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Review

ROS production in phagocytes: why, when, and where?

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ABSTRACT

In the phagocytosis field, ROS production by the phagocyte NOX has been associated with pathogen killing for the last 50 years. Since the discovery of nonphagocyte NOX, numerous other roles for ROS production have been identified. Oxidative stress and ROS-mediated signaling have received much attention in recent years. Much lower concentrations of ROS may be required for signaling compared with microbial killing. Based on the discoveries in nonphagocytic cells, it became logical to look for ROS functions distinct from pathogen killing, even in phagocytes. ROS are now linked to various forms of cell death, to chemotaxis, and to numerous modifications of cellular processes, including the NOX itself. ROS functions are clearly concentration-dependent over a wide range of concentrations. How much is required for which function? Which species are required for how much time? Is ROS signaling only a side effect of bactericidal ROS production? One major obstacle to answer these questions is the difficulty of reliable quantitative ROS detection. Signal transduction often takes place on a subcellular scale over periods of seconds or minutes, so the detection methods need to provide appropriate time and space resolution. We present examples of local ROS production, decreased degradation, signaling events, and potentially ROS-sensitive functions. We attempt to illustrate the current limitations for quantitative spatiotemporal ROS detection and point out directions for ongoing development. Probes for localized ROS detection and for combined detection of ROS, together with protein localization or other cellular parameters, are constantly improved.
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Introduction

"The extra respiration of phagocytosis" has been described 80 years ago [1]. This "respiratory burst" turned out to be the conversion of O_2 to $O_2^{\cdot -}$ by the phagocyte NOX and subsequent dismutation to H_2O_2 [2]. Formation of H_2O_2 and other ROS, such as $HO^{\cdot}$ and HOCl, may be catalyzed in enzymatic and nonenzymatic reactions [3]. A large number of papers have described the mechanism and the consequences of ROS production. The present review highlights a number of open questions that persist, despite the efforts of several decades. Quite often, answering these questions will require new experimental techniques. Part III of this review will deal with current techniques and future development. As this is part of a special issue on "The neutrophil in immunity," we will focus on PMNs, although many points also apply to macrophages, DCs, and other ROS-producing cells.

PART I: THE MULTIPLE FUNCTIONS OF ROS

ROS and pathogen killing

The first function of ROS to be discovered in neutrophils was their microbicidal activity. Indeed, a severe neutrophil killing defect was reported in patients with CGD and associated with the absence of the respiratory burst [4]. This genetic disease is a result of the absence or dysfunction of the proteins that constitute the phagocyte NOX [5]. Upon phagocytosis of pathogens, the ROS are produced by the NOX in the small volume of the phagosome. Few data are available concerning the actual amount of ROS produced in the phagosome. The production of $O_2^{\cdot -}$ in the phagosome has been estimated between 1 and 4 M and its steady-state concentration to be in the micromolar range as a result of its dismutation to H_2O_2 [6]. By modeling the reaction of superoxide in the phagosome, Winterbourn et al. [7] estimated steady-state concentrations to be

Abbreviations: $^1O_2$=singlet O_2, 3D=three-dimensional, APF=aminophenyl fluorescein, avGFP=Aequorea victoria GFP, $[Ca^{2+}]_c$=cytosolic-free calcium concentration, CGD=chronic granulomateous disease, Cl=chloride, cpYFP=circularly permuted YFP, DCF=dichlorofluorescein, $DCFH_2$=2′,7′-dihydrodichlorofluorescein, DHE=dihydroethidium, DHR=dihydrorhodamine, DPI=diphenyliodonium, DUOX=dual oxidase, EPR=electron paramagnetic resonance spectroscopy, Eu=europium, Fe=iron, FP=fluorescent protein, H_2O_2=hydrogen peroxide, $HO^{\cdot}$=hydroxyl radical, HOCl=hypochlorous acid, HPF=hydroxyphenyl fluorescein, HyPer=circularly permuted YFP inserted into the regulatory domain of the prokaryotic hydrogen peroxide-sensing protein, NET=neutrophil extracellular trap, $NO^{\cdot}$=nitric oxide, NOX=NADPH oxidase, O_2=oxygen, $O_2^{\cdot -}$= superoxide anion, $ONOO^-$=peroxinitrite, PCL-1=peroxy-caged luceferin-1, PTEN=phosphatase and tensin homolog, PTP=protein tyrosine phosphatase, PX=phox homology, RNS=reactive nitrogen species, roGFP=reduction oxidation-sensitive GFP, YVO_4=yttrium orthovanadate

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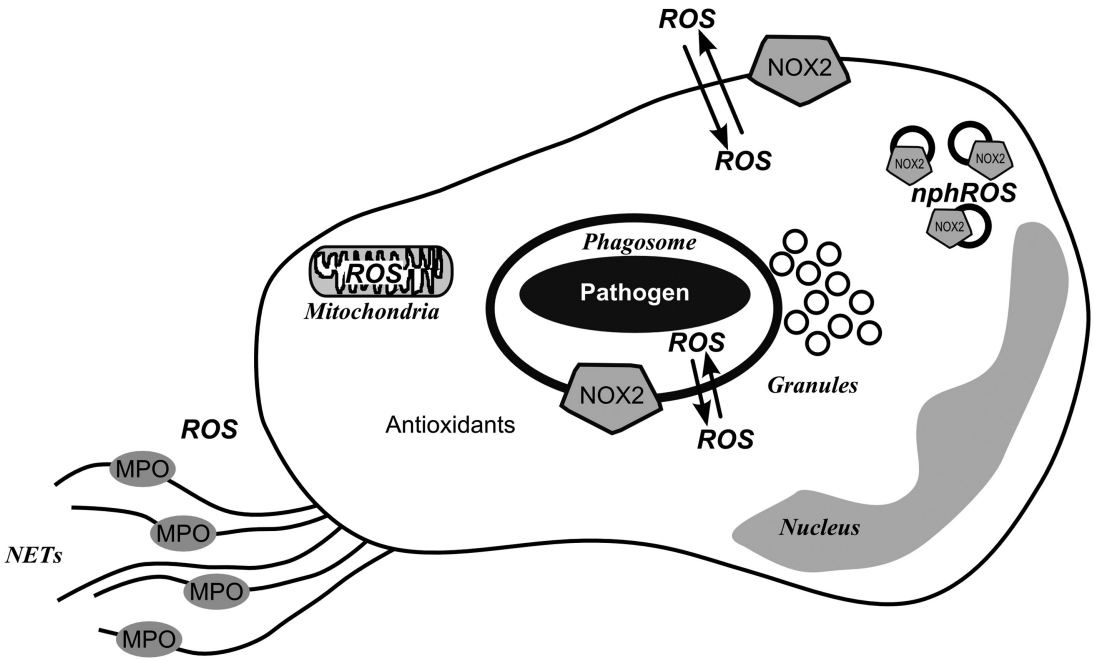

Figure 1. Sites of ROS production, diffusion, and action. A simplified scheme of a phagocyte producing ROS via NOX2 in three distinct locations: the plasma membrane, the phagosomal membrane, or the nonphagosomal intracellular organelles (nphROS). Certain ROS, mainly H_2O_2, diffuse across membranes. The cytosol contains a variety of antioxidants [12, 13]. The formation of extracellular NETs requires ROS, and the MPO is attached to NETs.

