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REVIEW

Role of Nox2 in elimination of microorganisms

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Abstract NADPH oxidase of the phagocytic cells (Nox2) transfers electrons from cytosolic NADPH to molecular oxygen in the extracellular or intraphagosomal space. The produced superoxide anion ($O_2^{\cdot-}$) provides the source for formation of all toxic oxygen derivatives, but continuous $O_2^{\cdot-}$ generation depends on adequate charge compensation. The vital role of Nox2 in efficient elimination of microorganisms is clearly indicated by human pathology as insufficient activity of the enzyme results in severe, recurrent bacterial infections, the typical symptoms of chronic granulomatous disease. The goals of this contribution are to provide critical review of the Nox2-dependent cellular processes that potentially contribute to bacterial killing and degradation and to indicate possible targets of pharmacological interventions.

Keywords Phagocytes · Neutrophilic granulocytes · Killing · NADPH oxidase · Reactive oxygen species · Ion movements

Introduction

Neutrophilic granulocytes (or PMN, for polymorphonuclear cells) play a key role in the antimicrobial defense of the body. These cells are able to detect, pursue, engulf, kill, and degrade various microbes. Final elimination of potentially pathogenic microorganisms is a complex process that requires directed migration, phagocytosis, production of toxic oxygen (and nitrogen) metabolites, and release of antibacterial proteins and enzymes stored in the different granule types. Human pathology indicates that proper functioning of all of these processes is vital as a deficiency in integrins or production of toxic oxygen metabolites or secondary granule proteins result in similar symptoms: recurrent, severe, often life-threatening infections [53]. Thus, elimination of microorganisms can not be ascribed to any single function or protein; it should rather be regarded as a joint action of several, equally important mechanisms.

The active NADPH oxidase enzyme of phagocytic cells consists of the transmembrane electron carrier (gp91 or Nox2) and four other subunits (p22, p47, p67 and Rac) that stabilize and activate Nox2. Only the properly assembled complex is able to transfer electrons across the membrane from cytosolic NADPH to molecular oxygen forming thereby superoxide ($O_2^{\cdot-}$) either in the extracellular or in the intraphagosomal space. In the present review, we refer to the enzymatically active protein complex as Nox2. The discovery that chronic granulomatous disease (CGD) is the consequence of a genetic alteration in any one of the five essential subunits of Nox2 indicates the vital function of the enzyme. There is, however, controversy as to which "product" of the enzymatic activity has essential function.

The chemical product of Nox2 activity is $O_2^{\cdot-}$. There is general agreement that $O_2^{\cdot-}$ itself is not too toxic but several of its derivatives are [44]. On the other hand, the transmembrane movement of electrons creates an electric potential that serves as driving force for secondary ion movements. It has been proposed that ion movements initiated by the electrogenic nature of Nox2 may contribute to the killing process [40, 120]. A new paradigm, developed in the recent years, suggests that the major—perhaps only—

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role of Nox2 is to provide driving force for ion (mainly K^+ and Cl^-) movements in and out of the phagosome creating thereby optimal conditions for the activity of granule enzymes [10, 110, 121, 123].

The purpose of this review is to summarize the experimental data on the role of Nox2 in the elimination of microorganisms. As the basic function of other Nox enzymes is also the transmembrane transport of electrons, most ideas are relevant to the entire Nox/Duox family of proteins.

Toxic effects of oxygen metabolites

The term "reactive oxygen species" (ROS) includes chemically reactive radicals and nonradical derivatives of oxygen. Radicals harbor one or more unpaired electrons, whereas nonradicals do not contain unpaired electrons. All oxygen radicals are ROS, but not all ROS are oxygen radicals [44]. Long before superoxide production by phagocytes has been discovered, the in vitro toxicity of hydrogen peroxide had been already known. Early reports suggested H_2O_2 to be the bactericidal agent in the antibacterial effect of notatin, a toxin from *Penicillium notatum* [25, 76]. The antibacterial effect of the xanthine oxidase system has been also shown [42]. But, the physiological importance of superoxide and ROS derived from superoxide was not recognized until phagocytosing polymorphonuclear leukocytes had been shown to produce superoxide anions [6]. Phagocytosing neutrophils undergo an abrupt increase in oxygen uptake, which was first called a "respiratory burst" but is not caused by mitochondrial oxidative metabolism. The phagocyte NADPH oxidase produces exclusively superoxide anions, which are the precursors of more distant ROS (H_2O_2, HOCl, $^1O_2$, $OH^\cdot$). ROS react with a wide range of biocompounds, including nucleic acids, proteins, lipids, carbohydrates and molecules of other structure.

Superoxide and hydrogen peroxide

Superoxide itself is only weakly reactive and reacts with a few macromolecules, and its direct toxicity is still controversial [118]. Superoxide is unstable, cannot reach very far from the site of its production, and is not able to cross lipid bilayers. Its reactivity, however, can be increased in a hydrophobic environment (e.g., in the proximity of the plasma membrane) or by lowered pH values since its protonated derivative (HO_2) is more reactive [72] and has been shown to penetrate into *Escherichia coli* and inactivate fumarase [79]. A toxic role of superoxide was shown in a few studies where in vitro killing of bacteria was achieved by addition of KO_2 or inhibited by superoxide dismutase (SOD; Table 1). However, it is a challenging task to prove that superoxide anions kill directly since superoxide spontaneously dismutates into hydrogen peroxide and two H_2O_2 molecules are able to produce distal ROS, for example highly reactive $OH^\cdot$. Inhibition of killing by SOD shows only the requirement of superoxide but does not reveal if superoxide kills directly or through distal derivatives. Two of these studies failed to show a clear direct role of superoxide in killing since they also showed complete or partial inhibition of killing by the H_2O_2 scavenger catalase and the $OH^\cdot$ scavenger mannitol [5, 114].

Hydrogen peroxide (H_2O_2) is formed by the spontaneous or SOD-catalyzed dismutation of superoxide. Two superoxide anions will produce one hydrogen peroxide molecule by incorporating two hydrogen ions and releasing one molecule of oxygen (Fig. 1, reaction no. 1). H_2O_2 is a well-known oxidizing agent capable of reacting with a wide range of macromolecules. Hydrogen peroxide is relatively stable and membrane permeable, so it can diffuse away from the site of its formation. Early works have shown already that hydrogen peroxide is toxic to a broad spectrum of microorganisms in vitro (Table 2). These studies differed both in the source of hydrogen peroxide and in the target bacterium used. Hydrogen peroxide can be added directly to the assay medium, can be produced directly by the enzymatic reaction of glucose by glucose oxidase (GO), or can be dismutated from superoxide created by xanthine oxidase (XO) and its adequate substrate (purine, hypoxanthine, xanthine, or acetaldehyde).

