_2O_2 concentrations, catalase is of critical importance in *Salmonella* resistance to killing, but at low cell densities and low H_2O_2 concentrations, DNA repair is essential, whereas catalase appears to be dispensable (36). The expression of antioxidant defense mechanisms, such as Dps, is growth phase dependent, such that logarithmic and stationary-phase bacteria exhibit very different levels of ROS susceptibility (56). Reduced levels of respiration enhance susceptibility to H_2O_2-mediated DNA damage during logarithmic phase by increasing NADH accumulation, resulting in the reduction of flavins and free iron (26, 31), while the inhibition of respiration is protective against H_2O_2 in stationary phase (57). Exogenous oxidative stress can be created by the simple addition of H_2O_2, chemically generated by the autooxidation of pyrogallol, or enzymatically generated by the xanthine oxidase/hypoxanthine system, but none of these methods can be said to precisely reproduce the stress induced by the sustained production of $O_2\cdot^-$ and the resulting ROS flux generated by NOX2 within an intracellular compartment.

Experimental variables also have a substantial effect on the antimicrobial actions of ROS in cultured cells and animal models, and these include timing, cell type, method of cellular activation, mode of cell entry, mouse strain, route of administration, and inoculum size. Timing is among the most important variables, as the respiratory burst is activated early and subsequently supplanted by other antimicrobial effector systems (58, 59). During *in vivo* infection, the nature of inflammatory cell populations evolves over time (60), and even cells of related lineages exhibit different levels of ROS production depending on their tissue of origin, with peritoneal macrophages producing greater quantities of ROS in response to standard stimuli than splenic or bone marrow macrophages (61, 62). Various agents may be used to prime or stimulate ROS release. Phorbol myristate acetate (PMA) triggers the phosphorylation and translocation of the p47phox component of NOX2 from the cytosol to the plasma membrane and is commonly employed to induce phagocyte ROS production. However, the plasma membrane localization of NOX2 in response to PMA differs from the phagosomal NOX2 localization observed after phagocytosis (49, 63), with likely functional consequences. Opsonization of bacteria prior to phagocytosis augments the respiratory burst, with both antibody and complement playing a role (64). Phagocytes from different strains of inbred mice exhibit various levels of ROS production upon stimulation, and one determinant is the presence of a functional Nramp1 (Slc11a1) locus, which influences innate susceptibility to intracellular pathogens, including *Salmonella*, *Mycobacterium*, and *Leishmania* spp. (65).

Finally, the route of administration and inoculum size determine the host cell populations initially encountered by microbes (60).

HOW DO HOST-DERIVED ROS DAMAGE BACTERIA?

In this light, recent new claims regarding ROS and *Salmonella* can be critically examined. Previous studies suggested that only periplasmic SodC among the antioxidant enzymes of *Salmonella* plays a specific role in virulence (36, 37, 43). Hébrard et al. revisited the role of antioxidant enzymes in *Salmonella* virulence and reported that a mutant strain lacking all three catalases and two of the putative peroxiredoxins ($HpxF^-$) is attenuated for growth in macrophages and virulence in mice (38). Those authors concluded that cytoplasmic antioxidant enzymes contribute to *Salmonella* virulence. It should be noted that Hébrard et al. actually confirmed earlier reports that catalases and the AhpC peroxiredoxin are individually dispensable for *Salmonella* virulence (36, 66). Reduced virulence was observed only in an $HpxF^-$ mutant lacking a combination of five antioxidant enzymes. However, this strain was also severely defective for aerobic growth in minimal medium and in macrophages treated with a NOX2 inhibitor, so it is difficult to attribute the virulence defect of an $HpxF^-$ mutant to a specific role of the cytoplasmic enzymes in detoxifying phagocyte-derived ROS.

