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Review

Regulation and termination of NADPH oxidase activity

T. E. DeCoursey[a,*] and E. Ligeti[b]

[a] Department of Molecular Biophysics and Physiology, Rush University Medical Center, 1750 West Harrison, Chicago, Illinois 60612 (USA), Fax: +1 312 942 8711, e-mail: tdecours@rush.edu
[b] Department of Physiology, Semmelweis University, P.O. Box 259, 1444 Budapest 8 (Hungary)

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Abstract. NADPH oxidase of phagocytes plays a crucial role in host defense by producing reactive oxygen species (ROS) that are intended to kill invading microbes. Many other cells produce ROS for signaling purposes. The respiratory burst oxidase in human neutrophils is the main but not exclusive subject of this review, because it is archetypical and has been studied most extensively. The activity of this enzyme must be controlled in phagocytes to prevent collateral damage, and in non-phagocytic cells to perform its signaling role. With many stimuli, NADPH oxidase activity is transient. Various forms of evidence indicate that sustained NADPH oxidase activity requires continuous renewal of the enzyme complex, without which rapid deactivation occurs. This review considers mechanisms that have been proposed to terminate the phagocyte respiratory burst. Changes in the phosphorylation state of $p47^{phox}$ and in the species of nucleotide bound to Rac seem to be the dominant factors in deactivation.

Key words. Respiratory burst; phagocytes; reactive oxygen species; leukocytes; neutrophil; Rac; G proteins; phosphatase.

Introduction

NADPH oxidase is a vital component of host defense. This enzyme produces superoxide anion, $O_2^{\cdot-}$, the precursor to a number of reactive oxygen species (ROS) that play essential roles in killing many types of bacteria and other invaders [1–5]. Mutations of any single component of the oxidase that prevents its function result in hereditary chronic granulomatous disease (CGD) [6, 7]. The CGD patient is susceptible to infection and the disease is generally lethal if not treated [8–10]. In a resting phagocyte, the NADPH oxidase complex is disassembled, with its components segregated into different parts of the cell. Upon stimulation, for example during phagocytosis, the half-dozen components of the enzyme complex assemble [11–13] and begin to work. The conversion of O_2 to $O_2^{\cdot-}$ increases the O_2 consumption of the cell by up to 100-fold [14]; whence the misnomer "respiratory burst [15]. Because O_2 consumption still occurs when mitochondria are poisoned, the "respiratory burst" does not reflect true cellular respiration; the O_2 is simply converted into $O_2^{\cdot-}$ and other ROS [16–19]. A huge literature delineates and elaborates the intricate process of activation and assembly of the NADPH oxidase complex [selected recent reviews: 9, 20–26]. The respiratory burst is transient [15, 27, 28], however, and less attention has been directed toward understanding the factors that limit and terminate the burst. Furthermore, abundant evidence indicates that there is continual turnover of oxidase components during the respiratory burst [20, 28–34]; thus NADPH oxidase activity in a cell may depend on a dynamic balance between the rate of assembly or activation and the rate of deactivation. The intent of this review is to examine the current state of knowledge on this question.

* Corresponding author.

Regulation of NADPH oxidase activity can be conceptualized in three stages: (1) initiation, the assembly and activation of the enzyme complex, (2) maintenance, a period of continuous turnover, and (3) termination or deactivation of the respiratory burst. During stage 2, various mechanisms presumably modulate the activity of the NADPH oxidase complex, but non-cataclysmically. Stage 3 mechanisms actually turn the enzyme off altogether. The main focus of this review is on stage 3 mechanisms, but because it is often difficult to distinguish these from stage 2 and even stage 1, we will discuss all mechanisms that are relevant to respiratory burst termination. We describe the termination of the respiratory burst as "deactivation" of NADPH oxidase activity, which should not be taken to imply any specific mechanism beyond "cessation of activity." Transcriptional regulation of the expression of various oxidase components occurs on a slower time scale than we consider here, and has been reviewed recently [25].

The ability of phagocytes to generate enormous quantities of ROS must be controlled. Control can be exerted at any of the three stages mentioned above, but deactivation is clearly a key process. Although phagocytes produce ROS with the intent to kill pathogens [35], too much $O_2^{\cdot-}$ or $O_2^{\cdot-}$ generated at an inappropriate location can cause serious damage to the host organism. For example, ROS are thought to contribute to the pathogenesis of atherosclerosis [36], ischemic stroke [37], ischemic liver necrosis [38], alcohol-induced liver disease [39], Parkinson disease [40], and aging [41].

The relevance of the subject of NADPH oxidase deactivation is not limited to the phagocyte respiratory burst. Many cells use ROS generated by an NADPH oxidase that is identical to or homologous with that in phagocytes, as signaling mechanisms. Cells that secrete ROS constitutively as signaling molecules must regulate its production carefully.

Methods of measuring of NADPH oxidase activity

There are several ways to measure the activity of NADPH oxidase, each of which has advantages and limitations. These include: (***i***) O_2 consumption, (***ii***) $O_2^{\cdot-}$ or H_2O_2 production by intact cells or in a cell-free system, and (***iii***) electron current. (***i***) Because O_2 is a substrate of NADPH oxidase, the increase over basal levels of O_2 consumption includes O_2 that is converted to $O_2^{\cdot-}$. Any increase in consumption of O_2 by mitochondria would lead to inflated estimates of NADPH oxidase activity. This error is 70% in monocytes but negligible in neutrophils [42]. In neutrophils, essentially all of the O_2 consumed during the respiratory burst is converted into $O_2^{\cdot-}$ [1, 18]. Nevertheless, oxygen electrodes are cumbersome and require a large number of cells. (***ii***) The detection of $O_2^{\cdot-}$ is constrained by two factors. First, only extracellular release is accessible to the standard and quantitative cytochrome *c* reduction assay. Despite early suggestions to the contrary, $O_2^{\cdot-}$ does not permeate cell membranes freely, except when mediated by anion transporters such as band 3 [43–46]. Detection of intracellular $O_2^{\cdot-}$ production requires measurement of chemiluminescence, and is more difficult to quantitate correctly. The second limitation is that the lifetime of $O_2^{\cdot-}$ is brief, especially when present at high concentration, and spontaneous dismutation can potentially reduce the signal that is captured. Detection of H_2O_2 has the advantages that H_2O_2 is relatively stable and is also membrane permeable. It is beyond the scope of this review to delve further into these techniques. The study of NADPH oxidase in a "cell-free system" has enabled enormous progress in understanding the function of this complex enzyme. The power of this reductionist approach is simultaneously a limitation for the present subject. When NADPH oxidase is reconstructed from its component parts, their individual functions can be dissected, but the mechanisms that terminate activity in this system may not apply to the oxidase in intact cells. (***iii***) Finally, measurement of electron current is a recently developed and extremely powerful approach [47]. Assuming that extraneous electrical "leak current" can be corrected, the electron current is a direct real-time reflection of the activity of NADPH oxidase in the cell. Electron currents can be detected in single cells or in patches of membrane excised from cells [48]. Dialysis of the cytoplasm in whole-cell measurements disrupts signaling pathways, but this can be overcome by use of the perforated-patch approach [49]. Use of excised patches of membrane provides profound opportunities to study regulation and interaction of the components, but being essentially a cell-free system, its strength is derived from its reductionism. In summary, Heisenberg's uncertainty principle applies also to biology: all measurements require disruption of the physiological system, and the nature and consequences of this disruption must be considered before extrapolating the results to living cells.

Composition and assembly of the NADPH oxidase complex

Figure 1 illustrates the main components of the NADPH oxidase complex. There is characteristically a delay between most stimuli that trigger a respiratory burst and the response [50, 51], which was detected as $O_2^{\cdot-}$ or H_2O_2 release (fig. 2b,c). During this delay, some or all of the following events must occur. (i) Agonist (e.g., an opsonized particle or a soluble agent such as fMet-Leu-Phe) binds its plasma membrane receptor. In the case of some artificial stimuli, such as phorbol myristate acetate

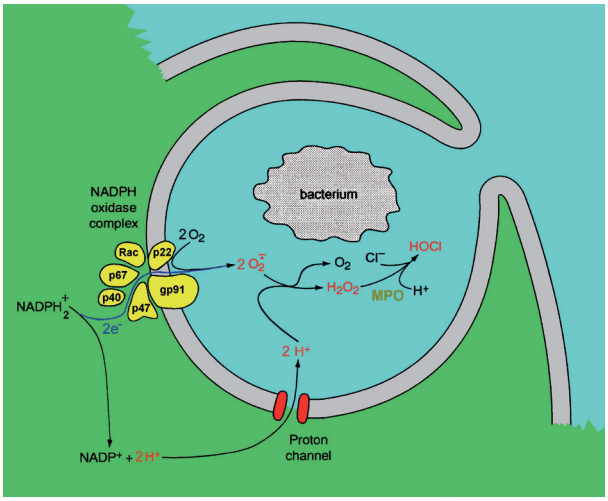

Figure 1. Components of the respiratory burst. NADPH oxidase comprises two membrane-bound proteins, gp91^{phox} and p22^{phox} that coexist as a heterodimer called flavocytochrome b_{558}. The cytosolic proteins are thought to exist partially pre-assembled in a heterotrimeric complex of p67^{phox}, p47^{phox}, and p40^{phox}, with Rac acting independently. The voltage-gated proton channel is a separate molecule [214, 215] that is, however, required for continuous function [78]. Reproduced from DeCoursey Ref. [67] with permission from the American Physiological Society.