More distal derivatives

Although hydrogen peroxide is reactive, its toxicity can be increased dramatically by forming further derivatives. One of the most abundant neutrophil proteins is myeloperoxidase (MPO), which is expressed only in polymorphonuclear leukocytes, monocytes, and certain types of macrophages in the human body. MPO resides in the primary granules of neutrophils and is released into the phagosome upon en-

Table 1 In vitro studies showing role of superoxide in killing of microbes

Source	Microbe	Gram	Buffer pH	SOD inhibition	Reference
Acetaldehyde/XO	*Staphylococcus aureus*	+	7.0	+	[114]
Purine/XO	*Staphylococcus epidermidis*	+	5.3	+	[5]
KO_2	*Escherichia coli*	−	7.3	−	[111]

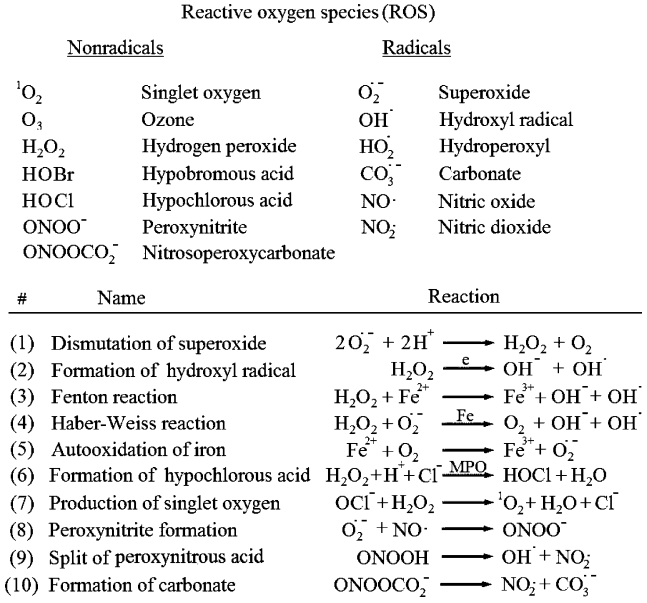

Fig. 1 Reactive oxygen species formed by phagocytes. The figure shows the chemical formulae of radical and nonradical ROS and the stoichiometric equations of chemical reactions leading to their formation

gulfment of bacteria. MPO catalyzes the oxidation of different substrates by hydrogen peroxide. The most common substrates are halides: chloride, bromide, fluoride, or iodide; MPO catalyzes their oxidation by hydrogen peroxide into hypohalous acids. The halides by themselves and the peroxidase on its own are not or only slightly antimicrobial, whereas the oxidized products are highly reactive and kill microbes easily [72]. The MPO/H_2O_2/I^- system has been extensively characterized in vitro [70]. *E. coli* were killed in an MPO-, hydrogen peroxide-, and halide-dependent manner. Iodination or chlorination of bacteria is inversely proportional to their survival meaning that killing must occur—at least in part—by reacting with hypoiodous/hypochlorous acid [20, 70]. Several studies proved the in vitro toxicity of the MPO/H_2O_2/halide system [51, 71, 90, 111] or HOCl itself [20, 45, 51, 87, 111]. The phagocytic NADPH oxidase is required for the iodination of bacteria since CGD neutrophils fail to do so [73]. Although iodide is the preferred substrate for MPO among the halides [71], due to high concentrations of chloride in the phagosome, hypochlorous acid is formed in the highest amounts. Hypochlorous acid is a strong oxidizing agent, it attacks a broad range of biologically relevant compounds, but the preferred targets are: thiols, thioesters, amines, phenols, unsaturated bonds; it is membrane permeable [45].

Although formation of hydroxyl radical ($OH^{\cdot}$), a very reactive radical occurs in vitro from hydrogen peroxide catalyzed by iron (Fig. 1 reaction nos. 2–4), its contribution to killing remains doubtful since the iron released into the phagosome by either the bacteria or the neutrophils is bound to lactoferrin [15], and studies using electron paramagnetic resonance failed to detect hydroxyl radical formation in neutrophils following engulfment of iron-rich bacteria [24].

Singlet oxygen is an electronically excited state of molecular oxygen. There are many pathways suggested to form singlet oxygen but the best established mechanism is the one by H_2O_2 and HOCl [115] (Fig. 1 reaction no. 7). Singlet

Table 2 In vitro studies showing role of hydrogen peroxide in killing of microbes

Source	Microbe	Gram	Buffer pH	Cat.	References
Acetaldehyde/XO	*Staphylococcus aureus*	+	7.0	+	[114]
Hypoxanthine/XO	*Bacillus subtilis*	+	Solid medium	+	[42]
Hypoxanthine/XO	*Micrococcus luteus*	+	Solid medium	+	[42]
Hypoxanthine/XO	*Moraxella catarrhalis*	−	Solid medium	+	[42]
Hypoxanthine/XO	*Proteus vulgaris*	−	Solid medium	+	[42]
Hypoxanthine/XO	*Pseudomonas aeruginosa*	−	Solid medium	+	[42]
Hypoxanthine/XO	*Staphylococcus aureus*	+	Solid medium	+	[42]
Hypoxanthine/XO	*Streptococcus pyogenes*	+	Solid medium	+	[42]
Purine/XO	*Escherichia coli*	−	5.3	+	[5]
Purine/XO	*Staphylococcus epidermidis*	+	5.3	+	[5]
Purine/XO	*Moraxella catarrhalis*	−	7.3	+	[54]
Purine/XO	*Neisseria gonorrhoeae*	−	7.3	+	[54]
Purine/XO	*Neisseria meningitidis*	−	7.3	+	[54]
Purine/XO	*Neisseria perflava*	−	7.3	+	[54]
Glucose/GO	*Neisseria gonorrhoeae*	−	7.3	+	[54]
H_2O_2 0.5 μM	*Escherichia coli*	−	5.0	n.m.	[70]
H_2O_2 1–100 mM	*Escherichia coli*	−	7,5; 6,5; 5,5	n.m.	[111]
H_2O_2 1–100 mM	*Staphylococcus aureus*	+	7,5; 6,5; 5,5	n.m.	[111]

Cat. Notes if the killing was inhibited by catalase, + inhibition, *n.m.* not measured

oxygen is a highly reactive and short-lived radical attacking a wide range of biomolecules. Although singlet oxygen has been suggested to be present in neutrophils' phagosome [2, 129], the contribution of singlet oxygen to killing in neutrophil phagosome is still difficult to interpret due to imperfect specificity of singlet oxygen scavengers [23].

The production of ozone, a highly reactive ROS, by IgG antibodies opsonizing bacteria has been suggested in phagocytosing neutrophils [7, 137], but doubts have been raised by others on the specificity of the probes used for ozone detection in those studies [68, 69].

Human neutrophils do not produce large amounts of nitric oxide (NO); they only do so when primed with a certain cytokine cocktail [138]. NO can react with superoxide to form peroxynitrite ($ONOO^-$; Fig. 1, reaction no. 8), which can be protonated (peroxynitrous acid) at physiological pH and split into two radicals, hydroxyl radical and nitric dioxide (Fig. 1, reaction no. 9). The reaction between peroxynitrite and carbon dioxide results in nitric dioxide and carbonate, two reactive radicals (Fig. 1, reaction no. 10). It has been shown that the latter species together can destroy molecules of biological origin and kill microbes [3, 98]. Nitrogen oxide and its oxygen-containing derivatives can be classified as both ROS and reactive nitrogen species.

ROS and myeloperoxidase: pro and contra

The efficiency of the $MPO/H_2O_2/Cl^-$ system to kill microbes of different origins has been shown in vitro without any doubt, but concerns have been raised if these experimental settings apply to the neutrophil phagosome as well.

Contra These reconstituted in vitro systems used much lower levels of MPO than the estimated concentration in the phagosome; they were conducted at acidic pH (instead of the close-to-neutral measured in the phagosome) and did not contain high amounts of granule proteins present under physiological conditions [111]. It has been proposed that the function of Nox2 in the neutrophil phagosome is to liberate and activate bound granule proteins (which would be responsible after all for killing), and ROS produced are only byproducts of the reaction, which need to be eliminated in order to protect the neutrophil itself [121]. High levels of MPO indeed protect *Staphylococcus aureus* from being killed by the $MPO/H_2O_2/Cl^-$ system in vitro [111]. The activity of MPO at the reported pH in the phagosome (7.0–8.0) is also very low compared to the optimal levels at pH 5.0–5.5 [70, 111]. These findings might raise the possibility that MPO helps to control the robust levels of ROS in the phagosome besides converting hydrogen peroxide into HOCl. Most of the targets of the MPO-mediated chlorination in the phagosome are indeed neutrophil and not bacterial proteins [20, 111].

