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EDITORIAL REVIEW

Holding back neutrophil aggression; the oxidase has potential

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In the previous issue, Rada *et al*. [1] demonstrated an aspect of the mechanism by which neutrophils regulate their cytosolic Ca^{2+}. They show a clear link between the activity of the nonmitochondrial (NADPH) oxidase and Ca^{2+}influx in neutrophils. In view of the importance of Ca^{2+} in regulating various neutrophil activities, this has important implications both for our understanding of chronic granulomatous disease (GCD), where the oxidase is totally inoperative, and, perhaps at first thought, paradoxically, for suppressing inflammatory disease. In this short overview, some of the theory underlying the results of Rada *et al*. [1] is briefly explained and the potential importance of this type of Ca^{2+} regulation for restraining neutrophil 'aggression' during inflammation is discussed.

A NEW FUNCTION FOR THE NEUTROPHIL OXIDASE?

It can be argued that the purpose of the programme of activity that begins with the neutrophil leaving the circulation and ends in phagocytosis of infecting bacteria is to bring the neutrophil nonmitochondrial oxidase in close proximity to the bacterium. The oxygen metabolites, such as superoxide ions (O_2^-), which are generated by this oxidase, are highly reactive, and thus have short life-times with consequently small diffusion distances. It is thus reasonable to assume that if the highly reactive oxygen metabolites generated are involved in the killing of the bacterium, they do so within the phagosome. As the product of the dismutation of superoxide ions is peroxide (H_2O_2), there would also seem to be a role for myeloperoxidase which is secreted into the phagosome after phagosomal closure, especially since its product, hypochlorite (OCl^-) is also highly toxic to bacteria. However, there are some arguments against this latter mechanism. For example, while dysfunction of the neutrophil oxidase has serious consequences for the patient (i.e. CGD), myeloperoxidase deficiency is relatively common and has no obvious clinical manifestations. Also, Reeves *et al.* [2] have argued that the main purpose of the oxidase may be as a proton pump, regulating the intraphagosomal pH or controlling K^+ concentration, both releasing the proteolytic activity of neutral proteases in the phagosome. Whatever the details, there is general agreement that the oxidase is involved in bacterial killing. However, could the oxidase have a second function not related to bacterial toxicity?

The key feature of the nonmitochondrial oxidase is its ability to transfer electrons from NADPH (the electron donor) to oxygen (the electron acceptor) across a membrane. While NADPH is within the cytosol, the oxygen acceptor is within the phagosome or external to the cell (Fig. 1a). There is consequently a vectorial movement of electrons (negative charge) across the phagosomal or plasma membrane. Although this movement of charge could be compensated for by an accompanying flow of positive charge (e.g. H^+), it has been shown that there is a significant current which results in a change in the potential across the membrane towards the positive [3–5]. Thus the neutrophil oxidase (like the mitochondrial oxidase) is electrogenic, i.e. it creates a change in the potential across the membrane in which it is operating. This raises the question of whether the electrogenic nature of the oxidase is merely an inevitable but unimportant accompaniment to oxidant generation or whether this aspect of its activity also has any biological consequences.

RESTRAINT OF CA^{2+} INFLUX BY THE OXIDASE-GENERATED MEMBRANE POTENTIAL

Although neutrophils are not electrically excitable cells, like nerve or muscle, and do not have voltage sensitive ion channels, the electrogenic effect of the oxidase is not trivial. When activated, the oxidase can result in the membrane potential rising to between + 30 and + 50 mV [3–5]. At these positive levels, it would be expected that the movement of charged ions, such as H^+, K^+ and especially Ca^{2+} would be affected. Ca^{2+} influx occurs when Ca^{2+} channels are open in the phagosomal and plasma membranes [6,7]. With the extracellular (or intraphagosomal) Ca^{2+} concentration at about 1 mM and the cytosolic Ca^{2+} at about 100 nM, there is a 10,000-fold difference in concentration. In the absence of any electrical effect, Ca^{2+} ions would flood in through the open Ca^{2+} channels (Fig. 1b). In fact, in the resting neutrophil, the membrane potential, which is negative (inside), exerts an even steeper electrochemical gradient. However, when the oxidase is activated