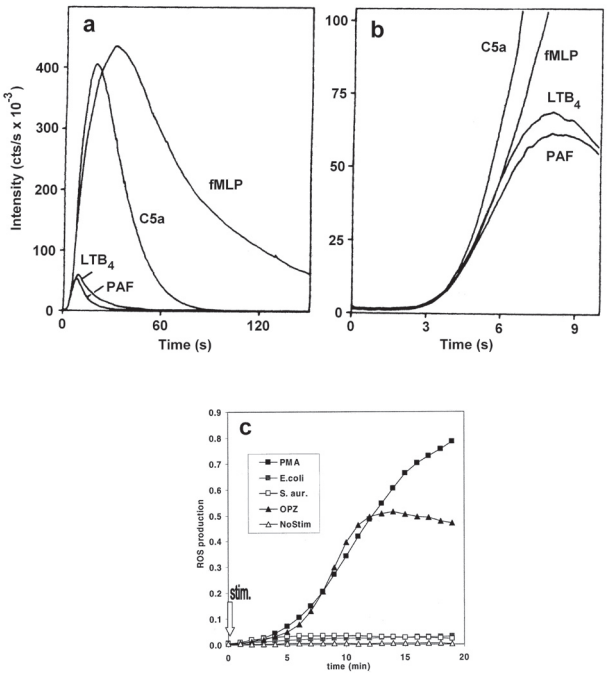

Figure 2. *(a, b)* Transience of the respiratory burst elicited by four physiological agonists: chemotactic peptide fMet-Leu-Phe (previously termed fMLP, an abbreviation that has been rendered obsolete and incorrect by the introduction of one-letter abbreviations for amino acids), complement C5a, leukotriene B_4 LTB_4, and platelet-activating factor PAF. H_2O_2 production was assessed by chemiluminescence. As emphasized by the enlargement in **b**, the initial time course is identical, but the onset of deactivation and the magnitude of response vary dramatically. Reproduced from Wyman et al. [217] with permission of the first author and of the *Journal of Biological Chemistry*, *(c)*. Time-course of the respiratory burst initiated by natural and artificial stimuli. Production of ROS was measured with lucigenin-based chemiluminescence and expressed in relative fluorescence units. Neutrophils were stimulated by a tenfold excess of opsonized *Escherichia coli* or *Staphylococcus aureus* or by the model stimuli 100 nM PMA or 1.2 mg/ml opsonized zymosan. B.K. Rada and E. Ligeti [unpublished data].

(PMA), no plasma membrane receptor is involved. (ii) Protein kinases – including protein kinase (PKC) [52] – phosphorylate several components, the most critical and extensively phosphorylated being p47^{phox} [12, 13, 53], which unfolds from an autoinhibitory conformation in which the interactive regions are cryptic to an extended shape receptive to binding to cytochrome b_{558} [54, 55]. (iii) Phospholipase A_2 is activated and generates arachidonic acid [56, 57], which activates NADPH oxidase [58]. (iv) The four cytosolic components comprising the heterotrimer of p67^{phox}, p47^{phox}, and p40^{phox} [59–62, but see also ref. 63], as well as Rac [64, 65], translocate to the phagosome or plasma membrane where flavocytochrome b_{558} is located, and assemble [11–13]. (v) NADPH binds to the assembled complex. (vi) At approximately the same time, proton channels are converted to a readily opened "activated" state [49, 66, 67], perhaps by arachidonic acid [68–73] or by phosphorylation by PKC [74, 75] or tyrosine kinase [76, 77]. Some of the mechanisms considered for deactivation of the respiratory burst are essentially reversal of steps that lead to activation.

Once the NADPH oxidase complex is assembled, control over the rate of enzyme activity could be exerted at a number of points. Processes that occur and thus could be rate limiting are illustrated in figure 3. The steps include: NADPH biosynthesis (1), NADPH binding to its binding site on gp91^{phox} (2), NADP release after loss of two electrons (3), electron transfer (4–7) from NADPH to FAD (4), FAD to the inner heme (5), inner to the outer heme (6), and heme to O_2 (7), O_2 binding to its pocket (8), $O_2^{\cdot-}$ release (9), and dismutation of $O_2^{\cdot-}$ to H_2O_2 (10), which might prevent reversal of electron flow.

Ultimately, the function of NADPH oxidase requires electron transfer across the membrane. If electron transfer is rate limiting under certain conditions, it could determine the turnover rate of NAPDH oxidase. Depolarization of the membrane potential to +200 mV turns NADPH oxidase off altogether, an effect hypothesized to reflect rate-limiting electron transfer between the two hemes (step 6 in fig. 3) [78, 79]. These hemes are located between two membrane-spanning alpha-helical protein segments, and are coordinated between pairs of His residues [80]. The amino acid separation between these heme-binding His residues is about 13 amino acids in NADPH oxidase, as well as in several other electron-transporting molecules that share this motif (table 1). Although NADPH oxidase-mediated electron current was strongly voltage

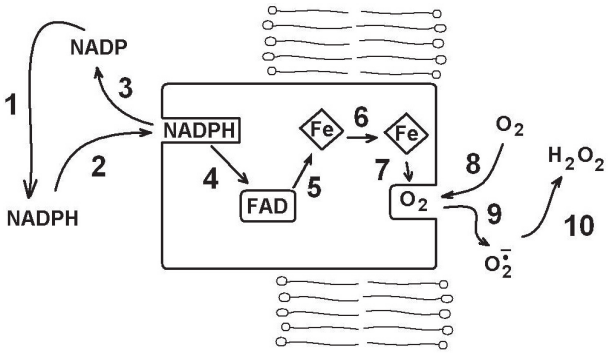

Figure 3. Potential rate-limiting steps in the electron transport cycle. Individual steps are described in the text. The general arrangement of these elements is based on a variety of reports [26, 80, 216].

sensitive at large positive voltages, it was practically voltage insensitive at more physiological potentials, thus some other process is most likely rate determining at physiological voltages [78]. This limitation may be the NADPH supply (steps 1 or 2 in fig. 3) [81].

Cross and colleagues have made several suggestions regarding stages at which electron transport might be regulated. They presented evidence for an intermediate state of activation of NADPH oxidase, in which electrons are transferred from NADPH to FAD, but not onward to heme [82]. A recent study using atomic force microscopy indicated that a conformational change in cytochrome b_{558} initiates electron transfer from NADPH [83]. Beyond these ideas, most views of the regulation and deactivation of NADPH oxidase do not involve specific steps in electron transfer.

Regulation of activity by availability of substrate (NADPH and O_2)

Because the O_2 concentration has little effect on $O_2^{\cdot-}$ generation by neutrophils until its partial pressure is reduced below 5 % of atmospheric levels [84], under normal conditions, the supply of O_2 would not limit the rate of NADPH oxidase function. The situation with NADPH is less clear. Cytoplasmic levels of NADPH are considered to be 50–100 µM [26, 85–88], which is comparable to its affinity for the oxidase; the K_m estimated in reconstructed cell-free systems is 26–263 µM [14, 86, 88–93]. Thus, changes in the concentration of NADPH likely would affect NADPH oxidase activity. This expectation is supported by observations of profound impairment of the rate of $O_2^{\cdot-}$ production in instances of hereditary low-affinity NADPH binding to the oxidase [94, 95]. It should be noted that NADPH must be synthesized continuously during the respiratory burst. A free NADPH concentration of 50 µM in an 8-µm-diameter cell would sustain NADPH oxidase activity in an eosinophil with 35 pA of electron current [96] for only 37 ms before being totally depleted. NADPH consumed during the respiratory burst in neutrophils is believed to be produced mainly via the hexose monophosphate shunt (HMS) [1, 14, 16, 17, 97–101]. Confirmation that the HMS provides most of the NADPH required for the *in vivo* respiratory burst comes from patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. When the deficiency is severe (<1% normal activity), O_2^- generation and bactericidal activity are impaired and the syndrome resembles CGD [85, 102]. Bactericidal defects are apparent with <20 % normal G6PD activity, and a large safety factor is deducible from the observations that subjects with >20 % G6PD are asymptomatic [85].