Pro On the other hand, many arguments support the importance of ROS and MPO as toxic agents [74]. Two studies have concluded that enough HOCl is produced in the phagosome of neutrophils to kill engulfed bacteria [45, 58]. Chlorination of neutrophil proteins leads to the formation of chloramines, which are still highly reactive and capable of reacting with microbes. It is also questionable if neutrophils do need to be protected against ROS since the cells die anyhow soon after the initiation of phagocytosis. *Helicobacter pylori* incapable of deoxyribonucleic acid (DNA) damage repair is sensitive to ROS-mediated killing [101]. OxyR-deficient *E. coli* are killed by neutrophils more efficiently than wild-type bacteria [128]. *S. aureus* capable of synthesizing antioxidant carotenoids has increased resistance to neutrophil killing [85]. Neutrophil cytoplasts (neutroplasts) do not contain nuclei and lost most of their granules (MPO too) but harbor an intact respiratory burst [78, 113]. Neutroplasts engulf but do not kill *S. aureus* unless the microbes are coated with MPO, which suggests that under these conditions, the respiratory burst by itself is not sufficient to kill the bacterium, but completed with MPO, it is [100].

The exact mechanism of oxidative killing of microbes in neutrophils and its players (NADPH oxidase, MPO, granule enzymes) is still a subject of debate. From an evolutionary point of view, the ROS-producing phagocytic NADPH oxidase is far too complex and dangerous if its primary purpose is simply to create membrane potential changes across the phagosomal membrane. Since CGD neutrophils can kill most bacteria as efficient as normal neutrophils and the list of pathogens in CGD patients is limited to only a few characteristic bacteria and fungi, most probably both ROS and the oxidase-induced membrane potential changes are very important but only two of the numerous elements of neutrophils' weaponry.

Nox2: an electrogenic transporter

Transfer of electrons via Nox2 results in net flux of negative charges across the membrane. This electron current can be measured by the patch clamp technique [120], and it leads to the change of the membrane potential. Resting PMNs have a membrane potential of about −60 mV that is quickly raised to approximately +60 mV or around 0 mV upon activation by PMA or the chemotactic agent formyl-methionyl-leucyl-phenylalanine (fMLP), respectively [56]. The electron current was shown to be dependent on the transmembrane potential difference and to cease when the membrane potential reached +160 to +200 mV [31, 104]. Taking into account the rate of $O_2^{\cdot-}$ generation and the membrane capacitance, it was calculated that the enzymatic activity of Nox2 would shut down in approximately 250 ms in neutrophils and in less than 20 ms in eosinophils [27, 31]. Evidently, the

duration of the oxidative burst is significantly longer, whether activation occurs by soluble or particulate agents [30]. Thus, the critical factor in maintaining $O_2^{\cdot-}$ production is adequate charge compensation.

Theoretically, compensation of the electron flux via Nox2 could occur by moving a cation in the same or an anion in the opposite direction or a combination of both processes (Fig. 2a). In the following paragraphs, we summarize the experimental data on movements of H^+, K^+, and Cl^- as the most probable ions for compensation of electron flux in PMN.

Charge compensation by H^+ efflux

H^+ efflux via electrogenic pathway(s) as charge compensation for $O_2^{\cdot-}$ production has been proposed more than 20 years ago [49]. Movement of protons parallel to electrons would avoid both strong acidification of the cytoplasm due to liberation of H^+ from NADPH and excessive alkalinization of the phagosomal space due to dismutation of $O_2^{\cdot-}$ ions (Fig. 2b).

Indeed, electrogenic H^+ transport has been detected and characterized in PMN both by chemical (following pH changes by fluorescent dyes) and electrophysiological (patch clamp) techniques ([29, 32, 66], reviewed in [27]). Although the molecular identity of the presumed H^+ channels has not yet been decided [27, 109, 117], there is general agreement that under resting conditions, H^+ conductance is very low. Several factors have been described that increase the opening probability and hence the H^+ current (Table 3). All these changes take place upon activation of PMN either by soluble or by particulate stimuli. In accordance with the concerted action of various factors upon the H^+ channels, in activated cells, the membrane potential was shown to correspond in a wide range to the equilibrium (Nernst) potential for H^+. This finding clearly indicates that in the activated state, H^+ conductance largely exceeds the conductance of any other ion [8].

Table 3 Factors affecting the H^+ channels in PMN

Activation	Inhibition
Plasma membrane depolarization [8, 29, 32, 64]	Zn^{2+}[8, 31, 60]
Intracellular acidification [22, 65]	Cd^{2+}[50, 60, 66]
Extracellular alkalinization [22, 49, 65]	
Arachidonic acid [21, 29, 48, 63, 65]	

However, there is no perfect synchronization between initiation of electron and H^+ flux as most of the factors leading to activation of H^+ channels (see Table 3) are consequences of Nox2 turnover. This is evident in changes of the plasma membrane potential: There is a rapid and drastic depolarization upon activation of PMN. After approximately 1 min, the membrane potential stabilizes or begins to reverse depending on the duration of the respiratory burst (see Fig. 3 of [41]). The fact that under identical conditions no change of the membrane potential can be detected in PMN from CGD patients [41] supports that depolarization of normal PMN is due to Nox2 activity. According to our quantitative analysis, there is a highly nonlinear relationship between the rate of $O_2^{\cdot-}$ production and extent of depolarization [107]: 50% of the maximal depolarization occurs when Nox2 turns only at 2% of its maximal activity. The membrane potential stabilizes when the enzyme activity reaches 10–20% of its maximal activity.

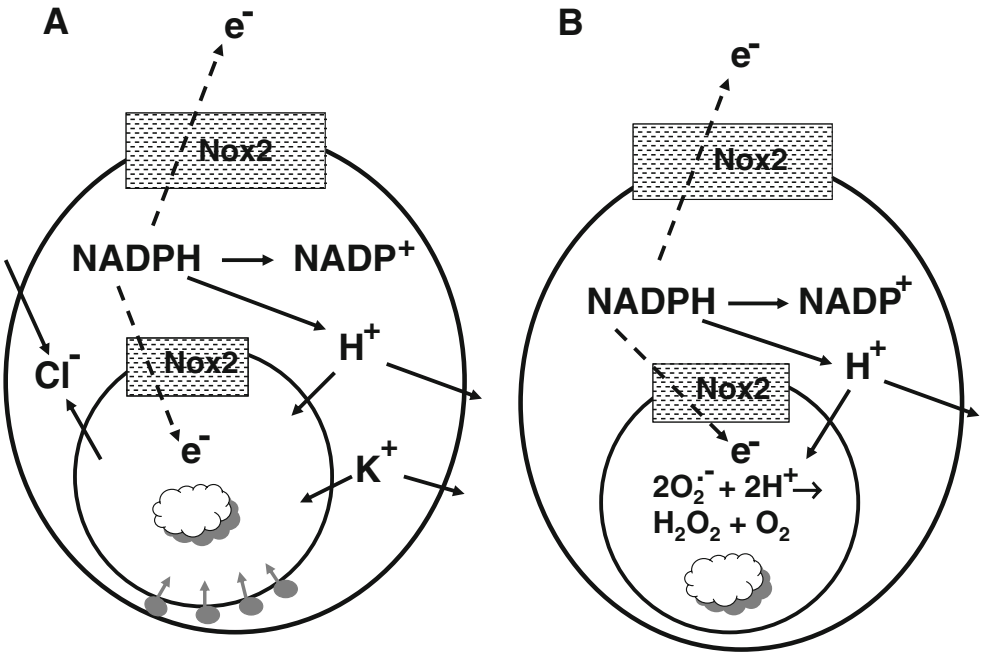

Fig. 2 Possibilities of charge compensation for electron transfer via Nox2. **a** summarizes the theoretical possibilities, whereas **b** shows the role of H^+ movements. Bacterium engulfed in the phagosome and release of protein content of different granules are represented with *symbolic figures*

