Published in final edited form as:
*Mol Microbiol*. 2011 May ; 80(3): 580–583. doi:10.1111/j.1365-2958.2011.07612.x.

# How does the oxidative burst of macrophages kill bacteria? Still an open question

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## Summary

Reactive oxygen species (ROS) are critical components of the antimicrobial repertoire of macrophages, yet the mechanisms by which ROS damage bacteria in the phagosome are unclear. The NADH-dependent phagocytic oxidase produces superoxide, which dismutes to form $H_2O_2$. The Barras and Méresse labs use a GFP fusion to an OxyR regulated gene to show that phagocyte-derived $H_2O_2$ is gaining access to the *Salmonella* cytoplasm. However, they have also shown previously that *Salmonella* has redundant systems to detoxify this $H_2O_2$. Although *Salmonella* propagate in a unique vacuole, their data suggest that ROS are not diminished in this modified phagosome. These recent results are put into the context of our overall understanding of potential oxidative bacterial damage occurring in macrophages.

Macrophages engulf and kill bacteria. Although the overall role of macrophages has been known for over 100 years, we understand surprisingly little of the actual mechanisms by which bacteria are destroyed. The cell biology of phagolysosomal formation is fairly well understood. Macrophages recognize and engulf bacteria into phagosomes, which subsequently acidify. These phagosomes mature into phagolysosomes upon vesicle-mediated delivery of various antimicrobial effectors, which include proteases, antimicrobial peptides, and lysozyme (Garin *et al.*, 2001)(Figure 1). The phagolysosome is also a nutrient-limiting environment. Reactive oxygen species and reactive nitrogen species are produced in this compartment. The multi-subunit NADPH-dependent phagocytic oxidase (Phox or NOX2) is assembled on the phagolysosome membrane and pumps electrons into the compartment to reduce oxygen to superoxide anion ($O_2^-$). The inducible nitric oxide synthase uses arginine and oxygen as substrates to produce nitric oxide (Fang, 2004).

Most bacteria are rapidly killed and degraded in the phagolysosome, making it difficult to dissect the mechanism of death. But a few bacteria have evolved to survive in macrophages. *Salmonella* use a type III secretion system to affect vesicular trafficking and maturation of the phagolysosome (Holden, 2002). It is presumed that the bacteria within this modified "*Salmonella* containing vacuole" (SCV) are subjected to a less intense antimicrobial response. However, the phagocytic arsenal still has a role in *Salmonella* pathogenesis and the bacteria must also be resistant to these antimicrobial factors. This balance between survival and killing makes *Salmonella* a powerful model to understand the mechanisms of action of the phagocytic effectors.

Reactive oxygen species (ROS) are critical weapons in the phagocyte arsenal. In theory, $O_2^-$ and nitric oxide can combine to form highly reactive peroxynitrite ($ONOO^-$). But the roles of Phox and iNOS are both temporally (Vazquez-Torres *et al.*, 2000a) and genetically (Craig and Slauch, 2009) separable during *Salmonella* infection, suggesting that $ONOO^-$ is irrelevant when combating this pathogen. Studies by Aussel et al. (2011), reported in this volume, provide important information regarding *Salmonella* resistance to the ROS

produced by Phox, and suggest that *Salmonella* relies less on blocking ROS formation than on scavenging.

## How do ROS damage the bacterial cell?

Our current understanding of potential mechanisms of ROS damage comes primarily from studies in *E. coli* that have, importantly, focused on cytoplasmic ROS/damage. Superoxide and hydrogen peroxide ($H_2O_2$) are produced inadvertently in the cytoplasm primarily when oxygen collides with various redox enzymes that have solvent-exposed flavins (Imlay, 2009). Superoxide can undergo spontaneous dismutation in a pH- and concentration-dependent reaction to yield $H_2O_2$ and $O_2$; the same reaction is catalyzed by superoxide dismutases. Both $O_2^-$ and $H_2O_2$ can damage a variety of biomolecules (Anjem *et al.*, 2009;Imlay, 2009). These species can oxidize solvent exposed 4Fe-4S clusters. They can also damage other enzymes, most likely via a Fenton reaction with iron that is bound as a non-redox cofactor. These types of damage result in metabolic defects, including auxotrophy for aromatic, branched-chain, and sulfur-containing amino acids. Damage to iron-sulfur clusters releases iron, which can undergo a Fenton reaction with $H_2O_2$ to yield hydroxyl radical, which damages any biological molecule, including DNA, in a diffusion limited manner. Fenton chemistry can also result in carbonylation of proteins. Hydrogen peroxide can directly react with cysteine residues, depending on the local environment of the sulfhydryl group. Superoxide cannot damage membranes in bacteria, as lipid peroxidation requires polyunsaturated fatty acids.