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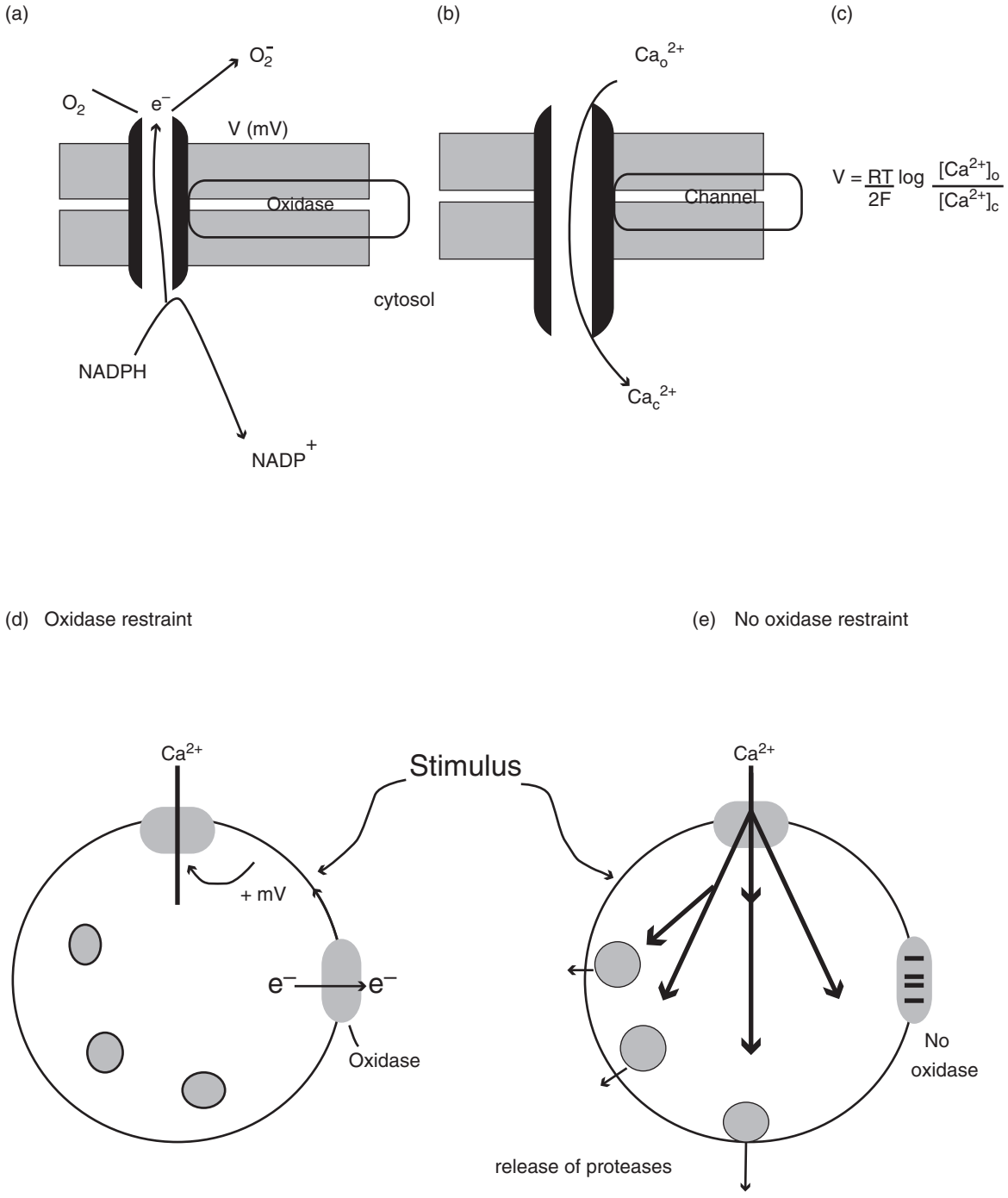

Fig. 1. Electrogenic effect of the oxidase on Ca^{2+} influx. The figure shows the schematic lay-out of (a) the oxidase, transporting electrons across the phagosomal or plasma membrane and generating a transmembrane potential (V) and (b) the open Ca^{2+} channel with Ca^{2+} ions moving against the electron flow. These opposite effects balance when the Nernst equation (c) is true, where R,T and F are the gas constant, the absolute temperature and the Faraday constant, respectively; 2 is the valency of Ca^{2+} and the square brackets denote the equilibrium concentrations of Ca^{2+} outside the cell and in the cytosol (denoted by the subscripts o and c, respectively) at the transmembrane potential, V. (d) shows the effect of a stimulus on an oxidase-competent neutrophil, causes Ca^{2+} influx, which activates outwardly directly electron transport via the oxidase. This results in an increase in membrane potential towards the positive, limiting Ca^{2+} influx. (e) shows the effect on an oxidase-defective cell, the Ca^{2+} influx restraint is lost and more Ca^{2+} enters the cell causing other cellular events including the release of proteases from stored granules to occur.