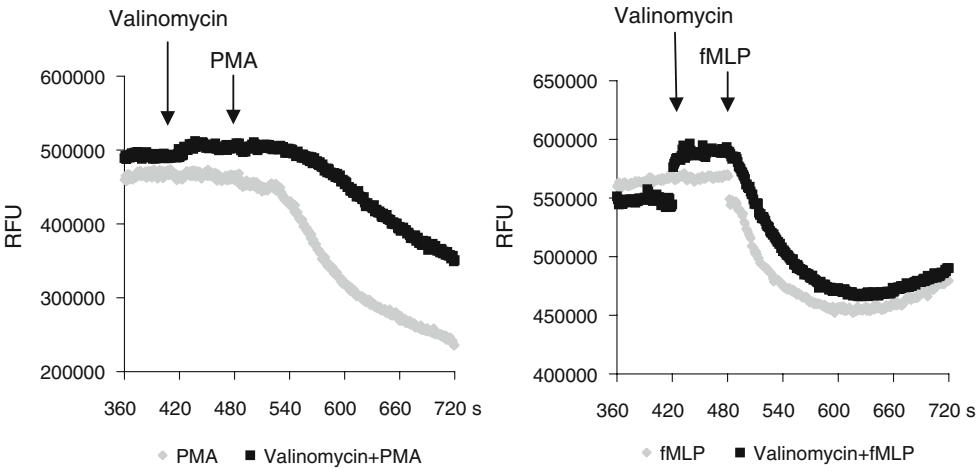

Fig. 3 Effect of valinomycin on the membrane potential of PMN. Membrane potential changes were followed by the potential sensitive fluorescent dye di-O-C_5 as described in [107]. Where indicated, valinomycin was applied in a concentration of 3 μg/ml; PMA at 100 nM, and fMLP at 1 μM. An increase in relative fluorescence (*RFU*) indicates hyperpolarization and a decrease in RFU signifies depolarization of the plasma membrane. Result of one representative experiment out of three similar ones is shown

Our experimental data correspond very well to the results of model calculations [95].

One study reported two-phase changes also in the intraphagosomal pH: Upon stimulation with PMA, an initial alkalinization was followed by acidification after approximately 2 min [122]. However, these observations could not be reproduced in another study [57].

The above data suggest that charge compensation is insufficient in the initial period or at a very low rate of $O_2^{\cdot-}$ production, and in this phase, the major compensating ion is probably not H^+. Stabilization of the membrane potential is reached when a new steady state is established, where electron transport is fully compensated by the movement of other ions. The fact that manipulation of either the H^+ permeability or the driving force for H^+ ions results in significant alteration of both the membrane potential and the rate of $O_2^{\cdot-}$ production (see Table 4) supports that in the steady state, the major compensating ion is H^+.

Charge compensation by K^+

Recently K^+ has been proposed as a cation responsible for partial compensation of the negative charges moved via Nox2 [1, 110]. In the same publications K^+ efflux from the cytosol of neutrophils has been interpreted as the key element of the killing process, and blocking of K^+ movement was suggested to prevent killing of microorganisms completely [1].

In the following paragraphs we list arguments which support or question the hypothesis on the central role of K^+ movements in microbial killing.

Data and considerations supporting the role of K^+ movements in bacterial killing

Most experiments concluded that the membrane potential of resting neutrophils is around −60 mV (for details, see [27]). This value is fairly close to the equilibrium potential calculated for K^+ ions (−89 mV; [96]) indicating that in the resting condition, K^+ conductance dominates. Earlier reports estimated the ratio of Na^+ to K^+ permeability in resting human PMN to be 0.1 [126]. It is thus reasonable to suggest that in the initial phase of $O_2^{\cdot-}$ generation, K^+ efflux could represent a significant component of charge compensation.

Indeed, K^+ or $^{86}Rb^+$ efflux has been measured upon activation of Nox2 [107, 110], and it is significantly enhanced by Zn^{2+} and Cd^{2+}, known inhibitors of the H^+ channels [1]. In our quantitative analysis, we found that a majority of K^+ movement takes place at low enzyme activity but gradually declines as depolarization progresses and H^+ channel activation increases [107]. This finding is in good agreement with earlier observations showing that H^+ conductance is dominant in the fully activated state [8].

In the case of maximal Nox2 activity, approximately 6% of the total charge movement was suggested to be compensated

Table 4 Consequences of alteration of H^+ movement through PMN plasma membrane

Primary alteration	Consequences
Inhibition of H^+ channels by Cd^{2+} or Zn^{2+}	Increase in depolarization upon stimulation with PMA or fMLP [8, 49, 106] Inhibition in $O_2^{\cdot-}$ generation stimulated with PMA or fMLP [31, 50, 66]
Increase in H^+ permeability by the mobile carrier CCCP	Prevention of PMA-induced depolarization ([49], Hably unpublished observation) Release of Zn^{2+} inhibition upon $O_2^{\cdot-}$ generation [31, 50]
Alteration of extracellular pH	At pH 6.6 increased depolarization upon PMA stimulation [49] At pH 8.3 decreased depolarization upon PMA stimulation [49]

by K^+ ions [121]. However, our data indicated that at the lowest measurable activity of Nox2 (2% of the maximal activity), 50% of the transported negative charge could be compensated by K^+ ions [106]. Thus, at low enzyme activity, K^+ movement may be critical. The major question is whether this K^+ flux has any relevance to the killing process.

In our experiments, we observed two phenomena that may indicate a role of K^+ movement in the elimination of bacteria. (1) In the presence of 10 μM Zn^{2+}, no increase of bacterial survival was observed in spite of a 30% reduction in $O_2^{\cdot-}$ generation [106]. Under these conditions, H^+ efflux is impaired resulting in decreased activity of Nox2 but increased depolarization and K^+ efflux [1, 106]. (2) The relationship between bacterial survival and Nox2 activity was steeper at low enzyme activity, when most of the K^+ flux took place, than at a high rate of $O_2^{\cdot-}$ generation when charge compensation occurred mostly by H^+ ions [107].

Both observations provide only indirect support for a role of K^+ ions in the elimination of microorganisms, and both refer to conditions in which Nox2 activity is limited. They may, however, model conditions where limited substrate supply—e.g., in hypoxic regions—allows only a low rate of $O_2^{\cdot-}$ generation.

Data and considerations questioning the central role of K^+ movements in bacterial killing

In an earlier publication, it has been claimed that bacterial killing was dependent on the opening or closing of one single type of K^+ channel [1]. If K^+ movements are so critical in the antimicrobial activity, then one would expect that alterations of the K^+ concentration of the environment have drastic effects on killing efficiency. However, in our hands, there was no difference in the killing activity of either human peripheral or murine bone marrow neutrophils whether the killing experiment was carried out in physiological solutions containing 5 mM K^+ or in nominally K^+-free media or in 145 mM KCl (Hably and Meczner, unpublished observations). These results are in agreement with earlier experiments, where alterations of K^+ concentration did not affect $O_2^{\cdot-}$ generation [50, 97, 130].