25 μM superoxide and 2 μM H_2O_2. The MPO released by the fusion of the granules with the phagosomes catalyzes the formation of HOCl from Cl and H_2O_2. In their model, Winterbourn et al. [7] estimated that HOCl is produced at a rate of 134 mM/min, and the concentration of phagosomal MPO was ~1 mM. $HO^{\cdot}$ and $^1O_2$ are also formed by a mechanism involving the MPO/H_2O_2/Cl system [4, 8], although in the phagosome, they are reported to be minor species [9].

The mechanism by which ROS kill pathogens is still a matter of debate. A predominant role has first been given to MPO. Indeed, HOCl can attack any oxidizable group. For example, it induces peroxidation of polyunsaturated lipids and reacts with sulfur containing amino acids and amines in amino acids. Reaction with amines generates chloramines, which retain oxidizing activity. It was observed that patients with MPO deficiency presented a higher occurrence of severe infections and chronic inflammation compared with normal subjects [10]. MPO knockout mice have also increased susceptibility to some microorganisms, such as *Candida albicans* [4]. Nevertheless, the consequences of MPO deficiency are not as drastic as expected. It was therefore proposed that MPO-independent mechanisms of killing exist or/and that organisms without functional MPO present an increase in the activity of such MPO-independent mechanisms [4]. A recent review proposed that MPO-dependent mechanisms of killing are mainly important during the early postphagocytic period and during infections with high pathogen load [9]. Another, yet-unanswered question is how much ROS reach the pathogens enclosed in the phagocytes. Although some bacterial-chlorinated products have been detected in the phagosome, most of HOCl, produced by MPO, is believed to react with neutrophil proteins [11]. This has a dual consequence: oxidation by HOCl will generate protein chloramines, which have cytotoxic actions, but also oxidize and inactivate granule proteases. It has been suggested that these reaction products may actually "extend microbial killing in time and space" [7, 9].

Production of HOCl requires H_2O_2, which also has several other functions. Accumulation of H_2O_2 in the phagosome is not only limited by peroxidases and other ROS scavenging reactions but also by diffusion out of the phagosome (**Fig. 1**). It was estimated that even in the absence of MPO, no more than 30 μM H_2O_2 would accumulate in the phagosome [7]. WT *C. albicans* is not killed by plating on medium containing 4 mM H_2O_2. On the other hand, sufficient H_2O_2 reaches bacteria inside phagosomes to provoke a transcriptional response (see below). Furthermore, *Escherichia coli* that lack H_2O_2 scavenging enzymes are killed by exposure to 1 μM H_2O_2 for a few hours [14]. Thus, the H_2O_2 concentrations needed for causing damage are extremely divergent and highly dependent on the microorganisms and their antioxidant defense. Although less reactive than HOCl, H_2O_2 may react with enzymes containing Fe or Fe-sulfur clusters. The release of Fe can enhance the Fenton reaction to generate $HO^{\cdot}$, which is a powerful oxidizing agent [9].

Neutrophils also produce extracellular ROS to attack pathogens that are too large to be phagocytosed, such as the hyphae of *Aspergillus fulmigatus*. Extracellular ROS are produced by the same NOX as phagosomal ROS (see below). The mechanism of this extracellular production is not elucidated completely but involves β2 integrin and class I PI3K [15].

ROS function as signaling molecules

ROS produced by the NOX could act as signaling molecules by modifying the redox state of proteins or lipids. One possible target of the ROS is the NOX itself. Superoxide and H_2O_2 added on NOX subunits have been shown to decrease ROS production but only when they were added before the assembly of the subunits [16]. A peroxidation of phospholipids was observed in differentiated HL60 cells upon stimulation by PMA or zymosan. The oxidase inhibitor DPI or the ROS scavengers SOD/catalase inhibited this lipid oxidation [17]. Oxidized phospholipids inhibit ROS production, but in a cell-free assay, this happened only when the oxidized lipids were added before the assembly of the NOX [18]. The mechanism of this inhibition is not known. The activities of some kinases and phosphatases have also been proposed to be regulated by ROS. The PTP superfamily contains a conserved cysteine residue in its catalytic domain that is susceptible to oxidation, and oxidation of this cysteine can inhibit the PTP activities in a transient way, only if the oxidation proceeds no further than sulfenic acid. Although several PTPs have been shown to be modulated by ROS [19, 20], the direct action of ROS on PTP inactivation has been demonstrated in only a few cases. In neutrophils, oxidation of the phophatase CD45 by ROS contributes to its inhibition [19]. The relevance of PTP oxidation in physiological conditions has not been established in neutrophils. Recently, ROS have been shown to oxidize the phospholipid phosphatase PTEN, which has a catalytic domain similar to that of the PTP superfamily. When neutrophils are stimulated by fMLP, a localized $O_2^{\cdot -}$ production at the leading edges of migrating neutrophils is observed [21] (1.5 nmol superoxide/5 min/10^6 cells). This induced the oxidation of PTEN, thereby decreasing its activity. A decreased PTEN activity at the leading edge of migrating neutrophils maintains a high concentration of phosphatidylinositol (3,4,5)-triphosphate and promotes migration [22]. The production of ROS in this case is extracellular, thus the ROS species that decrease PTEN activity—most likely H_2O_2—should cross the membrane and diffuse into the cytosol. In addition, a gradient of H_2O_2 should exist, such that only PTEN, near the membrane of the leading edge, is oxidized. PKC is also a good target for oxidation by ROS: cysteines in the regulatory and catalytic domains can be modified by oxidants. In vitro studies demonstrated that PKC activity can be modulated by H_2O_2 [23]. It would be interesting to know the relevance of this regulation in the neutrophil, as PKC isoforms are involved in NOX activation. The importance of ROS in signaling is, at the moment, not fully assessed. However, this may be a difficult task, as (1) ROS are one among several actors of protein or lipid regulation, (2) the action of ROS is local and thus, may concern only a small fraction of the target macromolecules, (3) different ROS have quite distinct reactivity, and (4) regulation by ROS may depend on the amount and type of ROS produced, as well as the amount and reactivity of ROS scavengers in the vicinity of the ROS targets. Intracellular ROS are scavenged by enzymatic reactions (SOD, glutathione peroxidase, catalase, peroxiredoxins), as well as nonenzymatic compounds, such as glutathione and ascorbate [12]. Peroxiredoxin I and II are abundant cytosolic enzymes that reduce H_2O_2 to H_2O rapidly, thereby effectively preventing any major rise in intracellular ROS concentration. However, peroxiredoxin I is inactivated by tyrosine phosphorylation, and local tyrosine kinase activation provides an elegant mechanism to raise H_2O_2 concentrations sufficiently to oxidize signaling targets [24].