NADPH synthesized during the respiratory burst is derived from glycogen breakdown [98]. Neutrophils have high glycogen content; when converted to glucose equivalents, on the order of 60 mM [103]. Glycogenolysis increases during the respiratory burst [16, 104, 105]. Furthermore, oxygen uptake during the respiratory burst is the same in the presence or absence of glucose [16], indicating that exogenous glucose is not a major source of NADPH. From all of these considerations, the NADPH concentration is clearly crucial to NADPH oxidase activity. Indeed, control of NADPH oxidase activity by substrate concentration has been explicitly proposed [51]. Recent data suggest that the NADPH concentra-

Table 1. Membrane proteins that translocate electrons *via* pairs of bis-His coordinated hemes

	1st pair	Separation (amino acids)	2nd pair	Separation (amino acids)	ΔE_m	Electron transfer time	References
FRE1	His^{294} His^{308}	13	His^{364} His^{378}	13	?	?	218
***bc*$_1$ complex**	His^{97} His^{111}	13	His^{198} His^{212}	13	140 mV	0.1 ms	219, 220
***b*$_6$*f* complex**	His^{82} His^{96}	13	His^{183} His^{198}	14 *	~0 mV	?	221, 222,W. A. Cramer [personal communication]
gp91*^{phox}*	His^{101} His^{115}	13	His^{209} His^{222}	12	–40 mV	≤ 3 ms	80, 218, 224, 225

* A 25º Phe kink brings the hemes closer together.

Table 2. Time course of the respiratory burst induced by various agonists in intact human neutrophils

Agonist	Delay (min)	Peak (min)	Duration (min)	Maximal rate (–pA)	Temp. (°C)	Method	References
PMA	0.3–2.0	1–4	∞	8.0	37	$O_2^{\cdot-}$	109, 226
PMA	3 ($t_{½}$)			2.3	20	I_e	49
AA	0	0.1		15	37	$O_2^{\cdot-}$	117, 226
AA	0+	1–3	3–≥6	8	20	I_e	73*
A23187, ionomycin	0.2–1.0	1.0–2.5	2.5–4.5	6.4–8.0	37	CL, $O_2^{\cdot-}$	109, 217
Opsonized zymosan	0.8		20	8.4	37	$O_2^{\cdot-}$	27, 228
Opsonized *Neisseria meningitidis*	15	25–30	90	0.30	37	DCF†	107
opsonized *Saccharomyces aureus*	0.17			0.51	37	H_2O_2	50
IgG-coated latex	0.37	0.85	1.2	5.62	37	O_2	106
Digitonin	1–2	>1.5		2.9	37	$O_2^{\cdot-}$	51
PAF	0.04	0.1	0.4		37	CL	217
fMet-Leu-Phe	0.04	0.7	2		37	CL	217
C5a	0.04		1.0	0.37	37	CL	227, 217
LTB_4	0.04	0.1	0.6	0.054	37	CL	227, 217
MBP	3–30	40–70	>100	0.37–0.54	RT‡	CL, $O_2^{\cdot-}$	227
Retinoid	4.6	~6	∞	10.1	37	$O_2^{\cdot-}$	225
Fluoride	10	20	25–40	3.1	37	$O_2^{\cdot-}$	116

Most values are approximate and vary from study to study as well as with the concentration of agonist; many were extracted from published figures. The delay is defined as the intersection of a tangent to the maximal rate with the baseline, as in previous studies [51, 226]. The duration is the time between stimulus and a decrease to ~10% of the peak rate. The maximal rate of $O_2^{\cdot-}$ production assessed by various methods ($O_2^{\cdot-}$ = cytochrome *c* reduction, O_2 = O_2 consumption, H_2O_2 = H_2O_2 release, I_e = electron current, or DCF = fluorescence of 2',7'-dichlorodihydrofluorescein diacetate) is expressed as the equivalent charge translocation rate in picoamperes. RT, room temperature; CL, chemiluminescence; PAF, platelet-activating factor; C5a, complement 5a; LTB4, leukotriene B_4; IL-8, Interleukin-8; MBP, eosinophil major basic protein.

* Eosinophils.

† This fluorescent dye monitors intracellular (phagosomal) $O_2^{\cdot-}$ production.

‡ Time course measured at room temperature, maximal rate at 37 °C.

tion may be rate determining at physiological levels and membrane potentials [81].

For the present purposes, the question is whether the respiratory burst ends due to dissipation of NADPH. Loss of substrate NADPH was ruled out as the cause of termination of the zymosan-stimulated neutrophil respiratory burst because addition of glucose did not prevent inactivation [27]. The inactivation of NADPH oxidase in a cell-free system is also insensitive to addition of NADPH or supplementary NADPH-generating systems [14]. Moreover, after termination of a burst initiated by opsonized latex beads, addition of more beads re-starts the burst [106]. A further argument against substrate limitation is that termination of the burst occurs in neutrophils stimulated with opsonized bacteria or zymosan, but not with PMA, even though PMA-stimulated cells produce $O_2^{\cdot-}$ at a much greater rate and hence must consume far more substrate [107]. In summary, the activity of NADPH oxidase during the respiratory burst in intact, living neutrophils is probably directly proportional to the NADPH (and not O_2) concentration, but deactivation is not due to depletion of NADPH.

Time course of NADPH oxidase activity

The respiratory burst stimulated by natural activators is transient [15]. Figure 2 illustrates the response in neutrophils, but the fMet-Leu-Phe-induced burst is transient in several types of phagocyte [108]. Characteristics of the time course, such as lag phase, maximal intensity, duration of $O_2^{\cdot-}$ production, differ profoundly for various agonists [91, 108, 109]. The time courses of the respiratory burst elicited by various stimuli are compared in table 2, which does not take into account factors like "priming" (enhanced response to an agonist as a result of pre-treatment with another agent that itself may produce no response) [110] and other history-dependent behavior (e.g., adherence and physical stress). Deactivation was found to be more rapid for NADPH oxidase assembled in the plasma membrane

than in the phagosome membrane [111]; the present review mainly considers deactivation at the plasma membrane. The variability of time course suggests that the mechanisms of deactivation may differ with different agonists.

Characteristics of $O_2^{\cdot-}$ production induced by different stimuli

Chemoattractants (fMet-Leu-Phe, C5a, LTB4, PAF) and chemokines (IL-8)

$O_2^{\cdot-}$ production achieved with these agents is directed to the extracellular space and can be detected by cytochrome *c* reduction assays. $O_2^{\cdot-}$ production is of varying, generally low, intensity and short duration 1–2 min at most, with a very short (e.g., 10 s) lag phase. In general, fMet-Leu-Phe is the most effective of this class; with several of these agonists, cytochrome *c* reduction is barely detectable. All of these agents bind to 7-TM (having seven transmembrane regions) receptors that undergo similar (rapid) inactivation and down regulation processes as do other 7-TM hormone receptors. The physiological relevance of $O_2^{\cdot-}$ production induced by these agents is questionable, because truly resting cells (e.g. cells prepared under strictly sterile conditions) that are challenged by these stimuli produce hardly any $O_2^{\cdot-}$ [112]. However, under inflammatory conditions, many substances are present. Thus, combinations of chemoattractants with other agonists may be more relevant to the physiological situation. If it is warranted to speak about $O_2^{\cdot-}$ production induced by these agents, then down-regulation of the receptors and thus cessation of the signal that stimulates continuous re-activation of the oxidase could be a mechanism of deactivation.

Integrins

Engagement of integrins per se is not sufficient to induce $O_2^{\cdot-}$ production, but in the presence of a "costimulant" such as an agonist for the tumor necrosis factor (TNF)-α receptor or for the low-affinity Fcγ receptor (such as IgG), there is long-lasting (20–30 min) $O_2^{\cdot-}$ production into the extracellular space, typically developing after a lag phase of a couple of minutes [113–115].

Phagocytosed opsonized bacteria

Bacteria are important "physiological" activators of the respiratory burst. In this case, $O_2^{\cdot-}$ production occurs exclusively in the intraphagosomal space and can thus be detected only by oxygen consumption or chemiluminescence. Signals are initiated via Fc and complement receptors, both involving tyrosine kinase activation. Receptor occupancy probably occurs for a fairly prolonged period (before degradation of bacteria and swelling of the phagosome begins). The burst has a fairly rapid onset, within 20–30 s, and gradually increases up to about 10 min (fig. 2c).