Another possibility for manipulation of K^+ movements is by application of valinomycin, an ionophore highly selective for K^+ and Rb^+. Valinomycin is the prototype for mobile carriers, and earlier investigations suggested that valinomycin doubles the K^+ permeability of human PMN [126]. The addition of valinomycin to resting neutrophils results in definitive hyperpolarization indicating an increase in K^+ permeability (Fig. 3). However, valinomycin has only a moderate effect on the rate or extent of depolarization induced by PMA or fMLP, i.e., when Nox2 is allowed to function at full activity (Fig. 3). Thus, K^+ flux carried by valinomycin is able to provide some—but far from full—charge compensation for electron transport via Nox2 (valinomycin is only able to provide significant charge compensation when Nox2 activity is mostly inhibited by diphenyliodonium [DPI] as in Fig. 4 of [41]). The important question is whether an increase of K^+ permeability has any effect on the killing activity of PMN. In our experiments, we could not detect any difference in elimination of *S. aureus* by human PMN in the absence or presence of the ionophore. Valinomycin did not affect the killing efficiency in K^+-free or K^+-rich medium either. Thus, even if valinomycin allows some charge compensation to occur via K^+, this does not seem to have significant impact on killing activity when Nox2 is turning at full capacity. Our findings are at variance with some previous data [110].

Our quantitative analysis also argues against an "all-or-none" role of K^+ movement in bacterial elimination. When Nox2 turned at 25% of its maximal rate, 70% of the total K^+ movement had already occurred, yet 90% of the bacteria survived [107]. An increase in the rate of $O_2^{\cdot-}$ generation resulted in gradual decrease in bacterial survival although only very little further K^+ transport occurred.

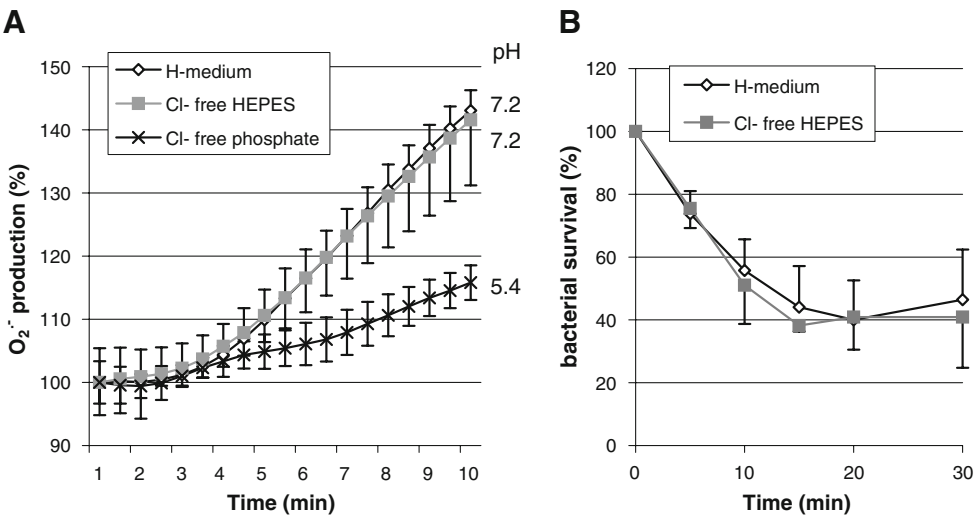

Fig. 4 Comparison of superoxide production and killing of *S. aureus* in media with high or low chloride concentration. In **a**, superoxide production was stimulated at time 0 by 100 nM PMA. pH of the solution at the end of the measurements is shown on the *right*. In **b**, killing of *S. aureus* was followed by the method described in [107]. In Cl^--free solutions, NaCl was replaced by 145 mM Na-HEPES or Na-phosphate pH 7.4, respectively. Mean±SD of three separate experiments is shown

A similar condition is known in human pathology in the form of "variant CGD" patients. In these cases, the mutation allows the expression of all the subunits, but the enzymatic activity is impaired so that the PMN of these patients are able to produce $O_2^{\cdot-}$ only at 10% to 20% of the normal rate [10, 127]. These patients present clinical symptoms, although their Nox2 activity could be sufficient to allow K^+ movements comparable to healthy cells.

K^+ channels in PMN

It is not clear through which kind of K^+ channels the detected K^+ flux occurs. Voltage-dependent, inwardly rectifying K^+ channels have been described in PMN and eosinophils, but these channels close upon depolarization [80, 130]. Thus, they probably do participate in the generation of the resting membrane potential but do not function under condition of intensive $O_2^{\cdot-}$ production. Indeed, blocking inwardly rectifying channels does not inhibit superoxide production [130]. Ca^{2+}-activated K^+ channels have also been demonstrated [77, 134], but their contribution is questionable, as in activated cells, H^+ current was the only detectable outward current [94].

The decisive function of large-conductance voltage- and Ca^{2+}-activated K^+(BK or maxi-K^+) channels has been proposed recently [1]. However, no trace of the existence or function of BK channels could be demonstrated by several groups (see Table 5).

If K^+ movement into the phagosome is important in the establishment of the optimal ionic environment for the function of granule enzymes, then it would be logical to increase K^+ conductance selectively in the phagosomal membrane. This goal could be achieved by the activation of some of the two pore domain K^+ channels (K_{2P}). Several members (e.g., TALK-1, TALK-2, TASK-1, TASK-2) of this family of K^+ channels are pH sensitive, showing increased conductance when the pH on the extracellular side becomes alkaline [33, 62]. In addition, TALK-1 and TALK-2 were shown to be activated by ROS [33]. Conditions prevailing in the phagosome (initial alkalinization, generation of $O_2^{\cdot-}$ and its metabolites) could be favorable for the opening of TALK-1 or TALK-2. With this idea in mind, we searched for the messenger ribonucleic acid (mRNA) of various K_{2P} channels. We could detect a message for TALK-2 both in peripheral blood PMN and in cultured PLB cells. The amount of TALK-2 mRNA was quantitated in real-time polymerase chain reaction experiments and estimated to be five orders of magnitude lower than that of GADPH. In view of this low level of mRNA, investigations at the protein level by electrophysiology or Western blotting were hitherto not feasible (Enyedi and Lakatos, unpublished observation).

Table 5 Experimental data arguing against a role of BK channels in bacterial killing (summarized in [28])

No BK current is detectable in patch clamp experiments [34, 37]
No BK channel is detectable by immunostaining [37]
IbTx does not inhibit detectable currents [34, 37]
IbTx does not inhibit degradation of bacterial phospholipids [37]
IbTx does not inhibit bacterial killing [28, 34, 37]
Presence of BK channels incompatible with measured depolarization [96]
Identical electrophysiological properties of $BK^{+/+}$ and $BK^{-/-}$ cells [34]
No change in bacterial clearence in $BK^{-/-}$ mice [34]

Charge compensation by Cl^-

The major mobile anion of both the extracellular and intracellular space is Cl^-. In a recent study, it has been proposed that 90% of charge compensation for Nox2 activity occurs via Cl^- flux from the extracellular or intraphagosomal space into the cytosol [121]. Further, it has been claimed that both the respiratory burst and microbial killing are abolished in the absence of Cl^- ions [121]. There are theoretical as well as experimental problems with Cl^- as the major charge-compensating ion. Theoretically, it is hard to envisage the movement of massive amounts of the osmotically active Cl^- ions and the consequent volume changes (for details, see [96]). Experimentally, Cl^- efflux has been detected during the respiratory burst, i.e., movement of Cl^- in the opposite direction than required for charge compensation [18, 89, 97, 125]. Recently, it has been reported that ClC-3-deficient mice have a defect in $O_2^{\cdot-}$ generation; nevertheless, these mice were fully competent in killing of various microbes [93].