## Are these cytoplasmic injuries relevant to what is happening in the phagosome?

The answer is apparently no. Phagocytes are estimated to produce relatively high amounts of $O_2^-$, on the order of 0.5 mM/sec (Imlay, 2009). Taking into account the rate of spontaneous dismutation and approximate volume of the phagosome, this is estimated to yield 50 μM $O_2^-$ at pH 7.4 or 2 μM at pH 4.5, the approximate pH of the acidified phagosome. Because the resulting $H_2O_2$ can diffuse across membranes, including out of the phagosome, the steady state concentration of the $H_2O_2$ would be approximately 1–4 μM. The $H_2O_2$ that diffuses into the bacterial cytoplasm could potentially cause damage. Indeed, dogma is that the phagocytic ROS kill bacteria by damaging DNA. But *Salmonella* produces three catalases and three hydroperoxide reductases, all cytoplasmic, that can scavenge $H_2O_2$. Hebrard et al. showed that *Salmonella* strains lacking catalases alone, or two of three peroxidases alone, remained fully virulent, whereas the mutant missing five enzymes was attenuated (Hebrard *et al.*, 2009). More recently, a third peroxidase was identified that also contributes to $H_2O_2$ scavenging (Horst *et al.*, 2010). These important results show that *Salmonella* is more than capable of handling the $H_2O_2$ that results from the oxidative burst. In *E. coli*, aromatic amino acid auxotrophy and DNA damage are observed at ~0.5 μM cytoplasmic $H_2O_2$. It is known that *Salmonella* mutants that are incapable of synthesizing aromatic amino acids are significantly attenuate. Thus we can infer that this pathway is intact and that the cytoplasmic $H_2O_2$ concentration is below 0.5 μM in the scavenging-competent bacteria.

In this volume, Aussel et al. (2011) further characterize *Salmonella* resistance to the oxidative burst by constructing a reporter strain containing an *ahpC* promoter-Gfp fusion. The *ahpC* gene encodes a peroxidase under the control of OxyR, which activates a series of genes in direct response to $H_2O_2$. They show that this fusion is induced when *Salmonella* are growing in macrophages and that induction is dependent on Phox. These data prove that $H_2O_2$ is gaining access to the bacterial cytoplasm at a concentration sufficient to induce OxyR, ≤ 100 nM (Imlay, 2009). Given the fact that the peroxide is being actively

scavenged, this is consistent with the estimates for $O_2^-$ and $H_2O_2$ production given above. Knocking out four of the six scavenging enzymes results in increased expression of the *ahpC* fusion, but this strain remains virulent. Moreover, although the OxyR regulon is induced in the SCV, it is not required; *oxyR* mutants are fully virulent (Taylor *et al.*, 1998), consistent with the redundant protection provided by the various detoxifying enzymes. Thus, there does not appear to be substantial damage to cytoplasmic contents in *Salmonella* caused by phagocyte generated $H_2O_2$.

## What of superoxide?

It is clear that phagocytic $O_2^-$ damages bacteria in the phagosome and that the periplasmic superoxide dismutase SodCI protects *Salmonella* specifically against this exogenous $O_2^-$ (Craig and Slauch, 2009, and references therein). Mutations in *sodCI* attenuate *Salmonella*, yet loss of periplasmic SOD confers no phenotype when *Salmonella* are grown in vitro or when the bacteria are infecting Phox$^{-/-}$ mice. Moreover, charged $O_2^-$ cannot cross membranes and the role of SodCI is genetically separable from enzymes involved in protecting the cytoplasm from superoxide-mediated damage, including cytoplasmic superoxide dismutases. Thus, the primary targets of phagocytic $O_2^-$ must be extracytoplasmic.