ROS and phagocyte death

Aged neutrophils die by spontaneous apoptosis. This apoptosis can be accelerated by phagocytosis or stimulation of death receptors, such as Fas or the TNF-αR. ROS have been involved in all types of apoptosis. However, the level of ROS seems not to be the same: during spontaneous apoptosis or apoptosis triggered by stimulation of FasR, the level of ROS production is low compared with the ROS production triggered by TNF-α [25, 26]. The rate of neutrophil apoptosis increases upon phagocytosis and is correlated with the bacteria:neutrophil ratio. This ratio also dictates the level of ROS produced [27]. In all of these studies, ROS are measured after 1–3 h of stimulation, whereas the apoptotic states of the cells are determined after 6–24 h, so that the direct implication of ROS is difficult to determine. However, to assess the role of ROS produced by NOX in apoptosis, the apoptosis of normal neutrophils was compared with neutrophils from CGD patients that do not produce ROS or determined after the addition of DPI or ROS scavengers to healthy neutrophils [25–29]. In the case of spontaneous apoptosis or Fas-induced apoptosis, CGD neutrophil apoptosis is only delayed compared with healthy neutrophils [30]; thus, in these two cases, ROS may be a minor activating pathway. Which ROS trigger apoptosis is still unknown. H_2O_2 is a good candidate, as it can diffuse from its site of generation to the site of action, and indeed, H_2O_2 added to the neutrophils triggers apoptosis [26]. H_2O_2 has also been shown to damage ferritin, which releases Fe [31]. An increase in Fe can induce further reactions, such as the Fenton reaction and the generation of highly reactive hydroxyl radicals. In most cases, ROS are believed to be upstream of caspases, and several mechanisms have been proposed; however, only a few ROS targets have been elucidated [29]. One identified target is the granule/lysosome membrane: H_2O_2 induces permeabilization of lysosome membranes, probably through the formation of $HO^{\cdot}$ by the Fenton reaction in the lysosome, which triggers the peroxidation of lipids. The subsequent release of cathepsin from granules provokes apoptosis in several cases [32, 33].

More recently, ROS have been involved in a new type of neutrophil death—NETosis. NETs are made of chromatin, decorated with granule proteins, such as elastase and MPO. NETosis is characterized by the loss of the nuclear envelope and the membrane of the granules, as well as decondensation of the chromatin. It involves elastase and MPO [34] and then, the disruption of the plasma membrane. The formation of NETs is induced in vitro by incubation for 3 h with PMA, LPS, and IL-8 or by infection with bacteria [35]. ROS are required for NET formation, as it was shown that DPI inhibited their formation and that the neutrophils of CGD patients did not produce NETs [35]. The ROS species involved may be dependent on the stimulus: NET formation with PMA or *C. albicans* required MPO [36], whereas NETs induced by the bacterium

Pseudomonas aeruginosa are independent of MPO activity [37]. It is not clear which MPO products are important for NET formation; short-lived MPO products have been proposed to be involved [36]. A role for $^1O_2$ in PMA-induced NET formation was proposed recently [38]. The targets of ROS during NET formation, which may also depend on the stimulus, have not been elucidated. NETs have been observed in tissues, such as skin and lung, and in vivo, in the liver sinusoids of mice after LPS administration and bacterial infection [39, 40]. In the latter study, NETs, by trapping bacteria, protected against bacterial dissemination in the organism, and their formation required interaction with platelets. This last point brings a first element of response to the question: which signals are responsible for inducing NETosis instead of apoptosis? Interactions with platelets may modulate signaling pathways. ROS are involved in both death pathways. Are their targets the same? If not, why? The amount of ROS species, the duration of their production, and the interplay with other signaling pathways need to be investigated further to better understand the fate of the neutrophils.

ROS effects in the environment of the phagocyte

ROS signaling effects are not limited to the ROS-producing cells. Extracellular ROS production and the diffusion of ROS, namely H_2O_2, across membranes enable ROS signaling in the extracellular space and in neighboring cells. As illustrated above, cysteines may be a prominent but not the only target [41]. Extracellular effects of the MPO/H_2O_2/Cl system include the activation of proteases by direct activation, as in the case of matrix metalloprotease 7 [42], or by inhibition of protease inhibitors, such as TIMP [43]. The consequences of ROS signaling in disease are not established easily. For example, ROS are often considered as a stimulator for inflammation and a mediator of inflammatory tissue damage; however, evidence for anti-inflammatory action of ROS is also accumulating [44, 45]. Whereas neutrophil-derived ROS are able to damage and kill cells at sites of inflammation, such as the liver, they also induce a transcriptional antioxidant response that may protect against further ROS damage [46]. Once again, the balance between intensity and timing of ROS production versus ROS scavenging appears to be critical for the outcome.

ROS-induced signaling in microorganisms

Numerous bacterial or fungal pathogens are known to mount a complex stress response upon exposure to ROS. For example, *Staphylococcus aureus* has developed several strategies to escape killing by neutrophils, including a sophisticated oxidative stress response [47]. Within 15 min of phagocytosis, the expression of more than 100 genes is up-regulated [48]. The bacterial agr system of virulence control appears to be critical in the response of phagocytosed *S. aureus* [49]. The multiple proteins of the stress response will insure survival and ultimately, killing of the phagocyte, possibly via membrane lytic toxins [47]. Some 400 fungal species are also pathogenic to humans [50]; they affect mainly immunocompromised hosts. *Candida glabrata* infections have increased substantially in recent years and account for 15–20% of all *Candida* infections [51]. Pathogenic fungi resist to phagosomal destruction and develop an important oxidative stress response. Fungi have several mechanisms to detect ROS inside and outside of its cell membrane. The sensor kinase Tco2 was identified in *Cryptococcus neoformans* to mediate the oxidative stress response via a MAPK pathway [50]. Oxidative stress detection in *C. albicans* occurs mainly through SSK1, which also signals through a MAPK pathway and enhances its resistance to killing by neutrophils [52, 53]. In *C. glabrata*, over 200 genes are up-regulated upon treatment with H_2O_2. The transcription factor CgYap1, the SOD CgSod1, and the catalase Cta1p are critical for survival in phagocytes [54, 55]. The importance of any individual protein in this multicomponent response remains difficult to evaluate.

Bacterial and fungal pathogens catabolize ROS or moderate their effects by production of numerous proteins, a few minutes after the engulfment. Among them, some are directly involved in detoxification, such as SOD or catalase. Therefore, time-resolved detection of ROS on the surface (outside) and inside each pathogen should help to dissect the role of the multiple components of the oxidative stress response. A good knowledge of the selectivity of the ROS sensor used in these experiments will help to characterize quantitatively and qualitatively the ROS produced and/or scavenged. In parallel, certain proteins of the pathogen's stress response might interfere with the ROS production itself, as in the case of *Helicobacter pylori* that divert ROS production from the phagosome to the plasma membrane of the phagocyte [56]. Granule fusion with the phagosome or NOX2 cytosolic subunits might be affected directly.

From the phagocyte point of view, it is critical to produce sufficient quantities of ROS to kill the pathogen before it can mount an effective oxidative stress response. Insufficient ROS production not only fails to kill the pathogen but also induces a stress response allowing the pathogen to resist to higher concentrations of ROS. Thus, under certain circumstances, low-level ROS might cause more harm than benefit for the host.