In view of the controversial data, we tested the ability of human peripheral PMN to produce $O_2^{\cdot-}$ in a Cl^--free environment (Fig. 4). Our results indicate that the ability of $O_2^{\cdot-}$ generation depends on the species of the anion replacing Cl^-: It is significantly decreased in the phosphate-based medium but not altered in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-based medium that is widely applied in patch clamp experiments. As high phosphate concentration is known to chelate di- and trivalent cations, resulting in strong acidification, we tested the pH of the solutions. We found a correlation between acidification and inability of $O_2^{\cdot-}$ generation. Accordingly, in the presence of adequate buffering capacity, a high rate of $O_2^{\cdot-}$ production could be restored also in the phosphate-based medium.

Thus, Nox2 activity per se does not seem to depend on the possibility of Cl^- influx. In accordance with this finding, we did not observe any difference in the killing activity whether the extracellular space contained Cl^- ions or not (Fig. 4b).

Alterations of Ca^{2+} household in CGD cells

The function of the electrogenic NADPH oxidase also affects the movement of another ion, calcium. Ca^{2+} does not have a

crucial role in regulating the membrane potential but does play an important role in many cellular processes and signaling events. When the phagocytic NADPH oxidase is activated, the depolarization prevents calcium ions from entering the cell, and it diminishes cytosolic calcium levels. Early studies could not detect any differences in fMLP-induced calcium signals between normal and CGD neutrophils [84]. However, in this report, the investigators used the calcium-sensitive dye, Quin-2, which has low affinity for calcium, and it could have disturbed the calcium signals in the cells. More recent studies of our laboratory using the ratiometric dye Fura-2 with high affinity for calcium have clearly shown that calcium metabolism is affected in neutrophils and neutrophil-like cells in the absence of a functional NADPH oxidase [41, 108]. In fMLP-triggered CGD neutrophils, the influx through store-operated calcium channels was increased and started earlier than in normal cells [41, 131]. In model cells lacking the NADPH oxidase, the fMLP-induced calcium signal was higher than in the normal cells [108]. The inhibition of the oxidase by DPI in neutrophils increased the cytosolic calcium transient stimulated by fMLP [108]. Extracellular production of superoxide by the xanthine/XO system did not have any effect on the calcium influx [41] showing that superoxide anions are not responsible for the difference. Hyperpolarizing the membrane potential in neutrophils after the addition of fMLP does not change superoxide production but increases cytosolic calcium levels immediately (Fig. 5a) due to the oxidase-induced membrane potential changes that increase the driving force for Ca^{2+} influx (Fig. 5b,c). Altered calcium signaling in CGD neutrophils might cause disturbed cytokine release, apoptosis, and killing [108].

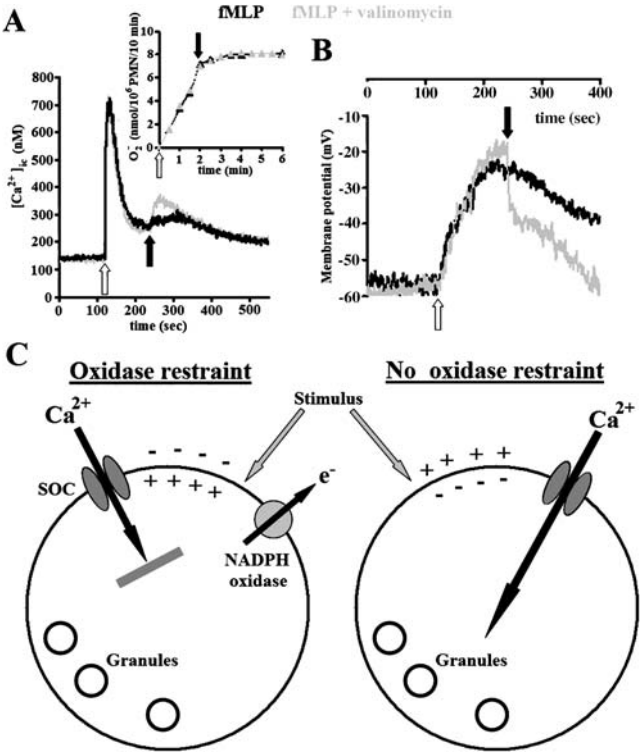

Fig. 5 The phagocytic NADPH oxidase inhibits calcium entry in human neutrophils. Human neutrophils were stimulated by fMLP (1 μM, *white arrow*), and 2 min later, the potassium ionophore, valinomycin (1 μM, *black arrow*), was added. Changes in cytosolic calcium concentration (**a**), superoxide production (**a**, *insert*), and membrane potential changes (**b**) were measured. Valinomycin-induced hyperpolarization did not influence fMLP-stimulated superoxide production but increased $[Ca^{2+}]_{ic}$. **c** Model showing the effect of the oxidase-induced depolarization on calcium entry. Result of one representative experiment out of four similar ones is shown

Effect of priming on electrophysiological properties of PMN

The effect of priming upon various PMN functions has been investigated in detail, and it is summarized in another paper of this issue (El Benna et al. 2008). However, there are two less well-known aspects that are related to the contribution of electrophysiological factors to the killing process.

Efficient priming during conventional preparation of PMN

In earlier studies, we observed that the release of secretory vesicles, the most easily mobilizable population of PMN vesicles, could only be detected if the cells were prepared under sterile conditions, in pyrogen-free solutions [91]; otherwise, they were eliminated already during the preparation process. We also found significant difference in fMLP-stimulated $O_2^{\cdot-}$ generation: Conventionally prepared PMN produced fourfold more $O_2^{\cdot-}$ than PMN prepared under sterile conditions did [61]. These data suggested that PMN might become primed during the conventional preparation process. Systematic investigation supported this suggestion (Fig. 6). PMN prepared under nonsterile conditions produced 3.5 times more $O_2^{\cdot-}$ upon stimulation with opsonized *S. aureus* than cells prepared under sterile conditions did (Fig. 6a). Nonsterile PMNs were also more efficient in killing of bacteria (Fig. 6b). We could partially mimic the effect of preparation under nonsterile conditions by treating sterile PMN with tumor necrosis factor-α (TNFα) or granulocyte macrophage colony-stimulating factor (Rada, unpublished observations). Thus, PMNs isolated by conventional techniques, i.e., not in solutions prepared with pyrogen-free water, are definitely primed, and their properties differ from those of resting cells.

Electrophysiological changes induced by priming

There was no difference in the resting membrane potential of PMN prepared under sterile or nonsterile conditions.