Model stimuli (phorbol myristate acetate opsonized zymosan, Ca^{2+} ionophores)

Certain stimuli produce intense and prolonged NADPH oxidase activity, and for this reason are favored by experimentalists who want large, reproducible responses. The popular phorbol ester, phorbol myristate acetate (PMA, or TPA) produces strong, continuous stimulation of a signaling element, PKC. Although PMA is rarely encountered by neutrophils *in vivo*, and is therefore an artificial stimulus, PKC activation is thought to occur during stimulation by more physiological agonists. However, PMA stimulation seems to be practically irreversible, and thus differs significantly from the responses to "physiological" stimuli. PMA induces NADPH oxidase assembly in the surface membrane, so that $O_2^{\cdot-}$ is released into the extracellular space. There is a long lag phase, the response is long-lasting, and very intensive (ten-fold higher than bacteria or fMet-Leu-Phe; fig. 2c). Calcium ionophores are weak agonists, but $O_2^{\cdot-}$ that is produced is released to the extracellular solution. Like PMA, opsonized zymosan (OPZ) produces a strong, continuous response, with a pronounced lag phase, but the burst is of shorter duration, and it is initiated by stimulation of surface receptors. Most of the $O_2^{\cdot-}$ is produced inside the cell where it is detectable by chemiluminescence measurements, but there is also some cytochrome *c* reduction, indicating $O_2^{\cdot-}$ in the extracellular space. The respiratory burst stimulated by OPZ is five to eight times larger than that obtained with opsonized bacteria (fig. 2c), although the same receptors are presumably involved. This disparity may reflect a larger total surface of contact or longer receptor contact due to the absence of a mechanism for degradation.

Activation of NADPH oxidase is reversible; re-activation is possible

Several types of data lead to the conclusion that the activated state of NADPH oxidase is reversible, and also that under some conditions, the oxidase can be re-activated. Neutrophils stimulated with fluoride produced $O_2^{\cdot-}$ but stopped upon washout, and resumed when fluoride or OPZ was added [116]. Indirect evidence from carefully designed experiments was interpreted to mean that not only were the same cells activated repeatedly, but that the same NADPH oxidase complexes were re-activated [116]. Neutrophils stimulated with arachidonic acid (AA) could be turned off with albumin (which binds AA) and then re-activated with AA [117]. Electrophysiological measurements of NADPH oxidase-mediated electron current show clearly that an individual neutrophil or eosinophil can be activated many times. Spontaneously active eosinophils can be inhibited with diphenylene iodonium (DPI), after which addition of PMA or AA restores electron current, as well as producing the

changes in proton channels that are associated with the activated state [118]. Eosinophils responded to multiple applications of AA with electron current and enhanced proton current, both of which were reversed slowly after washout of AA [73]. These single-cell measurements demonstrate unequivocally that the same cell can be re-activated; however, they do not resolve whether the re-activated NADPH oxidase complexes are the same or different molecules. Because only a small fraction of the total $p47^{phox}$ in the cell translocates at any one time [11, 31], there is a readily accessible pool of $p47^{phox}$, which complicates such interpretations.

Is assembly equivalent to activation?

Regardless of the stimulus, to function correctly *in vivo*, NADPH oxidase must require at least $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac, because mutations that result in the dysfunction or absence of any of these components results in CGD (enzyme dysfunction). Assembly of the NADPH oxidase complex per se is generally assumed to result in its activation. The time course of $O_2^{\cdot-}$ production by zymosan-stimulated neutrophils is similar to that of subcellular "particles" isolated from neutrophils at various times, and tested *in vitro* for activity [27], which suggests that the burst parallels the time course of assembled NADPH oxidase complexes. Tamura et al. [119] concluded that "continuous association of dissociable components (one of which is $p47^{phox}$) is required for maintenance of activity." Cross and Segal [26] complicated the picture somewhat by proposing a dynamic equilibrium between three states: inactive, intermediate, and active. In this model, electron transfer does not occur until a conformational change occurs in flavocytochrome b_{558}, which may result from interaction between cytosolic and membrane-bound components. Furthermore, Cross and colleagues found that $p67^{phox}$, and possibly Rac2, appeared to act catalytically, suggesting that a single $p67^{phox}$ molecule can activate several NADPH oxidase complexes sequentially [120]. In addition, PLA_2 knockout experiments suggest that the NADPH oxidase complex assembles normally without AA, but no $O_2^{\cdot-}$ is produced [121, 122]. The possibility must therefore be considered that assembly of the complex is not sufficient to initiate turnover of the enzyme, and that once the complex begins to work, it may continue despite partial disassembly.

Intrinsic deactivation mechanism revealed by evidence for continual reactivation of NADPH oxidase components

In their seminal study, Akard et al. [28] found that the covalent sulfhydryl-modifying reagent, N-ethylmaleimide (NEM) prevented activation or assembly of NADPH oxidase, but did not inhibit already assembled and active complexes in a cell-free system. However, NEM rapidly (within seconds) terminated NADPH oxidase activity when added to intact PMA-stimulated neutrophils. Deactivation induced (or revealed) by NEM is also very rapid (complete in 20 s) for NADPH oxidase assembled in the plasma membrane of neutrophils stimulated by fMet-Leu-Phe [111]. Akard et al. [28] interpreted these results to indicate that there is continual replenishment of active oxidase. A corollary to this conclusion is that when assembly is interrupted by NEM, the existing NADPH oxidase complexes proceed to deactivate by some intrinsic mechanism. NEM simply unmasks this intrinsic deactivation process [28].

Several other studies using quite different approaches provide additional strong evidence that there is continual turnover of NADPH oxidase complexes. (i) A phenomenon similar to that with NEM was reported recently for N-α tosyl phenylalanine chloromethyl ketone, which prevents NADPH oxidase assembly and which leads to rapid disassembly of already active NADPH oxidase complexes [34]. Similar results were obtained with tosyl-phenylalanine chloromethane [123]. (ii) van Bruggen et al. [33] found that recovery after photobleaching of green fluorescent protein-labeled Rac2 or $p67^{phox}$ occurred in <0.7 s, indicating that there is extremely rapid turnover of these components. (iii) Quinn et al. [31] found that the time course of translocation of Rac, $p47^{phox}$, and $p67^{phox}$ to the plasma membrane correlated closely to the resulting NADPH oxidase activity. With fMet-Leu-Phe, translocation was nearly complete after 1 min, and $O_2^{\cdot-}$ production ceased by ~3–4 min. The lag suggests that assembled NADPH oxidase complexes may remain active for ~1–2 min. With PMA, translocation continued over 20 min and $O_2^{\cdot-}$ production also persisted during this time period. (iv) Morgan et al. [96] observed a very rapid cessation of NADPH oxidase-generated electron current (time constant 5.6 s) in eosinophils studied using perforated-patch recording when the membrane patch was ruptured. Evidently, a cytoplasmic factor necessary for sustained NADPH oxidase function diffused out of the cell, very rapidly terminating activity. (v) As will be discussed below, there is ample evidence that continual phosphorylation is required for sustained activity. (vi) Dusi et al. [30] found that translocation of $p47^{phox}$ and $p67^{phox}$ was required for NADPH oxidase activity. In neutrophils stimulated with fMet-Leu-Phe, the burst terminates rapidly when translocation of additional $p47^{phox}$ and $p67^{phox}$ stops, even though they remain in the membrane. With PMA or concanavalin A, translocation continues long after the rate of $O_2^{\cdot-}$ production has plateaued. These observations indicate that continuous translocation is necessary to maintain NADPH oxidase in an active state. Evidently, the assembled NADPH oxidase complex remains active only for a relatively brief lifetime.

These studies indicate that to view the respiratory burst as the assembly of a static group of NADPH oxidase complexes which then function for an indefinite time period is incorrect. A somewhat creative interpretation of the data of Quinn et al. [31] leads to the idea that NADPH oxidase may function for at most only ~1–2 min after assembly. An even shorter life span of the functioning oxidase (a few seconds) is suggested by the time course of deactivation in studies in which assembly is suddenly interrupted [28, 34, 111], the cell is suddenly dialysed [96], or recovery after photobleaching green fluorescent protein-labeled Rac or p67^{phox} is examined [33]. A sustained respiratory burst requires continual replenishment of functioning enzyme complexes. These phenomena could reflect a range of mechanistic possibilities. At one extreme, there might be complete disassembly of NADPH oxidase complexes, with complete assembly of new complexes (either with or without recycling of "used" components). A less drastic possibility is a "switch," dissociation of a highly labile but necessary cytoplasmic component (e.g., AA, Ca^{2+}, or GTP) that might not even be one of the recognized components of the NADPH oxidase complex. Perhaps one or more components becomes dephosphorylated, with phosphorylation restoring activity. The deactivation mechanism needs only to disrupt any of the steps shown in figure 3, from NADPH binding (e.g., by a reduction in affinity), to electron transport, to O_2 binding and $O_2^{\cdot-}$ release.