Sublethal antibiotic treatment favors the emergence of multidrug-resistant bacteria. Kohanski et al. [57, 58] have shown that antibiotic treatment leads to oxidative stress in bacteria, which in turn increases the rate of mutation and possibly new antibiotic resistance. Could ROS, produced by the phagocyte, have a similar effect on bacteria? Kohanski et al. [57, 58] used 1 mM H_2O_2 much more than the estimated concentration in the phagosome (see above). However, a long duration of ROS exposure and multiple ROS species might compensate for the lower concentration. The idea that moderate phagosomal ROS production that does not lead to pathogen death, may actually lead to mutations in the pathogen needs more thought and experiments. In general, knowing the way pathogens block their destruction, escape the phagosome, or proliferate should open the path to efficient and innovative therapeutic approaches.

PART II: ROS GENERATION IN PHAGOCYTES

The phagocyte NOX is the most important source of ROS in phagocytes. This enzyme is formed from two integral membrane proteins ($gp91^{phox}$ or NOX2, $p22^{phox}$) and several cytosolic proteins ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, rac). In the resting state, these proteins are separated; upon cell stimulation, they assemble to form the active enzyme [59]. Enzyme activation is mediated by multiple protein kinases, including PKC and MAPK [59], $[Ca^{2+}]_c$ [60], and anionic phospholipids, such as phosphoinositides, phosphatidic acid, or phosphatidyl serine. In unstimulated neutrophils, $gp91^{phox}/p22^{phox}$ is localized in the plasma membrane or in the membrane of secondary and tertiary granules, as well as secretory vesicles (>80%), from where it translocates by granule fusion during activation [61, 62]. Soluble stimuli, such as chemotactic peptides or PMA, which activate PKC, trigger oxidase assembly in the plasma membrane and result in extracellular ROS production [63]. Intracellular ROS production induced by PMA is discussed below. Particulate stimuli, such as opsonized bacteria and fungi, trigger phagocytosis with concomitant oxidase assembly on the phagosomal membrane. The phagosomal membrane is derived from the plasma membrane; thus, its initial composition resembles the latter. The selective assembly on the phagosome, with little or no assembly on the bulk of the plasma membrane, requires local signaling cues. $[Ca^{2+}]_c$ is a good candidate for local signaling; however, calcium ions diffuse rapidly, and local calcium signals are hard to detect in small cells, such as neutrophils. A transient rise in $[Ca^{2+}]_c$ coincides with the onset of phagosomal ROS production but is not restricted to a particular region of the cell. Therefore, it has been proposed that "Ca^{2+} triggers time not space" of NOX activation [64].

The extent of phagosomal ROS production depends on several parameters: (1) the number of $gp91^{phox}/p22^{phox}$ complexes sequestered in the phagosomal membrane; (2) the number of active NOX complexes; and (3) the rate of oxidase inactivation. Assuming that $gp91^{phox}$ is distributed uniformly on the plasma membrane of resting phagocytes, it seems likely that $gp91^{phox}/p22^{phox}$ is somehow sequestered in the forming phagosome prior to its closure. The lateral mobility of $gp91^{phox}/p22^{phox}$ may be addressed in the future using FP-tagged $gp91^{phox}$ or $p22^{phox}$ [65]. In the neutrophil, fusion of the phagosome with secondary granules increases the number of $gp91^{phox}/p22^{phox}$ molecules in the phagosomal membrane. Several studies [66, 67] have reported the formation of granular structures with assembled and active oxidase that fuse with the phagosome. In addition, we do not know whether all phagosomal $gp91^{phox}/p22^{phox}$ is forming an active complex. It is likely that assembly can be modulated, and some pathogens have evolved sophisticated strategies to interfere with oxidase assembly [68].

The disassembly of the NOX is poorly understood, mainly because of lack of experimental tools. One of these problems resides in the difficulty to measure the true end of $O_2^{\cdot -}$ production, as discussed below. Several studies indicate that the oxidase complex has to be renewed constantly to sustain $O_2^{\cdot -}$ production [69]. However, recent experiments using fluorescence recovery after photobleaching with $p67^{phox}$-citrine suggest that the NOX2 complex on the phagosomal membrane may be stable once it is formed at the time of phagosome closure [70]. The concept of stable complex versus constant renewal has different signaling requirements. For example, a transient rise of $[Ca^{2+}]_c$ could trigger NOX2 assembly on the early phagosome. If the complex is stable, no more calcium would be required for continuous production. We need combined measurements of NOX assembly, signaling dynamics, and ROS production to integrate the events in time and space.

In addition to extracellular and phagosomal ROS production, there is growing evidence for nonphagosomal intracellular ROS production induced by various stimuli, independent of phagocytosis, as reviewed recently [63]. Nonphagosomal intracellular ROS appear to mediate signaling involved in endotoxin-induced priming [71] or in the repression of inflammation [72]. Most available detection methods for intracellular ROS are peroxidase-dependent. They will mainly detect compartments containing MPO, which resides in primary granules. Therefore, the site of intracellular production may be a new organelle created by the fusion of primary with secondary/tertiary granules; the latter contains $gp91^{phox}/p22^{phox}$. Alternatively, plasma membrane-derived NOX2 could be internalized by endocytosis [71]. Furthermore, PMA-treated macrophages appear to produce different ROS in three types of intracellular organelles of unknown origin [73]. Nonphagosomal intracellular ROS production has also been detected by electron microscopy using the reaction of H_2O_2 with cerium [74]. The investigation of intracellular ROS production would benefit further from targetable fluorescent ROS detectors (see Part III). The selective activation of the oxidase on the plasma membrane or on different intracellular sites requires localized signaling events. It may also imply a different organization of the NOX complex. In fact, the first description of a CGD variant, based on dysfunction of $p40^{phox}$, revealed that this protein is required for phagosomal ROS production but not for oxidase activation in the plasma membrane [75]. Thus, the subunit composition of the active NOX is not always the same. This distinct composition may be the result of different signals. Oxidase activity on the plasma membrane requires phosphatidylinositol (3,4,5)-triphosphate and phosphatidylinositol 3,4-bisphosphate, produced by class I PI3Ks [63, 76], and is strongly affected by mutations in the phosphoinositide-binding PX domain of p47 [77]. Phagosomal ROS production involves phosphatidylinositol 3-phosphate produced by class III PI3Ks and is sensitive to mutations in the PX domain of $p40^{phox}$ [75, 78]. These $p40^{phox}$ mutations have little effect on extracellular ROS production. Furthermore, plasma membrane and intracellular oxidase may be activated by distinct PKC isoforms [63].