and the membrane potential reverses, the inside of the cell becomes positive relative to the outside, so that the influx of positively charged ions, like Ca^{2+}, would be impeded, a phenomenon termed here 'oxidase restraint'. From the Nernst equation (Fig. 1c), it can be seen that at positive membrane potentials of 30–50 mV (those reported when the oxidase is active), the inward Ca^{2+} electrochemical gradient would cease when the concentration of Ca^{2+} on the cytosolic face of the Ca^{2+} channel was about 24–100 μM. In other words, despite the Ca^{2+} channels being open, there would be no further net flux of Ca^{2+} into the cell. The inward concentration gradient would be balanced by the opposing voltage gradient (Fig. 1d). This theoretical possibility has been tested by Ligeti's group [1,8]. They have shown previously with neutrophils from patients with GCD, which are unable to mount an oxidase response, that Ca^{2+} influx is exaggerated [8]. This result in CGD neutrophils has more recently been confirmed by another group [9]. Now Ligeti's group have also shown, both in a genetically modified cell-line devoid of the oxidase component $gp91^{phox}$ and in neutrophils in which oxidase activity was inhibited pharmacologically, that there is a demonstrable linkage between oxidase activity, its accompanying membrane depolarization and the extent of Ca^{2+} (or Mn^{2+}) influx [1]. There is thus now good experimental support for the theoretical basis of the 'oxidase restraint' model outlined here.

SIGNIFICANCE OF CA^{2+} INFLUX FOR NEUTROPHIL BEHAVIOUR

It is well known that neutrophil responses to a variety of stimuli are triggered by rises in cytosolic free Ca^{2+} [10]. This Ca^{2+} signal is often initiated by a release of Ca^{2+} from intracellular stores within the neutrophil, which is coupled, either within a few tens of milliseconds [11] or tens of seconds [12] to the opening of Ca^{2+} channels on the plasma membrane and a consequent influx of Ca^{2+}. While there is no clear role for the release of stored Ca^{2}, the accompanying Ca^{2+} influx is crucial for a number of neutrophil responses [10]. Ca^{2+} influx locally raises the cytosolic free Ca^{2+} just under the plasma membrane to over 50 μM [13], and is sufficient to activate submembrane μ-calpain to cleave β2 integrin from its cytoskeletal tether [14], with a kD for Ca^{2+} of about 30 μM. However, at higher submembranous cytosolic free Ca^{2+} concentrations (100–300 μM), exocytosis of myeloperoxidase-containing and protease-containing granules is also triggered [6,15]. This latter event would be pathogenic if uncontrolled. Fortunately, as Ca^{2+} influx is also important for activating the oxidase system [10], the accompanying voltage change would have a self-restricting effect on Ca^{2+} influx. For example, during phagocytosis, Ca^{2+} channels on the phagosomal membrane (presumably arising from the invaginated plasma membrane) are opened and Ca^{2+} effluxes from the phagosome into the cytosol [7], causing the oxidase to be activated, and the potential across the phagosomal membrane to increase. As the oxidase-generated membrane potential change approaches the Ca^{2+} reversal potential, the tendency for Ca^{2+} to influx into the cytosol would be reduced and so limit the possibility of pathogenic degranulation.

OXIDASE RESTRAINT ON NEUTROPHIL AGGRESSION

From the above discussion, it can be seen that as well as being a mechanism for intraphagosomal killing of bacteria, the oxidase may also be key to limiting the pro-inflammatory effects of cytosolic free Ca^{2+} within the neutrophil. In this issue, Rada *et al*. [1] suggest that this effect may underlie some of the pathology of CGD [16]. For example, while changes in cytosolic free Ca^{2+} are not necessary for chemotaxis by neutrophils [17,18], high cytosolic free Ca^{2+} causes them to become immobile [17]. In CGD this may result in the accumulation of immobile neutrophils at infection foci producing granulomas. This would be especially the case if high Ca^{2+} were also antiapoptotic [19]. Perhaps more importantly, if this mechanism exerts a limitation on neutrophil 'aggression', there are wider implications for this mechanism. Any reduction in oxidase activity, while perhaps not sufficiently severe to totally inhibit bacterial killing, may have a pathological consequence by failing to limit Ca^{2+} influx. Such cells would be easily activated, hyper-responsive and prone to degranulate, all conditions that may lead to inappropriate activation and inflammatory disease. It is thus interesting that hyperactivation of neutrophils has been described in CGD, and attributed to an accelerated Ca^{2+} influx [9]. Perhaps a more striking effect of the lack of 'oxidase restraint' is seen in a recent report that inflammatory arthritis in rats was linked to a polymorphism of a gene expressing neutrophil cytosolic factor (Ncf1), the analogue of human $p47^{phox}$ of the neutrophil oxidase, and that decreased neutrophil oxidase activity was associated with increased arthritic severity [20]. Strategies that increased neutrophil oxidase activity were found to be beneficial in reducing inflammatory tissue damage in this animal model [20]. Thus the activity of the neutrophil oxidase may be exerting a crucial role under inflammatory conditions in providing a restraint over Ca^{2+} influx and consequently acting as a brake on neutrophil aggression. Clearly, there is much to be done to establish this model and to identify ways in which it could be beneficially exploited. However, it is clear that the recent papers connecting the neutrophil oxidase, Ca^{2+} influx and neutrophil behaviour are already laying the necessary groundwork.

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