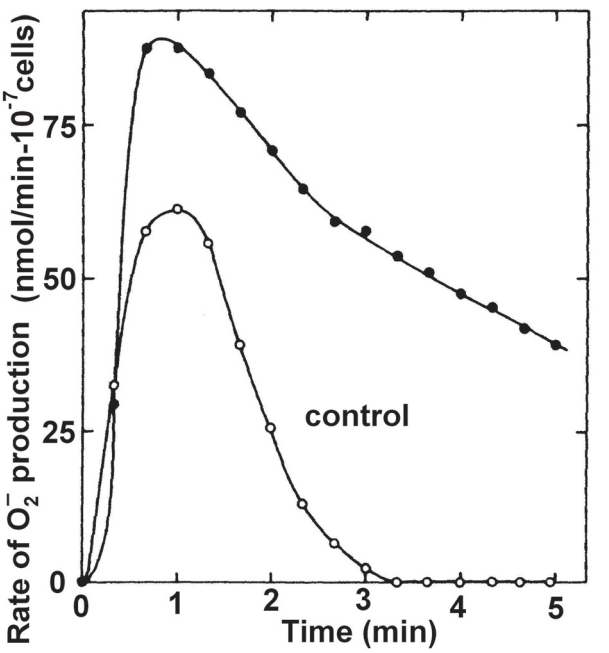

Figure 4. Prolongation of the fMet-Leu-Phe-stimulated respiratory burst by dihydrocytochalasin B in human neutrophils. Open symbols show $O_2^{\cdot-}$ generation measured as cytochrome *c* reduction in control granulocytes, solid symbols in the presence of dihydrocytochalasin B. Reproduced from Jesaitis et al. [130] with permission of the first author and Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

Evidence for and against several mechanisms of NADPH oxidase deactivation

A number of proposed mechanisms will be described. Not all of these are mutually exclusive. Furthermore, different mechanisms of deactivation may apply in different situations.

Myeloperoxidase deficiency prolongs the respiratory burst

The first major proposal to explain the transience of the respiratory burst, suggested by Jandl et al. [27], was based on observations by Klebanoff and coworkers in neutrophils from patients with myeloperoxidase (MPO) deficiency. MPO-deficient neutrophils have impaired bactericidal ability [124]. Their respiratory burst begins normally, but is greatly prolonged, whether stimulated by OPZ [125], opsonized *Staphylococcus aureus* [126], or fMet-Leu-Phe [127], perhaps because dissociation of p47^{phox} and p67^{phox} from the phagosome is delayed [24]. The PMA-stimulated respiratory burst is identical in MPO and normal phagocytes [128]; however, there is little deactivation of the PMA-activated burst to begin with. Similarly, inhibitors of MPO enhanced $O_2^{\cdot-}$ production in neutrophils stimulated with OPZ [27]. The implication of these observations is that a product of MPO (or some consequence of MPO activity) inactivates NADPH oxidase. Because inhibition of MPO with azide slowed but did not prevent deactivation, another mechanism that is independent of MPO must exist [27].

One straightforward idea is that ROS produced by MPO inactivate the oxidase. When formation of ROS by NADPH oxidase is prevented by activation under nitrogen atmosphere, $O_2^{\cdot-}$ production (measured in normal atmosphere) is greatly enhanced, to an extent comparable with MPO inhibition [27]. Another possibility is that without MPO, H_2O_2 accumulates (it being the substrate for MPO), and diffuses through the membrane into the cell where it inhibits phosphatases, thereby prolonging the lifetime of the NADPH oxidase complex. Several cytoplasmic factors, not necessarily derived from MPO, that may cause deactivation are discussed below.

The tangled web of cytoskeletal interactions

Cytoskeletal interactions occur both during assembly and deactivation of NADPH oxidase. During assembly of the NADPH oxidase complex, p47^{phox} and p67^{phox} associate with the cytoskeleton [12]. F-actin and the actin-binding protein coronin-1 accumulate in the vicinity of the phagosome. Colocalization of actin, coronin-1, and

p47^{phox} occurred both in normal and CGD neutrophils, but p47^{phox} and p67^{phox} rapidly dissociated from the phagosome in the CGD cells, presumably due to lack of cytochrome *b* [129].

The fMet-Leu-Phe-stimulated respiratory burst in neutrophils is notoriously transient (Fig. 2). However, the burst is sustained in adherent cells, or in cells pretreated with cytochalasin B (fig. 4) [130–132], both results suggesting that adherence is crucial [113]. The transience of the fMet-Leu-Phe-stimulated respiratory burst is not due to destruction of the agonist – addition of fresh fMet-Leu-Phe does not re-activate the burst after an initial stimulus [91]. Inactivation of the zymosan-stimulated neutrophil respiratory burst was largely prevented by cytochalasin B [27], a disrupter of the cytoskeleton. The cytochalasin B-treated cells were unable to internalize zymosan particles, and the possibility was suggested that the NADPH oxidase complex is located in the plasma membrane and is normally deactivated by internalization and degradation [27]. It has also been proposed that occupied receptors are desensitized by internalization; disruption of cytoskeletal interaction prevents internalization and hence prolongs the respiratory burst [130]. The implication is that the transience of the fMet-Leu-Phe-stimulated respiratory burst is due to internalization of occupied receptors. Compatible with this model, neutrophils from a patient with leukocyte adhesion deficiency type 1 exhibited impaired adherence and a depressed PMA- or OPZ-stimulated respiratory burst, but had a supernormal response to fMet-Leu-Phe [133]. Contrary to the model, ionomycin pre-treatment prevented desensitization, but the burst remained transient [134]. When fMet-Leu-Phe is displaced from its receptor by a competitor, the burst rapidly terminates [135]. However, okadaic acid prolonged the respiratory burst even after displacement of fMet-Leu-Phe from its receptors, indicating that the immediate cause of termination was protein dephosphorylation [135]. Finally, cumulative $O_2^{\cdot-}$ production by fMet-Leu-Phe-stimulated neutrophils was increased by more than ten-fold by cytochalasin B (both the rate and duration of the burst were increased), but no enhancement was seen in monocytes [131]. The cytoskeletal mechanism of deactivation thus appears not to occur in monocytes. Because the respiratory burst is similarly transient in both monocytes and neutrophils, but cytochalasin B has effects only in the latter, cytoskeletal interaction may not be relevant to the mechanism of respiratory burst termination in either cell type [131]. However, the reason the burst is transient may simply differ in these cell types.

Results nearly diametrically opposed to those in figure 4 were obtained in a cell-free system [136]. Actin-depolymerizing agents (DNase I, latrunculin A) *accelerated* deactivation of the respiratory burst. Exogenous actin prevented this effect. The authors concluded that actin stabilizes the NADPH oxidase complex and that actin polymerization/depolymerization regulates the duration of the burst. In this cell-free system, cytochalasins had no effect. This qualitative difference from the response of intact cells reinforces the proviso that extreme caution should be exercised in extrapolating mechanisms from one system to another.

The kinetics of the intracellular respiratory burst during phagocytosis is difficult to assess, due to asynchronous ingestion of particles [137]. In a synchronized system, cytochalasin B *accelerated* deactivation of intracellular $O_2^{\cdot-}$ production (into phagosomes) in neutrophils stimulated with opsonized yeast particles, and prevented a sustained burst [137]. Deactivation that occurs in the phagosome may thus differ from that at the plasma membrane. In summary, although cytoskeletal interactions appear to play numerous important roles in phagocyte function and dramatically modulate the kinetics of the respiratory burst, a clear, universal role in deactivation has yet to emerge.

Dephosphorylation or hyperphosphorylation of one or more NADPH oxidase components

Ample evidence supports a crucial role for phosphorylation in triggering the assembly of the NADPH oxidase complex. Because phosphorylation plays a central role in turning on NADPH oxidase, a logical question is whether dephosphorylation might terminate the burst. Phosphorylation probably influences both the affinity of the subunits for each other and the stability of the complex. Heyworth and Badwey [138] concluded that p47^{phox} was continuously phosphorylated and dephosphorylated, with the phosphorylation process dominating in "activated" cells. Evidence regarding the role of dephosphorylation in terminating the respiratory burst falls into several categories: (i) kinase inhibition terminates the burst, revealing constitutive phosphatase activity, (ii) phosphatase inhibitors prolong the burst, and (iii) phosphatase inhibitors can stimulate a respiratory burst. These will be examined in turn.