Other sources of ROS

Neutrophils from X-linked CGD patients show almost no increase in ROS production when stimulated with PMA (e.g., ref. [79]), and very little phagosomal ROS production is detected in gp91phox-deficient PLB985 cells [70]. These

examples indicate that NOX2 is, by far, the most important source of ROS in stimulated neutrophils. When phagocytes are not stimulated by phagocytic prey or PMA, low-level ROS production by sources other than NOX2 may be relevant for signaling; therefore, their potential origin will be discussed briefly. The phagocyte NOX2 is part of a larger family of similar enzymes—NOX1–5 and DUOX1 and -2. Expression of other NOX isoforms, in addition to NOX2, has been reported in macrophages but not in neutrophils [80]. Different types of macrophages have distinct expression patterns of NOX/DUOX enzymes; for example, liver macrophages (Kupffer cells) express NOX1, -2, and -4 and DUOX1 and -2 [81]. These NOX enzymes have quite different activation patterns and may therefore become relevant sources of ROS in various signaling situations [82]. For example, oxidized LDL enhanced macrophage apopotosis by inducing the expression of NOX4, whereas NOX2 was not involved [83]. Another report described NOX1 expression in LPS-stimulated macrophages [84].

iNOS is expressed in macrophages and to lesser extent, in neutrophils activated by proinflammatory cytokines [85]. Rodent neutrophils produce substantially more $NO^{\cdot}$ than human neutrophils [4, 19]. $NO^{\cdot}$ itself has multiple signaling roles. The reaction of $NO^{\cdot}$ with $O_2^{\cdot -}$ leads to $ONOO^-$, which has numerous signaling effects, is cytotoxic, and is involved in bacterial killing. $NO^{\cdot}$ and ROS interfere and complement each other [86], which is well-illustrated by the severity of infections in double iNOS and NOX2 knockout mice [87]. In humans, $NO^{\cdot}$ production from neutrophils has been implicated in a few pathologies [88]. However, most studies of ROS production in neutrophils conclude that $NO^{\cdot}$ does not significantly affect phagocytosis or the quantity of ROS production, even in mice (e.g., ref. [89]). Thus, the importance of $NO^{\cdot}$ in the phagocyte context highly depends on the type of phagocyte, the species, and the degree of cytokine activation.

A number of cellular enzyme reactions produce ROS, mainly H_2O_2 or $O_2^{\cdot -}$, as byproducts [90]. The enzymes, such as cytochrome P450 and xanthine oxidase, can be found in the cytosol or in different organelles—mitochondria, peroxisomes, and ER. For technical reasons, it is very difficult to determine the relative contribution to total ROS production of any of these enzymes and organelles [91]. Mitochondria produce superoxide in at least five steps of the respiratory chain and three other enzymatic reactions. In general, low ATP demand or lack of O_2 favor mitochondrial ROS production [92]. In vitro, 0.15–2% of the total O_2 consumption may be transformed into superoxide; however, the actual amount of mitochondrial superoxide or H_2O_2 produced in vivo is controversial [93]. Nevertheless, there is ample evidence for multiple regulatory roles of mitochondrial ROS in various cells [94]. As neutrophils have few mitochondria [95], their contribution to the overall ROS production is thought to be minimal. In macrophages, mitochondrial ROS are likely to be involved in ROS action outside of the phagosome. Furthermore, organelles, such as mitochondria and peroxisomes, not only produce but also degrade ROS through enzymes, such as catalase or peroxiredoxins [90]. Their net contribution to cellular ROS may be positive or negative. In conclusion, although NOX2 is most likely the predominant source of ROS during phagocytosis, other sources need to be considered in the absence of phagocytic prey.

PART III: ROS DETECTION

Many methods have been developed to detect the NOX activity. They can be divided into nonoptical and optical methods; the use of the latter encountered a large success in the past 30 years. There are numerous recent reviews on the detection of NOX activity [63, 96–98], and we will focus here on the ability of the available methods to afford spatiotemporal as well as qualitative and quantitative characterization of ROS production by phagocytes, even in closed compartments, such as their phagosomes.

Nonoptical methods

Electrochemical methods were developed since the middle of the 20th century to measure the consumption of O_2 [99]. In particular, amperometric detection using Clark's electrodes was widely used to observe the abrupt consumption of O_2 of the phagocytes. Smaller versions of those electrodes were developed to probe O_2 concentration in volumes down to 20 μl, i.e., with a lower amount of biological material (Lazar Research Laboratories, Los Angeles, CA, USA). In addition, there are examples of direct detection of O_2 concentration variations at the single cell level or in vivo using microelectrodes [100, 101].

More recently, electrochemical methods, inspired from neurosciences and taking advantage of ROS redox properties, were developed at the single-cell level using microelectrodes whose size (~10 μm) is comparable with the cell size [102]. The feasibility of the detection depends directly on the ROS redox half-wave potential on the electrode metal in aqueous oxygenated solution. The other decisive parameter is the lifetime of a given ROS that commands its diffusion inside and outside of the cells and eventually, toward the electrode that can be positioned outside of the cell in its close vicinity. Among the ROS described in this review, quantitative electrochemical detection was reported for superoxide anion and H_2O_2 [102]. More recently, nanometer size electrodes were developed successfully to measure ROS and RNS inside murine macrophages [103]. These new nanoprobes open the path to quantitative electrochemical detection of ROS inside cells, in combination with physiological stimuli. In addition, these electrochemical setups are fully compatible with a simultaneous and complementary fluorescence detection [104, 105], which can be dedicated to localized ROS detection or protein localization.

Optical methods—an overview

Optical methods for ROS detection are widely used and easy to perform (summarized in **Table 1** and reviewed extensively in refs. [96–98, 106]). Among them, SOD-inhibited cytochrome c reduction by superoxide anion is used

TABLE 1. Optical Methods for ROS Detection

Type	Name	Advantages	Limitations	References
Fluorescent dyes	Amplex Red (and Amplex UltraRed)	Very sensitive, red fluorescence, reacts with H_2O_2, peroxidase-dependent	pH-Sensitive below 7 (below 5.5 for Amplex UltraRed)	[96, 107, 108]
	CellROX	Different colors, low photoactivation	No precise information on the dyes' chemistry	[108]
	$DCFH_2$	Dye can be charged into cells or covalently attached to amine groups	Not specific for a particular ROS, risk of photooxidation	[70, 97]
	DHR	Membrane-permeant	Not specific for a particular ROS, lipophilic and may stick to membranes	[97, 109]
	HPF and APF	Specific for highly reactive ROS, APF-sensitive to $HO^{\cdot}$, HOCl, $ONOO^-$, HPF-sensitive to $HO^{\cdot}$, $ONOO^-$	Reduced sensitivity	[108, 110]
Fluorescent proteins (genetically encoded, targetable)	HyPer	Specific for H_2O_2, ratiometric detection	Highly pH-dependent, low dynamic range, reversibility depends on glutathione	[111]
	roGFPs	Reversible	Not really a ROS-sensitive probe but a redox state sensor	[112]
Nanoparticles	Rare earth nanoparticles (YVO_4:Eu)	Not cumulative, no photobleaching, quantitative	Not ROS-specific, need of a dedicated microscope with particular excitation source	[113].
Chemiluminescent probes	Isoluminol	Very low background signal, not cumulative, i.e., reveals ROS at a given time-point, only extracellular, peroxidase-dependent	Signal too weak for optical microscopy at cellular resolution, peroxidase-dependent	[63, 114]
	Luminol	As isoluminol but membrane-permeant		
	Lucigenin	Peroxidase-independent	Signal too weak for optical microscopy at cellular resolution, may generate superoxide	[63, 97]
Absorbance	Cytochrome c	Specific for $O_2^{\cdot -}$, only extracellular, calibration	Limited sensitivity, reversible reaction	[16, 115]