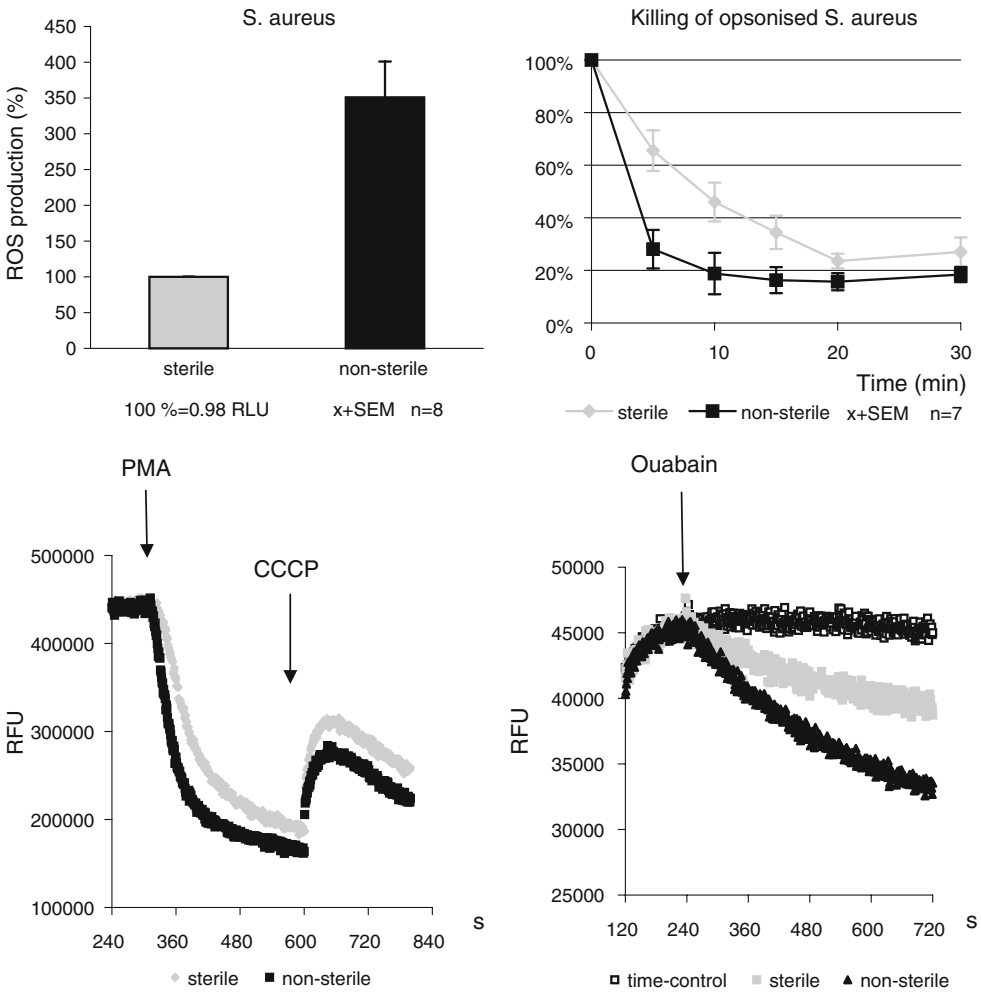

Fig. 6 Efficient priming of PMN during conventional preparation. ROS production (**a**), killing of bacteria (**b**), and rate of depolarization (**c** and **d**) were compared for PMN prepared under sterile or nonsterile conditions. In **c** and **d**, a decrease in RFU indicates depolarization. Mean±SEM of eight (**a**) or seven (**b**) separate experiments and the result of one representative experiment out of four (**c**) or eight (**d**) similar ones is shown

However, the rate of depolarization upon stimulation with PMA was increased in nonsterile cells, whereas the extent of depolarization showed no difference (Fig. 6c). These findings indicate that priming increased the activity of Nox2 but did not induce significant alteration in the activity of the charge compensating mechanisms.

Ionic composition of the intracellular space and thereby ionic movements are basically determined by the Na^+, K^+-ATPase. Inhibition of the Na^+/K^+ pump by ouabain results in gradual depolarization due to unopposed Na^+ influx. Following ouabain treatment of PMN, we observed significantly faster depolarization in nonsterile than in sterile cells (Fig. 6d), indicating an enhanced activity of the Na^+, K^+-ATPase in the primed cells. Activation of the Na^+, K^+-ATPase has been shown earlier to occur during differentiation of HL-60 promyelocytic cells [81] and upon chemotactic stimulation [9]. In renal collecting duct cells, it has been demonstrated that LPS and TNFα are able to induce the recruitment of Na^+, K^+-ATPase molecules to the plasma membrane [135].

Enhancement of the activity of the Na^+, K^+-ATPase as part of the priming process could increase the intracellular concentration of K^+ ions and their movement to the phagosomal space in compensation of the electron flux via Nox2. In this way, upregulation of the Na^+/K^+ pump may contribute to the observed improvement of the killing activity of primed PMN.

Medical significance of the mechanism of charge compensation for Nox2 activity

$O_2^{\cdot-}$ generation is vital in the efficient elimination of microorganisms. However, the production of $O_2^{\cdot-}$ at inappropriate sites or in an inadequate quantity can be deleterious to the host organism [112]. With the help of Nox2-deficient mice, it has been demonstrated that $O_2^{\cdot-}$ and its derivatives produced by phagocytes contribute to the pathogenesis of ischemia/reperfusion injury [103, 124], stroke [59, 136], neurodegenerative diseases [139], and alcohol-induced liver disease [77].

Thus, the inhibition of Nox2 could be advantageous in prevention or amelioration of several pathological conditions. At present, no efficient inhibitors are available that would be specific for any Nox or cell type [82] (the widely used inhibitor DPI interacts with almost any heme protein).

The inhibition of charge compensation has been shown to limit electron transfer via Nox2 [31, 66]. As ion channels have many isoforms and tissue-specific expression has been widely documented, careful investigation of the ion channels participating in charge compensation for electron flux through Nox2 may reveal new potential targets for pharmacological intervention.

On the other hand, if specific ion movement into the phagosome can—under certain conditions—indeed improve the efficiency of killing, then initiation of such ion movements may substitute or partially compensate for lacking O_2^- generation. This could provide a basis for new approaches in the therapy of CGD.

Alterations in CGD in addition to impaired phagosomal killing

The failure of CGD neutrophils to kill certain bacteria and fungi has been extensively studied, but numerous recent emerging reports suggest that the malfunction of the phagocytic NADPH oxidase in these patients has other consequences, as well.

It has been shown that CGD patients develop inflammatory granulomas in hollow organs without clinical signs of infections [19]; these granulomas are sterile, free of bacteria. Mice deficient in either $gp91^{phox}$ or $p47^{phox}$ show also exaggerated inflammatory reactions to fungus with enhanced accumulation of mononuclear cells and neutrophils [55, 95, 105]. These data suggest that disregulated inflammatory processes contribute to the symptoms of CGD. Neutrophils leave the blood stream and migrate to the site of infection by following the chemical gradient of inflammatory cytokines and bacterial products. On the site of bacterial invasion, neutrophils undergo apoptosis soon after the initiation of phagocytosis and are phagocytosed by macrophages. Delayed apoptosis and disregulated cytokine production could explain the experienced abnormalities in CGD.

Apoptosis and cytokine production in CGD

Many studies suggested that ROS induce apoptosis in neutrophils [4, 16, 35, 36, 46, 67, 86, 119, 140] and showed delayed onset of apoptosis in CGD neutrophils [26, 35, 39, 102, 116].

Several reports have shown that CGD neutrophils and CGD macrophages have altered production of inflammatory cytokines and mediators to a range of stimuli and also showed increased expression of inflammatory molecules in the lung of X-linked CGD (X-CGD) mice (Table 6). Hydrogen peroxide produced by the phagocytic NADPH oxidase has been presented to inhibit fMLP-triggered interleukin-8 (IL-8) production in human neutrophils in vitro, and this inhibition was absent in PMNs from CGD patients [83]. The altered calcium homeostasis of CGD neutrophils discussed above might also contribute to delayed apoptosis and altered cytokine production since calcium has been shown to be important in both processes in neutrophils.