Kinase inhibition terminates the burst, revealing constitutive phosphatase activity

As illustrated in figure 5, the PMA-stimulated respiratory burst can be terminated rapidly (*i.e.*, within 1–2 min) by addition of the PKC inhibitors staurosporine or H-7 [138]. This study identified p47^{phox} as a target that is phosphorylated by PMA and then dephosphorylated with an appropriate time course. This result suggests that continuous phosphorylation by one or more PKC isoforms is required to sustain the burst, and also that either phosphatases or spontaneous dephosphorylation are poised to terminate the burst when active phosphorylation stops. The preferred mechanism seems to be phosphatases, because protein phosphatase antagonists (okadaic acid or calyculin A) prevent the H-7-mediated termination

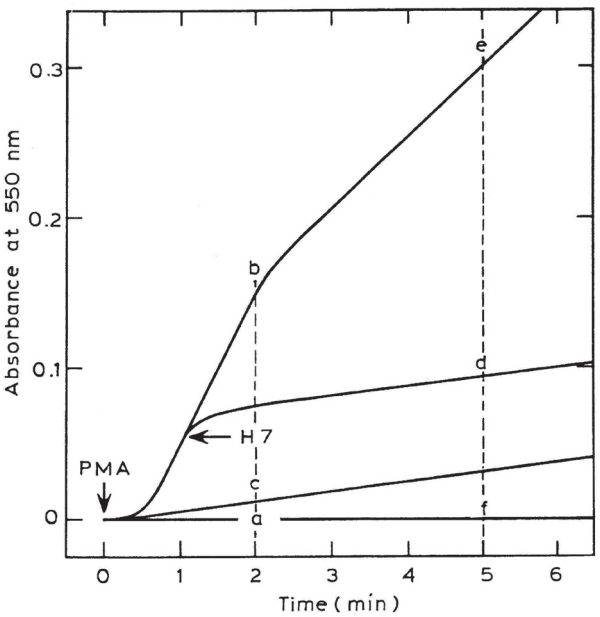

Figure 5. Termination of the respiratory burst (measured as $O_2^{\cdot-}$ production) after PKC inhibition. Human neutrophils were stimulated with PMA (**b**–**e**), PMA then H-7 1 min later (**d**), pre-treated with H-7 before PMA (**c**), or given no stimulus (**a**). Reproduced from Heyworth and Badwey [138], with permission of the first author and Elsevier.

as well as the loss of phosphate from $p47^{phox}$ [29]. Two interpretations are discussed by Ding and Badwey: (i) assembled $p47^{phox}$ might be dephosphorylated, resulting in disassembly of the complex, or (ii) the phosphatases attack "free" $p47^{phox}$, thus preventing assembly, which implies that continuous assembly is required. The first interpretation was supported by Curnutte et al. [139], who found that PKC inhibition of activated neutrophils resulted in disassembly of the complex, which was prevented by phosphatase inhibition.

A similar phenomenon was reported recently for N-α-tosyl phenylalanylchloromethylketone (TPCK), which prevents NADPH oxidase assembly by preventing $p47^{phox}$ phosphorylation and hence translocation [34]. In addition, TPCK results in disassembly of already active NADPH oxidase complexes, suggesting that (i) continuous phosphorylation appears to be required to sustain the respiratory burst, and (ii) phosphatases are evidently ready to pounce on phosphorylated oxidase components given the slightest opportunity. The implication is that a balance between kinases and phosphatases continuously establishes the current level of NADPH oxidase activity.

Phosphatase inhibitors prolong the burst stimulated by fMet-Leu-Phe but not PMA

If sustained respiratory burst activity requires constant turnover of enzyme complexes, there must be sustained kinase activity. The burst stimulated in neutrophils by fMet-Leu-Phe lasts only ~1 min, but is greatly prolonged by pre-treatment with the phosphatase inhibitor okadaic acid [29, 132, 135, 140, 141], suggesting that dephosphorylation by phosphatases limits the duration of the fMet-Leu-Phe-stimulated respiratory burst. In contrast, okadaic acid inhibited the burst in neutrophils stimulated with opsonized yeast particles [141] or PMA [135, 140], or in Kupffer cells stimulated with PMA [142]. A reasonable conclusion is that the fMet-Leu-Phe-stimulated burst may be terminated by phosphatase-mediated deactivation.

The selectivity of the effect of phosphatase inhibitors is surprising – one would imagine that once the oxidase complex is assembled, it ought to deactivate by a pathway that is independent of the method of activation. Harbecke et al. [141] concluded that the state of phosphorylation had no direct effect on the oxidase. Gay et al. [143] concluded from its concentration dependence that calyculin A exerts additional effects on phospholipase D (PLD).

Phosphatase inhibitors stimulate a respiratory burst

Kinases may have constitutive activity that is held in check by constitutively active phosphatases. Constitutive kinase activity is suggested by finding that phosphotyrosine phosphatase inhibitors stimulate a respiratory burst [144, 145]. One contrary report found that the tyrosine kinase inhibitor herbimycin A inhibited constitutive $O_2^{\cdot-}$ generation in B lymphocytes [146], but signaling pathways regulating constitutive activity may differ from those that trigger the respiratory burst. In contrast, inhibition of serine/theonine phosphatases with okadaic acid [135] or calyculin A [143] did not stimulate neutrophils, indicating a lack of constitutive activity of protein phosphatases 1 and 2A. These studies support the existence of a constitutively active tyrosine kinase that is normally held in check by phosphatase activity.

Hyperphosphorylation

Although substantial evidence supports the idea of a balance between phosphorylation and phosphatases, the story with respect to deactivation may be more complicated. In neutrophils stimulated with opsonized *Neisseria meningitides,* phosphorylation of $p47^{phox}$ did not change upon burst termination [107]. In fact, additional phosphorylation of membrane-associated $p47^{phox}$ appeared to precede dissociation of the $p47^{phox}$/$p67^{phox}$ dimer. The removal of $p47^{phox}$/$p67^{phox}$ coincided with burst termination. Thus, although phosphorylation of $p47^{phox}$ is a necessary step in initial assembly of the complex, termination occurs when $p47^{phox}$/$p67^{phox}$ dissociate (or are degraded), an event triggered by hyperphosphorylation of $p47^{phox}$ [107].

Rac-related mechanisms

Role of Rac

GTP-binding proteins function as molecular switches that limit protein-protein interactions in time. The in-

dispensable role of the small GTPase Rac in NADPH oxidase function has been shown amply both in cell-free systems [64, 147] and in Rac2-deficient animals [148] and patients [149]. Detailed biochemical and crystallographic analysis revealed that specific amino acids (A27, G30, D38) of the switch I region of Rac play a critical role in its binding to the tetratricopeptide repeat domain of $p67^{phox}$ [150–152]. It is thus feasible that Rac could play a critical role in the regulation of NADPH oxidase.

Activation and catalytic activity of NADPH oxidase require the GTP-bound form of Rac [153, 154]. Diebold and Bokoch [155] showed that suppression of the interaction between RacGTP and $p67^{phox}$ allows the first step of activation (electron transfer from NADPH to FAD, step 4 in fig. 3) to occur, but precludes electron transfer via the hemes to oxygen (step 7 in fig. 3). In accordance with these data, replacing GTP by GDP in the active, electron-transporting enzyme complex resulted in a precipitous fall and termination of $O_2^{\cdot-}$ production [156, 157]. The latter experiments were carried out under conditions in which the active, superoxide-producing enzyme complex was separated from the soluble factors, so that re-assembly could not occur [156], and where Rac was the only GTP-binding protein present, so that the interaction of another small GTPase could be excluded [157]. As in other small GTPases, amino acids of both the switch I and the switch II region of Rac interact with the γ-phosphate group of GTP, thereby stabilizing the active conformation of the G protein [158] and probably of the entire NADPH oxidase complex. Hydrolysis of GTP releases the restraint within the Rac molecule, and the increased mobility of the switch regions [159] may destabilize the oxidase complex and hinder electron flow. Thus, an increase in the rate of GTP hydrolysis on Rac could provide an effective mechanism for termination of $O_2^{\cdot-}$ production.

GTPase-activating proteins deactivate NADPH oxidase

GTPase-activating proteins (GAPs) are able to accelerate the hydrolysis rate of GTP by several orders of magnitude. The human genome contains approximately 70 proteins that could function as GAPs for Rac [160]. The observation that the number of regulatory proteins largely exceeds the number of small GTPases of the Rho/Rac family suggests that the role of individual GAPs is restricted to very well-defined, specific interactions. In neutrophils, three RacGAP proteins – p190RhoGAP, Bcr, and p50RhoGAP – have been identified and the presence of further RacGAPs was indicated in a radioactive overlay assay [161]. Studies carried out on genetically modified animals indicate a role for Bcr in the regulation of $O_2^{\cdot-}$ production, as Bcr-deficient mice reacted to endotoxin challenge with a much higher production of $O_2^{\cdot-}$ than the wild-type controls [162]. These functional data do not, however, provide information on the site of action of Bcr, as Rac proteins (Rac1, Rac2, and Rac3) have been shown to regulate multiple key elements of phagocyte function, such as PI3K, p21-activated kinase (Pak), and cytoskeletal reorganization.