frequently for specific and quantitative extracellular superoxide detection in phagocyte populations. The reduced form of cytochrome c presents a specific absorption band at 550 nm associated with a rather low extinction coefficient of 19 mM^{-1} cm^{-1} [98]. Long periods of incubation should be avoided, as cytochrome c can be reoxidized, leading to an artificially diminished absorbance. Another classical dye is NBT, which forms an insoluble precipitate upon reaction with ROS that can be readily detected by light microscopy. Despite their widespread use, both methods are not very useful for dynamic, quantitative single-cell experiments. Luminescence, generated from the reaction of superoxide anion with specific dyes, mostly luminol, isoluminol, and lucigenin, is a very sensitive method for the detection from small numbers of cells (reviewed in refs. [63, 114]). Even if the chemistry of these dyes is rather complex [97], their major advantage is that they allow a good readout of the kinetics of NOX activity; the start, the amplitude, but also the end of ROS production can be monitored. Attempts to detect luminescence by light microscopy on a single cell have had little success with one exception [121].

To our knowledge, the most important recent developments in ROS detection concern fluorescent probes. Indeed, dedicated tools for fluorescence detection are now common in laboratories, such as fluorimeters or microplate readers that analyze the average behavior of a cell population (typically, 10^4–10^6 cells) and fluorescent microscopes or flow cytometers that allow the measurement of one or several parameters for each individual cell. Cytometry experiments yield information on many individual cells in a population (typically, 10^4), whereas microscopy offers an unequaled spatiotemporal resolution of the fluorescent signals but only for a few cells. The latter may appear laborious, but it is the only one able to discriminate individuals with close but different behavior [122]. Some dyes are only compatible with a few detection modes, essentially for sensitivity reasons. Ideally, to dissect the oxidative response of

phagocytes, the fluorescent dye should have the following properties:

- The fluorescence has to be modulated by a specific reaction with one type of ROS whose chemistry must be clear and fully understood. The dynamic range of the variations should be large. The reaction with ROS could change the absorption, the fluorescence wavelengths, the extinction coefficient, the quantum yield, or the fluorescence lifetime of the dye. In some cases, ratiometric approaches that allow more accurate and quantitative measurements can be considered.
- The rate of the reaction with ROS needs to be characterized and be fast enough to follow accurately the production rate that should be the limiting step of the process. If the rate is too slow, endogenous antioxidants might also react with ROS before their reaction with the probe.
- As some ROS might diffuse away from their production site, the dye should be targetable to locally probe the ROS concentration.
- The dye quantity should be in good adequacy with the amount of ROS produced to avoid any saturation of the recorded signal.
- Ideally, the signal should be reversible to monitor variations in the production (increase as well as decrease or stop).

Classical fluorescent probes

The most widely used commercial organic dyes usually refer to small molecules that are indeed redox probes; nonfluorescent in their reduced form, they become fluorescent after oxidation. This property provides them an unequaled, dynamic range of their fluorescence response. Those so-called "leuco" dyes belong to four families of molecules: (1) fluoresceins, e.g., $DCFH_2$; (2) rhodamines, e.g., DHR; (3) phenoxazines, e.g., Amplex Red; and (4) ethidines, e.g., DHE [97]. The chemical reactivity, including specificity and reaction rates, was reviewed in detail [96–98]. None of these dyes fulfills all of the criteria of an ideal probe mentioned above. For example, they present a cumulative fluorescent signal that is not easily compatible with the precise monitoring of a decrease or the end of the production. Therefore, they are not fully adapted to the dissection of the phenomena associated with the end of the ROS production in phagocytes, for which many open questions are remaining. Nevertheless, these commercial and thus, accessible probes proved their efficiency in many biological studies for characterizing the overall ROS production, as illustrated for $DCFH_2$ [116].

$DCFH_2$ is most often used in a cell-permeant form, in which polar groups of the dye are protected by esters. After diffusion of the dye into the cell, esters are hydrolyzed by cellular esterases. $DCFH_2$ that does not react with superoxide and H_2O_2 alone is oxidized efficiently to DCF by a number of different conditions, including H_2O_2 in the presence of peroxidases or by $HO^{\bullet}$ [97]. In addition, in phagocytes that produce $NO^{\bullet}$, the oxidation by peroxynitrite itself or its decomposition products may considerably contribute to the overall DCF signal [98]. We also identified a new oxidation product in the presence of HOCl—Xfluo [122]—whose photophysical properties differ significantly from DCF. Despite the difficulties that can be encountered with the use of $DCFH_2$ (reviewed in ref. [97]), it remains an interesting global ROS indicator if great care is taken for data acquisition and interpretation using general guidelines for control experiments [98, 123]. Finally, $DCFH_2$ exists as succinimdyl ester, a very convenient form for phagocytes studies, considering that it allows covalent labeling of internalizable objects: IgG [118], zymosan [64, 117], beads [124], and yeast [70, 122]. After phagocytosis, the dye is localized inside of the phagosome, and its oxidation rate reflects exclusively the kinetics of phagosomal ROS production as a result of the initial production of superoxide anion by the NOX [122]. With this method, we showed that the time course of phagosomal ROS production depends on the type of opsonization using complete serum or complement-depleted serum [70]. We also showed that the dye, after its immobilization on the yeast, was much less sensitive to photo-oxidation than the free dye. It is also possible to encapsulate the fluorescent ROS probe in a polymer that forms nanoparticles to exploit the advantages of these nanometric objects [125]. Indeed, DCF diacetate, a nonfluorescent lipophilic precursor of $DCFH_2$, was trapped successfully in a hydrophobic polymer that formed nanoparticles [126]. Despite an important pH sensitivity of the nanoparticle's response, the properties of the polymer control the accessibility of the reduced dye and increase its selectivity toward H_2O_2. The grafting of the fluorescent probe on an object, such as nanoparticles, zymosan, beads, or yeast, offers many additional possibilities, such as the simultaneous observation of different parameters (ROS, pH, protease activity) via probes of different colors or the development of ratiometric approaches using a ROS-insensitive reference probe on the same object [127–129].

DHR, like $DCFH_2$, does not react with superoxide anions and H_2O_2 alone, whereas it forms the fluorescent rhodamine 123 after reaction with $HO^{\bullet}$ or HOCl. However, DHR reacts with H_2O_2 in presence of peroxidase [97]. The lipophilicity of the reduced form facilitates its diffusion across membranes. After oxidation, rhodamine 123 has a positive charge and is trapped effectively in the cells. The fluorescence increase upon cell activation can be monitored by flow cytometry or fluorimetry [97, 109]. In addition, a DHR flow cytometry-based assay is commonly used with phagocytes as a diagnostic screening test for CGD [79].