Neutrophil extracellular traps

Recently, a very important novel function of ROS has been suggested in the formation of Neutrophil extracellular traps (NETs) [38]. NETs are extracellular fibers made by activated but not by naïve neutrophils. NETs contain smooth stretches and globular structures and are not surrounded by membrane [13]. DNA is the structural backbone of NETs, but it also contains proteins (histones and proteins from primary, secondary, and tertiary granules) [13]. The formation of NETs is an active process and can be initiated by different stimuli (PMA, IL-8, lipopolysaccharide [LPS], bacteria). NETs have been shown to colocalize with and kill bacteria extracellularly through their granular proteases [13]. NETs also capture and kill fungi [132]. As the formation of the NETs, a novel cell death program ("netosis") has been suggested [38]. During NET formation, stimulated neutrophils change their shape, homogenization of the hetero- and euchromatin occurs, later the membrane of the nucleus and the granules disintegrate, and their content becomes mixed [38]. Subsequently, the plasma membrane breaks, and the extracellular traps are released. The authors claim the mechanism for NET formation being different from both, necrosis and apoptosis, involving ROS formed by the phagocyte NADPH oxidase [38]. CGD neutrophils failed to form NETs when stimulated with stimuli that were effective in normal PMNs (*S. aureus*, PMA) but elicited NET formation when hydrogen peroxide was provided enzymatically by the glucose/GO system [38]. Type I and II interferons have been suggested to prime neutrophils for subsequent NET formation after stimulation by complement factor 5a [88]. With the discovery of NETs, the long-described antimicrobial effect of the histones and their derivatives also gain physiological importance [14, 52]. Beyond the in vitro studies, NETs have already been shown to be important in vivo in human preeclampsia [43], appendicitis [13], and streptococcal infections [92] resulting in necrotizing fascitis [17] and pneumococcal pneumonia [11]. NET formation revealed a new mechanism by which neutrophils kill microbes but also by which they might contribute to destruction of host tissues or to both at the same time, e.g., in the pathogenesis of sepsis [133].

Our view on the function of neutrophilic granulocytes in the human body has been changing tremendously recently. This cell type, previously thought to be very simple, fulfills a central role in coordinating the inflammatory process between the innate and the adaptive immunity [99] and goes through a second major burst of transcriptional and protein synthetic

Table 6 Altered expression and release of inflammatory mediators in chronic granulomatous disease

Cells, tissue	Stimulus	Mediator	↑/↓	Measured	Reference
Human neutrophils	Spontaneous	IL-8	↑	Cytokine release in the supernatant	[47]
	LPS	IL-8	↑	Cytokine release in the supernatant	[47]
	SAA	IL-8	↑	Cytokine release in the supernatant	[47]
	Spontaneous	TNF-α	↑	Cytokine release in the supernatant	[47]
	LPS	TNF-α	↑	Cytokine release in the supernatant	[47]
	SAA	TNF-α	↑	Cytokine release in the supernatant	[47]
	Spontaneous	PGD_2	↓	Release in supernatant	[16]
	fMLP	IL-8	↑	Expression and production	[83]
Human macrophages	Spontaneous	TNF-α	↓	Release in supernatant	[16]
		PGE_2	↓	Release in supernatant	[16]
		IL-10	↓	Release in supernatant	[16]
		PGD_2	↓	Release in supernatant	[16]
	Nonopsonized, apoptotic PMNs	TGF-β	↓	Release in supernatant	[16]
		PGD_2	↓	Release in supernatant	[16]
	Opsonized, apoptotic PMNs	TNF-α	↓	Release in supernatant	[16]
		PGE_2	↓	Release in supernatant	[16]
		IL-10	↓	Release in supernatant	[16]
		PGD_2	↓	Release in supernatant	[16]
Mouse lung	*Aspergillus fumigatus hyphae*	IL-1β	↑	Expression levels in total lung RNA	[95]
		TNF-α	↑	Expression levels in total lung RNA	[95]
		KC	↑	Expression levels in total lung RNA	[95]
		$TGF\text{-}\beta_1$	↑	Expression levels in total lung RNA	[95]

The table summarizes published data on altered levels of cytokine and inflammatory mediator expression and production measured either in isolated CGD neutrophils and macrophages or in whole-X-CGD animals (mice) in comparison to their normal countermates.
Proinflammatory mediators: *TNF-α* tumor necrosis factor alpha, *IL-1β* interleukin-1β, PGE_2 prostaglandin E_2, *IL-8* interleukin-8, neutrophil chemoattractant. Antiinflammatory mediators: *TGF-β* transforming growth factor β, PGD_2 prostaglandin D_2, *IL-10* interleukin-10. *LPS* Lipopolysaccharide, *SAA* serum amyloid A, *KC* murine homologue of the human GROα, growth related protein, *fMLP* formyl-methionyl-leucyl-phenylalanine, bacterial tripeptide, neutrophil chemoattractant. ↓ Reduced levels in CGD, ↑ enhanced levels in CGD

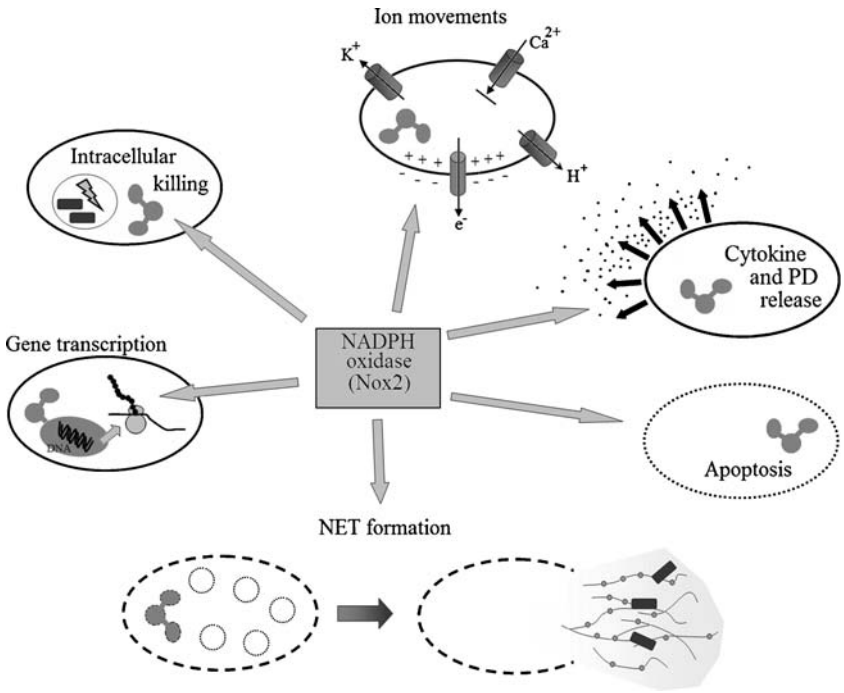

Fig. 7 Possible roles of the Nox2-based NADPH oxidase in PMN function

activities when leaving the blood stream [12]. Besides the novel roles of the best-studied neutrophil enzyme, the NADPH oxidase in bacterial killing (activation of certain granule enzymes and NET formation), a report investigating global gene expression changes in phagocytosing healthy and X-CGD neutrophils showed more than 200 genes whose expression levels are dependent on ROS produced by the NADPH oxidase [75]. CGD neutrophils have increased expression of proinflammatory and lowered expression of anti-inflammatory genes compared with normal neutrophils. These data of NADPH oxidase-dependent transcriptional changes of numerous genes support the observations that neutrophils deficient in $gp91^{phox}$ show prolonged inflammation and delayed resolution of the inflammatory process.

Conclusion

The experimental findings detailed above can be summarized in a very simple message: Nox2 plays multiple important roles in elimination of microorganisms (Fig. 7). Intraphagosomal killing is the result of concerted action of (1) various ROS formed from the primary product of Nox2, (2) different granule enzymes and antimicrobial peptides, and (3) ion transport that allows sustained Nox2 activity and provides optimal microenvironment. In addition to this classical function, ROS participate in induction of apoptosis, influence cytokine synthesis and secretion, and modify gene expression. Last but not least, they also play a role in extracellular killing of various microorganisms. Intensity and relative importance of these processes may differ in the case of different microorganisms or under different conditions (such as hypoxic environment), but apparently, they all contribute to the complex clinical picture that characterizes the immundefficiency in CGD.

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