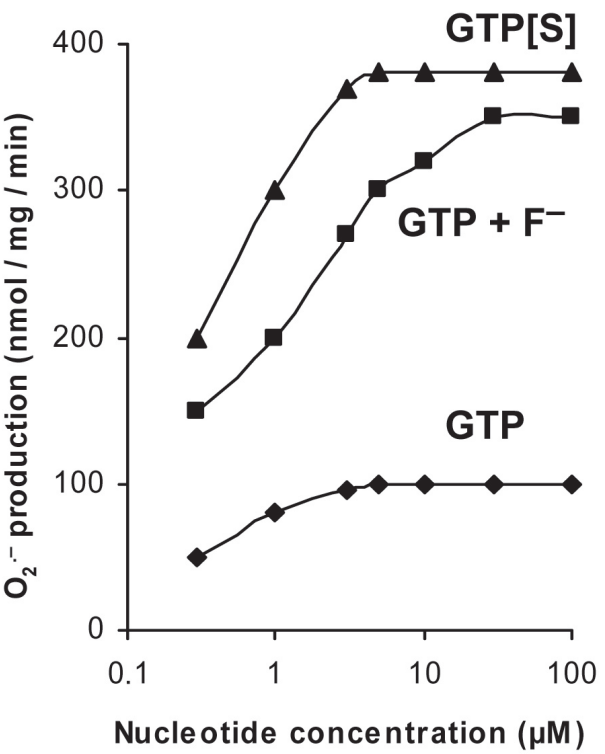

Figure 6. Prevention of GTP hydrolysis on Rac reveals continuous GAP activity in a cell-free system. Rate of $O_2^{\cdot-}$ production induced by different guanine nucleotides in a cell-free activation system. The indicated concentration of GTP (♦) or GTP[S] (▲) or GTP plus 20 mM NaF (■) was present during the activation phase. Ligeti et al. [unpublished data].

Experiments carried out in the cell-free activation system indicate a clear role for GAPs both in the assembly and in the catalytic function of the NADPH oxidase. Using crude membrane and cytosol preparations, the rate of $O_2^{\cdot-}$ production achieved in the presence of GTP is only 20–30 % of the maximal rate obtained in the presence of a non-hydrolyzable GTP analog such as GTPγS (figure 6). However, application of fluoride, an inhibitor of GAPs [163–165], results in a concentration-dependent increase in the rate of GTP-elicited $O_2^{\cdot-}$ production approaching the values obtained with GTPγS (fig. 6). It is remarkable that even a large excess of GTP is not able to overcome the effect of GAPs (fig. 6),

Activation of the oxidase under cell-free conditions can also be achieved by replacing the cytosol or the membrane fractions with recombinant $p47^{phox}$, $p67^{phox}$, and Rac or purified cytochrome b_{558}, respectively. Comparing the total GAP activity of the various forms of the cell-free activation system and the fraction of $O_2^{\cdot-}$ production that could be obtained with GTP revealed a clear inverse

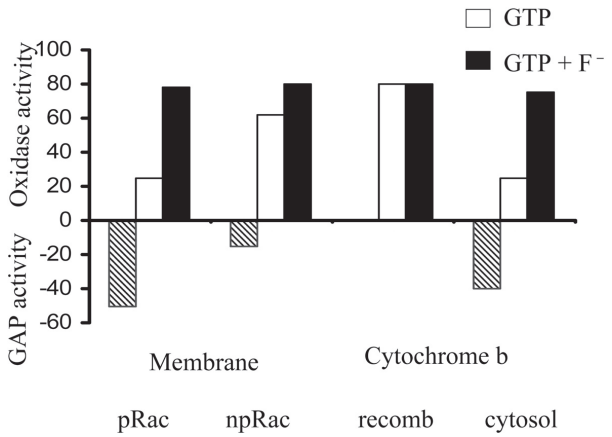

Figure 7. Relation of total GAP activity and $O_2^{\cdot-}$ production in different versions of the cell-free activation system. GAP activity (hatched columns) is expressed as the percentage of protein-bound radioactive GTP hydrolyzed in 5 min. $O_2^{\cdot-}$ production was measured in the presence of 10 μM GTP alone (white columns) or in combination with 20 mM NaF (black columns). The cell-free activation assay contained either washed (*i.e.,* Rac-depleted) neutrophil membranes and recombinant $p47^{phox}$, $p67^{phox}$, and prenylated (pRac) or non-prenylated Rac (npRac) loaded with GTP, or purified cytochrome *b* plus recombinant $p47^{phox}$, $p67^{phox}$, and prenylated Rac loaded with GTP ("recomb") or crude cytosol ("cytosol"). Both the membrane and the cytosol fractions contain GAP activity which correlates inversely with $O_2^{\cdot-}$ production attained in the presence of GTP. Full activation of the enzyme (100%) was measured in the presence of 10 μM GTP[S]. Ligeti et al. [unpublished data].

relationship: the higher the GAP activity, the lower the rate of $O_2^{\cdot-}$ production in the presence of the natural nucleotide, GTP (fig. 7). However, identical values were detected in the presence of fluoride, when GAPs were inhibited. Thus, GAPs present both in the membrane (e.g., p50RhoGAP) and in the cytosol (e.g., Bcr, p190 RhoGAP) are constantly active in reducing the amount of RacGTP and in down regulation of the NADPH oxidase activity.

All three Rac/RhoGAPs identified in neutrophils were able to reduce the rate of $O_2^{\cdot-}$ production if they were added to the cell-free system during the activation phase in the presence of GTP [161, 166], but they had no effect upon the assembled, electron-transporting enzyme complex. In contrast, fluoride was still able to increase the enzyme activity in the assembled state [157]. These data indicate that soluble (cytosolically localized) GAPs are able to interfere with RacGTP during its translocation to the membrane and thus they can influence the rate of re-assembly of the enzyme. In the assembled complex, RacGTP is in an unexposed position to which soluble GAPs do not have access. However, membrane-localized GAP(s) seem to be able to act on RacGTP even in the molecular complex and decrease or terminate electron flow. Information available at present on the crystal structure of Rac-$p67^{phox}$ [152] and Rac-p50RhoGAP [167] complexes is compatible with the formation of a ternary complex between Rac, its target $p67^{phox}$, and its regulatory protein p50RhoGAP.

Taken together, several RacGAPs may interfere with the activation of the phagocytic NADPH oxidase at different steps. GAPs are large proteins with a wide variety of potential interacting domains that could be targets of multiple regulatory actions [168]. Most recently, two different mechanisms were revealed, which modulate the RacGAP activity of the p190 and p50 proteins [169, 170]. Interaction of different GAPs with Rac in different phases of the oxidase activation allows fine and selective regulation of $O_2^{\cdot-}$ production, and may account for some of the differences observed with different stimuli.

Inhibition of Rac by integrins

Adherence of neutrophils produces an initial suppression of $O_2^{\cdot-}$ generation, followed by enhancement. Apparently, enhanced tyrosine phosphatase activity dephosphorylates a critical nucleotide exchange factor, Vav1, which then prevents activation of Rac2 [114]. Stimulation of β_2 integrins was also shown to activate p190GAP and potentially other GAPs in neutrophil granulocytes [171, 172] and may contribute to the temporary suppression of $O_2^{\cdot-}$ production.

Disassembly of the oxidase complex (how stable is the complex?)

In principle, the removal of any required component of the NADPH oxidase complex might terminate its function. Useful information has been obtained from study of cell-free systems. NADPH oxidase is highly labile in a cell-free system, but is stabilized by cytosolic factors, which may include $p47^{phox}$ and a putative NADPH-binding protein [119]. Evidently, $p47^{phox}$ readily dissociates from the active complex, which terminates function, whereas $p67^{phox}$ either does not dissociate or is no longer needed once assembly is complete [119]. When cytosol was removed, NADPH oxidase activity disappeared with a half-life of 2 min, which was extended to 120 min by a chemical cross-linker, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [119]. The stabilizing effect of EDC was attributed to prevention of flavin exchange [173].

When $p67^{phox}$ and $p47^{phox}$ are fused together artificially in a cell-free system, the NADPH oxidase complex continues to generate $O_2^{\cdot-}$ for prolonged periods, whereas activity of the NADPH oxidase complex formed with normal non-fused proteins decays with a half-life of 16 min [174]. A constitutively active Rac construct, RacQ61L stabilized the cell-free system six-fold [175].

Edgar Pick's group [personal communication] have created a "trimera," a triple chimera consisting of "active domains" of $p47^{phox}$, $p67^{phox}$ and Rac1 fused into a single molecule. The trimera behaves as a *bona fide* GTPase. When, in a cell-free system consisting of phagocyte membranes incubated with a mixture of $p47^{phox}$, $p67^{phox}$,

and Rac1-GMPPNP (a hydrolysis-resistant GTP analog), the mixture of cytosolic components is replaced by the trimera-GMPPNP, the NADPH oxidase is activated by anionic amphiphile and remains active upon the removal of the amphiphile, with a calculated half-life of about 9 h, at room temperature. In comparison, a complex generated with individual cytosolic components has a half-life of about 10 min. Specifically, p47^{phox} appears to be essential for the maintenance of an active complex, because a complex generated by exposing membranes to a mixture of free p47^{phox} and a dimeric [p67^{phox} – Rac1]-GMPPNP fusion protein exhibits low stability, with a half-life of only 3 min.

All of these studies indicate that the assembled NADPH oxidase complex remains active only so long as its essential components (in particular p47^{phox} and cytochrome b_{558}) remain together, and that at least in a cell-free system, there is a tendency for spontaneous dissociation.