Amplex Red [107] readily reacts with H_2O_2 in a 1:1 stoichiometry in the presence of peroxidases to form the bright fluorescent resorufin whose fluorescence is highly pH-sensitive below pH 7. The more recent Amplex UltraRed (Molecular Probes, Life Technologies, Carlsbad, CA, USA) is more sensitive to ROS and pH-insensitive down to pH 5.5. It is possible to evaluate the concentration of H_2O_2 produced through an external calibration using known concentrations of H_2O_2, which is a great advantage in terms of quantification of the oxidative response. Amplex Red does not penetrate inside of the cell and is used in combination with HRP; thus, H_2O_2 detection is limited to the extracellular production. In combination with another H_2O_2 sensor (blue or green) that can be targeted to a specific subcellular localization, one can imagine following the diffusion

of H_2O_2 in and out of the cell. Amplex Red is commonly used in microplate readers. Details on its reactivity, advantages, and drawbacks can be found elsewhere [96].

New fluorescent dyes to detect superoxide anion, H_2O_2, and HOCl

Increasing the specificity of the ROS-sensitive fluorescent dye, along with the available color palette, is a great challenge for the chemist. Indeed, the ability to monitor simultaneously one ROS at different locations in the presence of several ROS in the same compartment is of great interest to improve the understanding of the phagosome chemistry, the different roles of MPO, the amount of ROS that escapes the phagosome, and the phagocyte itself or the interplay among the different actors of the microbicidal activity. To this end, several groups recently reported new dyes described as more selective for superoxide anion, H_2O_2, or HOCl. In their actual form, none of those new dyes are easily targetable to the phagosome; derivatives with amine- or thiol-reacting groups would be useful for covalent attachment to proteins and particles, as described above for $DCFH_2$. A combination of ROS-sensitive dyes with the SNAP-tag technology provides another promising approach for intracellular targeting of the probes [130]. We adopted a classification based on the selectivity of those new dyes in the following paragraphs to give several examples of their properties, propose improvements, and expose their applications for phagocyte biology.

Detection of superoxide anion

To increase the selectivity toward $O_2^{\cdot -}$, a strategy based on the nucleophilic character of $O_2^{\cdot -}$ and on a protection-deprotection chemistry of phosphinate or benzenesulfonyl fluorescent derivatives was proposed recently [111, 131–133]. Quantitative kinetic data are not always available, but their reaction rate with the superoxide anion is most probably slower than the reaction rate of the widely used DHE [133]. An example of a particular interest is a phosphinate-based probe that specifically reacts with $O_2^{\cdot -}$ and produces a bright red fluorescence signal after deprotection [132]. Such a red dye at the edge of the visible spectrum is of particular interest, as it is compatible with the simultaneous detection of several other parameters using multilabeling approaches. A fluorescence increase was also observed in macrophages loaded with the dye after PMA stimulation [132]. Despite the clear selectivity of the dye, a loading before the activation itself, as well as controls with inhibitors of the enzymatic systems involved in the ROS production, would increase the impact of this report. Finally, further chemical improvements are needed to reduce the pH sensitivity of this red dye in the pH 7–8 range.

Detection of H_2O_2

Among all of the strategies developed to detect H_2O_2 with nonredox dyes [97, 98], we will focus on dyes based on chemoselective boronate deprotection by H_2O_2 [134]. This broad family, developed during the last 10 years, offers probes for H_2O_2 detection with a large dynamic range, even at low levels and in many conditions. Several probes were engineered to cover the entire visible spectrum (useful for multicolor approaches), to be used as ratiometric probes for quantitative analysis of H_2O_2 production, to be targetable using the SNAP-tag methodology (for spatial detection), to detect simultaneously two parameters, such as H_2O_2 and pH, or to detect H_2O_2 in vivo [134]. H_2O_2 is not very polar and may easily escape the phagosome or other sites of production. It will be responsible for oxidative damage or signaling in the phagocytes (eventually after chemical transformation in the cytosol) or outside of the cell. It is thus of great interest to measure selectively H_2O_2 at different locations and at widely different concentrations. Despite the relatively slow reaction rates of those dyes with H_2O_2 [73, 135], they offer the possibility to follow the kinetics of H_2O_2 production during several minutes [136]. Recently, the reactivity of $ONOO^-$ toward small boronate molecules has been studied [137], which questioned the selectivity of aryl boronate toward H_2O_2. This possible side reaction has to be kept in mind in phagocytes, where NO synthases can be activated, such as macrophages. Nevertheless, very interesting results concerning the spatial ROS production can be obtained with such boronate sensors. For example, an orange probe, PO1, was tested in combination with a green probe, APF, specific for HOCl, $HO^{\cdot}$, and $ONOO^-$ in macrophages. Probes were localized inside of the cell, and the staining was homogeneous. When H_2O_2 or HOCl were applied outside of the cell, the fluorescence increase in each color channel was ROS-specific. After activation with PMA, three distinct classes of vacuoles were observed with distinct fluorescence color: some produced only H_2O_2, others only HOCl, $HO^{\cdot}$, and $ONOO^-$, and some produced both [73]. This result highlights the heterogeneity of the signals at the cellular levels and the need of specific dyes to understand the interplay between the different ROS and their production pathways.

Detection of HOCl, $HO^{\cdot}$, and $ONOO^-$

New molecules for the detection of HOCl alone were developed in the past years and tested in living cells with microscopy techniques. These dyes are of particular importance for phagocyte studies, as they might allow addressing specifically the role of MPO and HOCl in the phagosome chemistry and more generally, in phagocytes. Most of those molecules described until now are off-on probes, based on a rhodamine scaffold [138–140]. These probes are cell-permeant and can detect cellular HOCl production efficiently in neutrophils or macrophages. Nevertheless, some of them present a pH sensitivity that might prevent an accurate HOCl quantification [138, 140]. For in vivo exploration of HOCl production, far-red to near-infrared fluorescent probes offer many advantages, including low phototoxicity or better penetration of excitation beams. Such HOCl probes were developed recently [141, 142] and applied successfully to detect HOCl in a peritonitis mouse model stimulated with zymosan and PMA [136] or with thioglycollate [142]. Based on the same scaffold but with another reaction mechanism, a promising near-infrared probe was developed recently [143]. It is not specific and detects HOCl, $HO^{\cdot}$, and $ONOO^-$, but its reversibility is a great advantage that needs to be exploited further.

Commercial dyes

All of the new fluorescent dyes described in the previous paragraphs are promising and should become available in cell-permeant or targetable forms for testing in various biological settings. A few of them are now commercial (Molecular Probes), such as APF and HPF, developed by the Nagano's group [110], which are specific for HOCl, $HO^{\cdot}$, and $ONOO^{-}$, despite a high cellular leakage and a low sensitivity [106]. Molecular Probes also developed its own dyes, for example, the CellROX dyes that exist in three colors: green, orange, and deep red. The first binds to DNA, whereas the latter two are cytosolic, opening the way for multiparameter studies. Further information on their chemistry might be useful for the research community.