A study by Craig and Slauch took a different experimental approach, employing mixed murine infections with various *Salmonella* mutant strains to determine the contribution of specific genetic loci to virulence (67). The studies were performed with BALB/c mice that lack a functional Nramp1 locus and are exquisitely susceptible to *Salmonella* (intraperitoneal 50% lethal dose [LD_{50}] $<$ 10 CFU) (68). Craig and Slauch did not investigate catalases or peroxiredoxins but rather evaluated the contribution of the SodCI periplasmic superoxide dismutase in *Salmonella* strain backgrounds deficient in cytoplasmic superoxide dismutase activity or DNA repair. They concluded that since SodCI does not exhibit synthetic effects on the *in vivo* competitive index with cytoplasmic SOD or DNA repair, the antimicrobial effects of host-derived ROS are the result primarily of damage to an extracytoplasmic target, rather than to DNA. Of note, the authors reported a 5- to 8-fold attenuating effect of a *sodCI* mutation in wild-type *Salmonella* but a 32-fold effect in a *recA* mutant deficient in recombinational DNA repair. This might be interpreted to indicate that periplasmic SOD protects *Salmonella* from DNA damage repaired by RecA and is consistent with other studies indicating that DNA is a major target of ROS (55). Craig and Slauch rejected this interpretation because SodCI had only a 5-fold effect in a *ruvAB* mutant that lacks the RuvAB resolvase, which is also involved in recombination. However, mutations in *recA* and *ruvAB* are not equivalent. For instance, RecA is essential for repair of double-strand breaks, whereas RuvAB can be functionally replaced by RecG (69). Additional observations suggest that DNA is an important target of phagocyte-derived ROS. Periplasmic SodC deficiency potentiates H_2O_2 killing of mutant *Salmonella* lacking the DNA-protective protein Dps (70). DNA repair-deficient *Salmonella* strains are sensitive to killing by ROS-producing macrophages, and this is dependent on ROS production (71, 72). Furthermore, recombinational DNA repair is essential for the ability of *Salmonella* to withstand ROS at low cell densities, resist killing by ROS-producing macrophages, and cause lethal systemic infection in NOX2-producing mice (36, 73). Mutant *Salmonella* lacking the Fpg enzyme responsible for removal of oxidized guanine and formamidopyrimidine residues exhibits an enhanced mutation rate during murine infection despite the inhibition of nitric oxide synthesis (74), suggesting that ROS production by the host during infection is sufficient to damage bacterial DNA. Lastly, it should be noted that *fur* mutant and ferritin-deficient *Salmonella* strains with elevated intracellular free-iron levels exhibit attenuated virulence in mice, which suggests that cytoplasmic Fenton chemistry is an important determinant of susceptibility to host defenses (44).

DOES THE SPI2 T3SS PROTECT *SALMONELLA* FROM NOX2?

Most recently, Aussel et al. utilized a green fluorescent protein (GFP) transcriptional fusion to the *Salmonella ahpC* peroxiredoxin gene as a biosensor of oxidative stress experienced by *Salmonella* during infection (75). Those authors observed that *ahpC* expression was dependent on host ROS production and the presence of catalases and peroxiredoxins but not the expression of the SPI2 T3SS. That study, supported by an accompanying commentary by Slauch (76), concluded that the contribution of SPI2 to *Salmonella* pathogenesis is unrelated to an interaction with NOX2. In addition, Aussel et al. cited a recent study by Helaine et al. which indicated that SPI2 promotes bacterial replication rather than resistance to killing during infection (77).