This extracytoplasmic damage is the result of $O_2^-$ per se and not some downstream ROS. Spontaneous or enzymatic dismutation of $O_2^-$, in theory, yields the same amount of $H_2O_2$. SodCI simply lowers the steady state concentration of $O_2^-$ in the periplasm. Aussel et al. (2011) used their *ahpC*-GFP fusion strain to monitor the amount of $H_2O_2$ that enters the cytoplasm of wild type *Salmonella* versus a mutant lacking periplasmic SOD. Surprisingly, they found that the $H_2O_2$ concentration was higher in the wild type cell. Simplistically, this suggests that in the SOD mutant, $O_2^-$ is substantially consumed by some reaction that does not result the production of $H_2O_2$. SodCI normally prevents this latter reaction by driving the $O_2^-$ to $H_2O_2$. Since both spontaneous dismutation and substrate oxidation convert $O_2^-$ into $H_2O_2$, this result suggests that $O_2^-$ is acting as a reductant in the periplasm. This is somewhat surprising since targets with the required redox potential are expected to be very limited. Further study will be required to resolve this conundrum and identify the most vulnerable target(s) of $O_2^-$. But this superoxide-mediated extracytoplasmic damage is at least as critical as the potential cytoplasmic damage caused by $H_2O_2$ and there is no reason to think that this is unique to *Salmonella*. Thus, identifying the targets of exogenous $O_2^-$ is key to understanding the overall mechanism of oxidative damage in phagocytes.

## Is *Salmonella* special in its ability to handle the oxidative burst?

*Salmonella* injects a series of proteins into the macrophage cytoplasm via the SPI2 type III secretion system leading to alterations in vesicular trafficking and the establishment of the SCV (Holden, 2002). Two groups have provided data suggesting that the assembly of Phox on the SCV membrane is also inhibited by SPI2 effectors (Gallois *et al.*, 2001;Vazquez-Torres *et al.*, 2000b). Clearly this proposed exclusion of Phox is not 100%, or there would be no role for SodCI. But, does this inhibition significantly lower the amount of $O_2^-$ created in the SCV? Aussel et al. (2011) show that mutants defective in SPI2 secretion are equally attenuated in congenic wild type and Phox$^{-/-}$ mice, although both wild type and mutant bacteria propagate more readily in the Phox$^{-/-}$ host. These results suggest that SPI2 has a role that is functionally unrelated to the delivery or assembly of Phox. Both results could be correct, in that alterations in vesicular trafficking could decrease the delivery of Phox, but any decrease in phagosomal ROS formation may be too moderate to affect bacterial survival. These new results will certainly generate further study, but they do emphasize the

power of competition assays in sorting out complicated host-pathogen interactions (Craig and Slauch, 2009).

Mice and humans defective in Phox are clearly more susceptible to *Salmonella*. Aussel et al., for example, recovered >1 log more *Salmonella* from the spleens of $Phox^{-/-}$ mice than from those of wild type mice. Does this mean that the oxidative burst is indeed killing a large fraction of the wild type *Salmonella*? This is not clear. There is ample evidence that $O_2^-$ and downstream ROS not only directly damage bacteria, but also are critical for signaling and activation of other antimicrobial effectors (Gwinn and Vallyathan, 2006;Forman and Torres, 2002). In other words, additional attenuation in the wild type mouse does not prove that this is the result of direct damage by phagocytic $O_2^-$.

## Does superoxide act alone?

Antimicrobial peptides in the SCV can partially disrupt the outer membrane of *Salmonella* and allow access of phagocytic proteases to periplasmic proteins (Kim *et al.*, 2010). Importantly, this is occurring in *Salmonella* cells that survive this assault and go on to kill the animal. Indeed, data suggest that SodCI is both tethered within the periplasm and protease resistant, allowing it to detoxify $O_2^-$ in the face of this combined attack. Additional in vivo evidence of synergism between phagocytic effectors is surprisingly limited. Rosenberger et al. showed that the addition of protease inhibitors to macrophages in culture also blocked proteolytic processing and activation of antimicrobial peptides (Rosenberger *et al.*, 2004). But one can imagine more intimate synergy at the level of damage. Perhaps antimicrobial peptides are required to facilitate $O_2^-$ access to a periplasmic target. Alternatively, superoxide-mediated damage could make the cell susceptible to some other antimicrobial effector.

## What's next?

These recent studies have emphasized the differences in our understanding of endogenous versus exogenous ROS-mediated damage. In effect, they have told us what is NOT damaged by phagocytic ROS; we still have not identified the pertinent targets of the phagocytic oxidative burst. The problem is complicated by our inability to produce $O_2^-$ in the laboratory at concentrations that approach, even perhaps within an order of magnitude, those apparently produced in the phagosome (Craig and Slauch, 2009). The good news is that the sophisticated molecular and genetic tools available in *Salmonella* should enable us to address these questions.

## Acknowledgments

I thank Jim Imlay for helpful discussions and comments. This work was supported by AI080705 from the National Institutes of Health.