FPs as ROS biosensors

FPs from the avGFP family consist of an 11-stranded β-barrel with a coaxial α-helix, bearing the chromophore that results from an autocatalytic cyclization of three residues (Ser65-Tyr66-Gly67 in avGFP) that occurs spontaneously after the folding of the protein. In the past 15 years, avGFP has been engineered to give birth to a large palette of FPs with various colors and/or chemical properties, including sensitivity to ROS. In particular, roGFPs [112], HyPer [111], and cpYFP [120] have been reviewed in detail recently [98]. The responses of roGFP and HyPer and in particular, their reversibility are closely related to the glutathione system thus not being fully appropriate to measurements in phagosomes. cpYFP was proposed as a selective superoxide biosensor, whose fluorescence increases in the presence of the anion [120]. However, there is, unfortunately, no explanation yet for the changes of the fluorescence properties induced by superoxide, and the specificity of cpYFP is the subject of an ongoing debate [144–146]. Other attempts to use the direct reaction of FPs with ROS for biosensing were also reported [120, 147–149]. Among all reactive species discussed in this review, the fluorescence properties of EGFP were particularly altered by HOCl, probably as a result of the direct chromophore oxidation [148]. This observation was used to monitor MPO activity and HOCl production in phagosomes with GFP-tagged *S. aureus* [150]. As a major advantage, FPs with an appropriate signal sequence offer a specific targeting to different subcellular locations of the phagocyte or the pathogen itself (in its cytosol, at its surface, etc.), opening the path to a better understanding of the host-pathogen interplay. Furthermore, using cell-specific promoters, theses sensors can be introduced into the genome to generate transgenic animals with cell or tissue-specific sensor expression. Nevertheless, phagocytotic cell lines are not easy to transfect, the expression level of the sensor can be low in comparison with ROS production, and the sensor may become rapidly saturated.

Finally, HyPer, cpYFP, and likely, all GFPs and YFPs are pH-sensitive [151]. Thus, despite their resistance to proteases [152], green and yellow variants might not be fully appropriate for measurements in phagosomes, where the pH may vary by >1 pH unit around physiological pH [56]. There are now cyan [153–155] or red [156] pH-insensitive FPs, and they should be used advantageously to develop new ROS sensors. In addition, the use of the these proteins for ROS detection will leave free a large part of the visible spectrum and thus, is spectrally compatible with the combined detection of other parameters in the same cells using different biosensors, also based on FPs.

Nanoparticles

Rare earth nanoparticles (YVO_4:Eu) were used for the quantitative and dynamic measurement of H_2O_2 [113]. Eu^{3+} ions, trapped in the nanoparticles, display a strong fluorescence at 617 nm. They can be reduced to Eu^{2+} through a photoinduced mechanism requiring a relatively high intensity delivered by a specific laser source at 466 nm. Subsequent application of H_2O_2 leads to the oxidation of Eu^{2+} to Eu^{3+} and recovery of fluorescence at 617 nm. Many cycles of reduction-oxidation can be performed without any loss of the fluorescence properties, as this kind of nanoparticle is usually not amenable to photobleaching. The amplitude and speed of the fluorescence recovery depend on the H_2O_2 concentration, opening the path to its quantitative and time-resolved detection with a dynamic range of 1–45 μM and a temporal resolution of 30 s. YVO_4:Eu nanoparticles were used to detect H_2O_2 production by vascular smooth muscle cells after stimulation by an endothelin-1 or a platelet-derived growth factor. After rinsing the stimuli solutions, the decrease of H_2O_2 production was also observed. This reversibility is a unique feature of these rare earth nanoparticles that could be very useful in characterizing the end of the ROS production in phagocytes as well. Phagocytosis of these particles might be possible after opsonization, and their attachment to antibodies or microorganisms needs to be explored. The fluorescence of single nanoparticles offers a spatial resolution comparable with the particle size, i.e., 50 nm. We should note that the oxidation of Eu^{2+} to Eu^{3+} can also be triggered by other oxidants present in the phagosome, such as HOCl, and with a lower efficiency by superoxide anion as well.

In vivo detection of ROS

If ROS do more than kill pathogens in closed phagosomes, then we would like to detect them in their physiological environment, for example, an inflamed tissue. In vivo imaging has taken big steps forward with more sensitive cameras, two-photon excitation, and multicolor FPs in living cells or tissues. The use of red (>600 nm) light excitation and emission reduces absorption in animal tissues. In terms of in vivo ROS detection in whole animals, at least three promising approaches have been explored.

$DCFH_2$ has been used to detect ROS in animals [157]. The fluorescent biosensors HyPer and glutaredoxin 1-roGFP2 were expressed in the transparent animal *Caenorhabditis elegans* for successful in vivo ROS detection [158]. Luminescence has the advantage of an extremely low background, as no illumination is required. ROS- or RNS-dependent luminescence was detected in whole mice following LPS-induced inflammation after injection of the luminescent probe L-012, which turned out to be much more sensitive than luminol [159]. Another

luminescence approach used a caged version of luciferin—PCL-1. Reaction with H_2O_2 releases luciferin from PCL-1. In the presence of luciferase and ATP, luciferin is oxidized in a red light-emitting reaction. In mice expressing firefly luciferase, PCL-1 allowed detection of intracellular and extracellular ROS in a wide variety of tissues [160]. In combination with cell- or tissue-specific luciferase expression, this method opens numerous opportunities for site- or cell-specific ROS detection in whole mice. EPR, also called electron spin resonance, is a noninvasive technique for the detection of paramagnetic substances in vitro and in vivo. EPR imaging is used to determine the redox status of various tissues in small animals [161]. For example, a 3D redox map of the mouse brain after LPS injection was obtained every 3 min with a spatial resolution (x–y) below 1 mm [162]. For detection of particular ROS, so-called spin traps are added to the biological sample to react with the short-lived radicals and form more stable paramagnetic adducts that are detectable by EPR [163, 164]. The selectivity and the half-life of the spin trap in the sample is critical for the sensitivity and specificity of the ROS detection. With more spin traps to come, EPR may, in the future, provide time-resolved 3D maps of particular ROS in small animals. The spatial and temporal resolution of these whole animal techniques is much lower than for single-cell microscopy. Issues of specificity are at least as complicated as for cellular techniques. Nevertheless, in vivo imaging of ROS becomes reality and opens a whole new field of ROS-related research.

CONCLUSION

ROS production in phagocytes serves multiple purposes, from cell signaling to microbial killing. Numerous reactive species over a wide range of concentrations are involved. Timing and location of ROS production, diffusion, and scavenging are critical for the outcome. To illustrate this complexity, we referred the reader to reviews that deal with fundamental aspects of phagocyte ROS production wherever possible, and we apologize to those whose work is not mentioned explicitly. The phagocyte community needs new probes for high-level (phagosomal) ROS detection, as well as low-level (signaling) detection. The recent interest in redox biology has pushed the development of new detection methods with improved specificity, better sensitivity, and new ways to localize the detector with cells. The harsh conditions of the phagosome (pH, proteases, level, and diversity of ROS) are particularly challenging to any detection method, and the specificity of any new dye should be tested under these conditions. Subcellular targeting of FPs and the SNAP-tag technology for organic dyes are good candidates to improve the spatial resolution of ROS detection. The end of ROS production may be addressed with nanoparticles. The spatiotemporal correlation of ROS production with signaling events will be addressed by combining dyes of different color that become available now. We are convinced that new organic compounds, as well as FPs, will increase our choice to explore in more detail the why, when, and where of phagocyte ROS production.

AUTHORSHIP

All authors contributed equally to this review.

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