Loss of ATP or GTP, or Rac2

Sustained NADPH oxidase activity has been proposed to require the presence of the GTP-bound form of Rac [157]. Addition of excess GDP terminates activity, presumably by replacing GTP bound to Rac [157]. Furthermore, NADPH oxidase activity in excised membrane patches, detected as electron current, is reportedly dependent on the presence of ATP and GTPγs for sustained activity [48]. However, in similar experiments, electron current was sustained for >20 min in the absence of these factors [D. Morgan, V. V. Cherny and T. E. DeCoursey, unpublished data]. In excised patches, many cytoplasmic factors are absent. For example, the Cdc42 GTPase has recently been shown to be a competitive antagonist of Rac at the level of flavocytochrome b_{558} [176]. In the absence of Cdc42 or some analogous inactivating component, NADPH oxidase activity might be sustained.

Loss of AA

During the respiratory burst, phospholipase A_2 (PLA_2) is activated and generates AA [56, 57, 121], which activates NADPH oxidase [58, 177]. AA also modulates proton channels, greatly promoting their opening [68, 69, 71–73]. PLA_2 knockout experiments suggest that the NADPH oxidase complex assembles normally without AA, but no $O_2^{\cdot-}$ is produced [122]. However, recent studies indicate that PLA_2 is not required for NADPH oxidase activation [178, 179]. Reconstitution of NAPDH oxidase activity in a cell-free system requires either AA [180] or some other detergent, such as sodium dodecyl sulfate [181]. AA has been proposed to be the final step in activating NADPH oxidase [68, 122]. One proposed mechanism is that AA catalyzes the assembly of NADPH oxidase [182], possibly by inducing the same conformational change in p47^{phox} as does phosphorylation [54, 183, 184]. Another proposal is that AA interacts with heme to facilitate its reaction with O_2 [23, 185]. A third possibility is that by shifting the threshold for opening voltage-gated H^+ channels [69, 70, 73, 186], AA enables charge compensation, which is indispensable for continuous electron transfer [78]. If AA is a component of, or plays a critical role in the functioning of the assembled, active complex, then its dissociation ought to terminate the respiratory burst. Experimentally, removal of AA results in deactivation of NADPH oxidase activity [73, 117] as well as reversal of AA-induced enhancement of proton channel properties [73]. However, this would be an unwieldy mechanism *in vivo*, where, given the lipophilicity of AA, it would tend to stay in the membrane. Most likely, this mechanism occurs *in vitro* but not *in vivo*.

Loss of something else

NADPH oxidase generates electron currents in cells stimulated in the "perforated-patch" configuration. In this technique, electrical access is achieved by pore-forming antibiotics (e.g., nystatin or amphotericin B) in the pipette solution to allow electrical recording from the whole-cell membrane without disrupting the cytoplasm. When the membrane patch was ruptured, NADPH oxidase-generated electron current in eosinophils shut down very rapidly (time constant 5.6 s) [96]. Including ATP and GTPγs in the pipette did not prevent this rapid shut-down of oxidase function. Perhaps a cytoplasmic factor necessary for sustained NADPH oxidase function diffused rapidly out of the cell. The identity of this mysterious cofactor is unknown.

Regulation by p40^{phox}

The role of p40^{phox} in NADPH oxidase activity is controversial. It is expressed exclusively in hematopoietic cells [187], including basophils and mast cells that produce little or no $O_2^{\cdot-}$ but do express proton channels [67]. The other NADPH oxidase components can function in its absence, for example in the COS-7 expression system [188], and in cell-free systems [60, 65, 189, 190]. Addition of p40^{phox} to a cell-free system that includes the other components down-regulates NADPH oxidase activity [191]. Transfection of p40^{phox} into K562 cells down-regulated NADPH oxidase activity in one study [192], but up-regulated activity in another study [193]. Other studies in cell-free systems produced evidence that p40^{phox} enhances $O_2^{\cdot-}$ production [194–196]. Intriguingly, an antibody to p40^{phox} that causes dissociation of p40^{phox} from p67^{phox} reduced $O_2^{\cdot-}$ production, but not when added after assembly [194]. This result argues against a role for p40^{phox} in deactivation of the oxidase. A recent study indicated that p40^{phox} down-regulates NADPH oxidase activity, but only when it is phosphorylated [197]. Because most of these studies were done in cell-free systems, their results cannot be immediately applied to the situation in intact phagocytes.

Recently, the main NADPH oxidase components including elements of the fMet-Leu-Phe signaling pathway were transfected into the non-hemopoietic COS-7 cell line. Exogenous expression of $p40^{phox}$ enhanced the fMet-Leu-Phe response [198]. In summary, $p40^{phox}$ appears not to be required for NADPH oxidase function, and whether it enhances or suppresses function, it does so in a modulatory manner. Therefore, $p40^{phox}$ does not appear to be involved in the deactivation of NADPH oxidase.

Changes in affinity of the NADPH oxidase complex for NADPH

The affinity of the NADPH oxidase complex for NADPH has been proposed to increase dramatically (about ten-fold) during phagocytosis [199]. This mechanism was called into question when Curnutte et al. [200] showed that in the presence of Mn^{2+}, the rate of NADPH oxidation is artificially enhanced by a free radical chain reaction unrelated to NADPH oxidase. Nevertheless, there are cases of CGD in which the primary defect seems to be a reduced affinity of NADPH for the oxidase [94, 95, 201]. In a cell-free system, two different forms of active NADPH oxidase were reported, the higher-affinity form having three rather than two cytosolic components bound [202]. In a cell-free system, AA increased the affinity of NADPH for the NADPH oxidase complex about five-fold [203]. Ravel and Lederer [204] proposed that assembly of cytosolic components with cytochrome b_{558} results in a conformational change that greatly increases the affinity of the enzyme for NADPH. Recent evidence suggests a conformational change in cytochrome b_{558} that initiates electron transfer from NADPH [83], although not necessarily one that changes NADPH affinity. It is difficult to draw any firm conclusions from these intriguing shards of evidence.

A mysterious cytosolic deactivator

Theories postulating a cytosolic deactivating substance are the counterpart to theories in which deactivation occurs when some essential component or factor dissociates from the NAPDH oxidase complex. Evidence consistent with the existence of a necessary and readily dissociable cytosolic component came from the classical NEM experiment of Akard et al [28]. Because NEM does not cause comparable deactivation in a cell-free system, it has been argued that there must be a cytosolic deactivating factor [205]. Eklund and Gabig [206] isolated just such a putative deactivating molecule from neutrophil cytosol, a thiobis ester lipid, similar to or identical with propanoic acid 3,3'-thiobis(didodecyl) ester, which reversibly deactivated NADPH oxidase in a cell-free system. Although this lipid is present at appropriate concentration in neutrophils [206], whether, how, and under which conditions it might actually function as a deactivator of NADPH oxidase *in vivo* is not clear.

A neutrophil-derived lipid, presqualene diphosphate, can partially inhibit $O_2^{\cdot-}$ generation by neutrophils [207]. As with the thiobis ester, assessing the physiological role of this molecule is difficult.

Ceramide accumulates as the respiratory burst is ending, and exogenous ceramide inhibits H_2O_2 production, leading to the suggestion that ceramide may contribute to termination of the burst [208]. In contrast, in the cell-free system, high concentrations of ceramide had no effect [209].

ATP accelerates NADPH oxidase deactivation in a cell-free system [210]. The effect appears to be due to chelation of Mg^{2+} which consequently destabilizes actin and hence the complex [211]. On the other hand, ATP can enhance NADPH oxidase activity by re-phosphorylating GDP to make GTP, which is required for Rac to bind and do its job [212].

An endogenous peptide, PR-39, inhibits NADPH oxidase by preventing assembly, but requires prolonged pre-incubation [213].

In summary, although a variety of substances can terminate NADPH oxidase activity, convincing proof identifying a physiologically relevant culprit remains elusive.

Concluding remarks

Experimental data summarized in this review strongly indicate that in phagocytes, the NADPH oxidase complex is continuously reactivated, and the stability of the catalytically active, electron-transporting conformation of the assembled complex is limited. The actual time-course of $O_2^{\cdot-}$ production is therefore the result of a fine balance between activating mechanisms and those leading to deactivation (destabilization and eventual disassembly) of the complex. Changes in the phosphorylation state of $p47^{phox}$ (dephosphorylation and/or hyperphosphorylation) and in the species of the nucleotide bound to Rac seem to be the dominant factors in deactivation. Constitutive phosphatase and GAP activity contribute to continual down-regulation of the oxidase that results in deactivation whenever the forces of activation pause. Both protein phosphatases and GAPs are targets of multiple regulatory actions providing suitable mechanisms for selective modulation of oxidase activity by different stimuli and leading to termination of $O_2^{\cdot-}$ generation with different time-courses.

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