## References

Anjem A, Varghese S, Imlay JA. Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. Mol Microbiol. 2009; 72:844–858. [PubMed: 19400769]

Aussel L, Zhao W, Hébrard M, Guilhon AA, Viala J, Henri S, Chasson L, Gorvel JP, Barras F, Méresse S. *Salmonella* detoxifying enzymes are sufficient to cope with the host oxidative burst. Mol Microbiol. 2011 MMI-2010-10662.R1.

Craig M, Slauch JM. Phagocytic superoxide specifically damages an extracytoplasmic target to inhibit or kill *Salmonella*. PLoS ONE. 2009; 4:e4975. [PubMed: 19305502]

Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol. 2004; 2:820–832. [PubMed: 15378046]

Forman HJ, Torres M. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. Am J Respir Crit Care Med. 2002; 166:S4–S8. [PubMed: 12471082]

Gallois A, Klein JR, Allen LA, Jones BD, Nauseef WM. *Salmonella* pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane. J Immunol. 2001; 166:5741–5748. [PubMed: 11313417]

Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, et al. The phagosome proteome: insight into phagosome functions. J Cell Biol. 2001; 152:165–180. [PubMed: 11149929]

Gwinn MR, Vallyathan V. Respiratory burst: role in signal transduction in alveolar macrophages. J Toxicol Environ Health B Crit Rev. 2006; 9:27–39. [PubMed: 16393868]

Hebrard M, Viala JP, Meresse S, Barras F, Aussel L. Redundant hydrogen peroxide scavengers contribute to *Salmonella* virulence and oxidative stress resistance. J Bacteriol. 2009; 191:4605–4614. [PubMed: 19447905]

Holden DW. Trafficking of the *Salmonella* vacuole in macrophages. Traffic. 2002; 3:161–169. [PubMed: 11886586]

Horst SA, Jaeger T, Denkel LA, Rouf SF, Rhen M, Bange FC. Thiol peroxidase protects *Salmonella enterica* from hydrogen peroxide stress in vitro and facilitates intracellular growth. J Bacteriol. 2010; 192:2929–2932. [PubMed: 20304995]

Imlay, JA. Oxidative stress. In: Böck, A.; Curtis, R3.; Kaper, J.; Karp, PD.; Neidhardt, FC.; Nystrom, T., et al., editors. EcoSal-Escherichia coli and Salmonella: Cellular and Molecular Biology. Washington, DC: ASM Press; 2009.

Kim B, Richards SM, Gunn JS, Slauch JM. Protecting from antimicrobial effectors in the phagosome allows SodCII to contribute to virulence in *Salmonella enterica* serovar Typhimurium. J Bacteriol. 2010; 192:2140–2149. [PubMed: 20154132]

Rosenberger CM, Gallo RL, Finlay BB. Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular *Salmonella* replication. Proc Natl Acad Sci U S A. 2004; 101:2422–2427. [PubMed: 14983025]

Taylor PD, Inchley CJ, Gallagher MP. The *Salmonella typhimurium* AhpC polypeptide is not essential for virulence in BALB/c mice but is recognized as an antigen during infection. Infect Immun. 1998; 66:3208–3217. [PubMed: 9632587]

Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. J Exp Med. 2000a; 192:227–236. [PubMed: 10899909]

Vazquez-Torres A, Xu Y, Jones-Carson J, Holden DW, Lucia SM, Dinauer MC, et al. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science. 2000b; 287:1655–1658. [PubMed: 10698741]

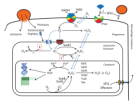

**Figure 1. Reactive oxygen species in the *Salmonella* containing vacuole**

The phagocytic oxidase (Phox) produces $O_2^-$ which enters the periplasm through porins or perhaps crosses the outer membrane that is partially permeabilized by antimicrobial peptides. This superoxide potentially kills or inhibits cells by reducing or oxidizing unknown targets. SodCI protects the cell by dismuting superoxide to hydrogen peroxide, which can freely diffuse across membranes into the cytoplasm. This species can cause the same damage as endogenously produced peroxide, all via Fenton chemistry. However, *Salmonella* produces six catalases or peroxidases that are capable of keeping the peroxide levels below 5 μM. The SCV is created by the action of SPI2 effector proteins injected into the macrophage cytoplasm that block vesicular trafficking and lessen the delivery of antimicrobial compounds. The assembly of Phox on the phagosomal membrane might also be decreased, but not to an extent that has phenotypic consequences. The shown chemical reactions are not necessarily balanced.