It is uncontroversial to state that some contributions of SPI2 to *Salmonella* virulence are NOX2 independent. The expression of SPI2-related virulence phenotypes in nonphagocytic cells lacking the high-output generation of ROS has been noted previously (49, 78, 79). However, the observations of Aussel et al. do not exclude a role for SPI2 in opposing the antimicrobial actions of NOX2. One limitation of the study by Aussel et al. is the use of a stable GFP derivative (80), which might not have detected effects of the SPI2 T3SS on the temporal dynamics of oxidative stress *in vivo*. Another concern is the reliance of these investigators on *ahpC* expression as an indicator of oxidative stress. AhpC expression is elicited by low endogenous levels of H_2O_2, and given that steady-state H_2O_2 accumulation is limited, it cannot be assumed that the *ahpC-gfp* reporter is capable of sensing enhanced intraphagosomal H_2O_2 fluxes in the absence of SPI2, even though oxidative cellular damage might be increased. In addition to the aforementioned researchers (47–50), Suvarnapunya and Stein used a different type of biosensor to demonstrate that macrophages inflict increased oxidative DNA damage in *Salmonella* mutants that lack SPI2 (81). Those authors additionally observed that the timing of SPI2 expression is dependent on experimental conditions, which thereby determine the observed relative contribution of SPI2 and DNA repair to intracellular *Salmonella* survival. It is also important to note that the bacterial inoculum size used by Aussel et al. to infect mice was significantly different from the inoculum sizes used by earlier investigators. For technical reasons relating to their novel *gfp* reporter system, Aussel et al. infected mice with large bacterial inocula (ca. $10^4 \times LD_{50}$) that may have overwhelmed innate immune defenses and obscured an interaction between the SPI2 T3SS and NOX2. After administration of these large inocula, Aussel et al. observed that most *Salmonella* cells were contained within neutrophils, which contrasts with the predominant role of macrophages when smaller inocula are administered (60). This is of potential importance because neutrophils generally generate higher quantities of ROS than macrophages and because it is unknown whether SPI2 can affect NOX2 trafficking in neutrophils, which occurs via a mechanism different from that in macrophages

(52). Earlier studies to investigate the interaction between SPI2 and NOX2 used inoculum sizes approximately 1,000-fold lower than those used by Aussel et al. and Helaine et al. Finally, in comparison to the peritoneal or human monocyte-derived macrophages used by earlier investigators (47–50), bone marrow-derived macrophages, which were used by Aussel and Helaine et al., exhibit reduced anti-*Salmonella* activity (82). The failure to observe a significant effect of SPI2 on *Salmonella* killing might simply reflect the poor bactericidal activity of these cells. Aussel et al. stimulated their macrophages with PMA, which, as previously mentioned, targets NOX2 to the plasma membrane rather than the phagosome (49, 63). Numbers of bacterial CFU were not reported by Aussel et al., so it is unclear how effectively SPI2 promoted intracellular *Salmonella* survival under these conditions. Thus, differences in both methodology and interpretation may contribute to the discrepancies between the recent studies and earlier observations.

Further experimentation may help to reconcile some of the present uncertainties. For example, it would be of interest to repeat some of the relevant studies using a wider range of inoculum sizes, cell types, biosensors, *gfp* derivatives (83), and other experimental conditions to determine which of these variables is most important. However, one must also consider that the desire for simple reductionist explanations (84) may be futile when considering the complex antimicrobial actions of ROS. Experimental observations in apparent conflict might each be valid but also limited in their relevance to specific stages or types of host-pathogen interactions. As the Bob Dylan song (85) goes,

Half of the people can be part right all of the time
Some of the people can be all right part of the time
But all of the people can't be right all of the time
I think Abraham Lincoln said that.

CONCLUSIONS

ROS can attack diverse targets to exert antimicrobial activity, which helps to account for their versatility in mediating host defense against a broad range of pathogens. The observable actions of ROS and the contribution of various microbial antioxidant strategies to resist them are highly dependent on the experimental methods employed. Under certain conditions, ROS may be bacteriostatic or bactericidal for *Salmonella*, may attack extracytoplasmic or cytoplasmic targets (in particular iron-sulfur centers and DNA), and may be opposed by antioxidant enzymes or the SPI2 T3SS. The experimental conditions most relevant to natural host-pathogen interactions are presently uncertain. Nevertheless, available evidence suggests that the effects of host-derived ROS on microbial pathogens are complex. Simple explanations regarding the mechanisms and roles of ROS during infection on the basis of individual experimental models should be regarded with caution.

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