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The role of C-kinase in the physiological activation of the neutrophil oxidase

Evidence from using pharmacological manipulation of C-kinase activity in intact cells

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The role of C-kinase in the triggering of the neutrophil oxidase by two stimuli (latex beads and the chemotactic peptide fMet-Leu-Phe), representative of endocytotic and exocytotic routes of activation, were investigated by using experimental agents that activate, or inhibit C-kinase, in intact cells. The activation by the phagocytotic stimulus latex beads (i) was mimicked by C-kinase activators giving the same characteristic lag (20–30 s), followed by a constant oxygen consumption rate with the same maximum rate and affinity for oxygen (K_m approx. 13 μM), (ii) competed with activation by PMA (4β-phorbol 12-myristate 13-acetate) in a simple common-target manner, and (iii) was inhibited by retinal, an inhibitor shown to inhibit activation by PMA. In contrast, activation by chemotactic peptide (i) was not mimicked by C-kinase activation alone, chemotactic peptide inducing biphasic oxygen consumption with a K_m for oxygen of the second prolonged phase of 3.9 μM, (ii) did not compete with activation by PMA, and (iii) was not inhibited by retinal. However, PMA and retinal produced slight enhancements of activation by chemotactic peptide and production of monophasic oxygen consumption. It was concluded that C-kinase activation plays a simple central transducing role in activation of the oxidase by latex beads, but that its role in activation by chemotactic peptide is a part of a more complex set of interactions that involve other Ca^{2+}-activated and non-Ca^{2+}-activated processes.

INTRODUCTION

The activation of the neutrophil oxidase during phagocytosis may play a role in the killing of endocytosed micro-organisms (Babior *et al.*, 1973). The oxidase is also activated during chemotaxis, and may mediate the pathogenesis of some inflammatory diseases (Fridovich, 1979). Whereas the molecular mechanisms of oxygen reduction by the activated oxidase are becoming clear (Cross *et al.*, 1985), the mechanisms which trigger the activation during these two physiological events remain obscure. It has been established that, for chemotactic stimulation of the oxidase, there is an absolute requirement of a rise in intracellular Ca^{2+}, whereas for phagocytotic stimulation, no change in intracellular Ca^{2+} was detected, and activation occurred in the presence of intracellular Ca^{2+} buffering (Campbell & Hallett, 1983; Lew *et al.*, 1984). A further complication was that activation of the oxidase during chemotactic stimulation occurred at far lower intracellular Ca^{2+} concentrations than that required when stimulating with calcium ionophore or the membrane-pore-forming attack complex, complement (Hallett & Campbell, 1982, 1984). These experiments led to the conclusion that other intracellular messengers beside Ca^{2+} were operating in these cells. Recently, a mechanism has been suggested that would account for these apparent paradoxes and suggest the nature of the other messenger, namely, the involvement of the Ca^{2+}- and phosphatidylserine-dependent C-kinase (Nishizuka, 1984). Although this enzyme has been implicated in neutrophil oxidase activation, (i) by the association of phosphorylation of certain proteins with chemotactic stimulation (Huang *et al.*, 1983) and (ii) by the predicted synergistic effect of the Ca^{2+} ionophore and the C-kinase activators PMA and OAG (Dale & Penfield, 1984; Kajikawa *et al.*, 1983), no direct demonstration that C-kinase is causally involved in physiological stimulation has yet been made. The aim of the present paper, therefore, was firstly, to define the effects on oxidase activation of experimental agents that activate and inhibit C-kinase activity. The second aim was to determine whether the interaction of these agents with two stimuli representative of the physiological routes, namely phagocytosis and chemotaxis, was consistent with a physiological involvement of C-kinase.

EXPERIMENTAL

Methods

Rat neutrophils were prepared from peritoneal exudate 20 h after intraperitoneal injection of sodium caseinate (12%, w/v) as previously described (Hallett *et al.*, 1981). This procedure produced (1–3) $\times 10^8$ cells/rat with a final purity and viability greater than 99%. Unless otherwise stated, cells were suspended in modified Krebs–Ringer Hepes buffer, containing 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM-$MgSO_4$, 1.2 mM-KH_2PO_4, 1.3 mM-$CaCl_2$, 0.1% (w/v) bovine serum albumin and 25 mM-Hepes, adjusted to pH 7.4 with NaOH.

Oxygen consumption measurements. Oxygen consumption was measured by using a Clark oxygen electrode chamber (Rank Bros., Bottisham, Cambridge, U.K.) held

Abbreviations used: PMA, 4β-phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol.

at a polarizing voltage of 0.6 V. The output was measured with a 'home-built' nanoammeter calibrated by use of a digital gas mixer (Edwards *et al.*, 1983). The response time of the electrode was less than 0.1 s. Latex beads were added as an aqueous suspension. All other reagents were dissolved in dimethyl sulphoxide, producing a final concentration in the cell suspension of less than 0.3%. All oxygen-consumption rates reported here were insensitive to cyanide, azide or indomethacin.

Oxygen-affinity measurements. The oxygen affinity of the activated oxidase was measured as described previously (Edwards *et al.*, 1983) and the K_m defined as the oxygen concentration reducing the respiratory rate by half.

Materials

Sodium caseinate (nutrose) was purchased from Difco, Detroit, MI, U.S.A., and PMA, OAG, retinal, fMet-Leu-Phe (chemotactic peptide) and cytochalasin B from Sigma Chemical Co. (Poole, Dorset, U.K.). A sample of OAG was kindly provided by Professor Y. Nishizuka, Department of Biochemistry, Kobe University Medical School, Kobe, Japan. Latex beads (diameter 1.01 μm) were purchased from Poly Science Ltd. (Northampton, U.K.), Ficoll–Hypaque from Pharmacia, trifluoperazine (stelazine) from Smith, Kline and French, Welwyn, Herts., U.K., and all other reagents were of AnalaR grade from BDH, Poole, Dorset, U.K.

RESULTS

Stimulation of neutrophil oxidase by C-kinase activators

The characteristics of neutrophil stimulation by three C-kinase activators, PMA, mezerein (Daphnetoxin) and OAG, were compared (Fig. 1). Other activators of C-kinase *in vitro*, dipalmitoylglycerol, dilinoleoylglycerol and dioleoylglycerol, had no effect on neutrophil activation. The three agents that caused activation did so with similar characteristics, namely: (i) a detectable lag after addition of the agent, before the onset of oxidase activation, of approx. 25–30 s, followed by (ii) a constant rate of oxygen consumption that continued for at least 30 min; (iii) the maximum rate of stimulation was approx. 2.5 nmol of O_2/min per 10^6 cells; (iv) K_m for oxygen of 13.3 ± 0.6 μM. These characteristics were similar for activation by non-opsonized latex beads, where the lag was 30–40 s, with a prolonged constant rate of oxygen consumption that had a maximum at approx. 2.5 nmol of O_2/min per 10^6 cells and a K_m of 12.6 μM-O_2 (see Fig. 4*a* below). This was in contrast with stimulation by fMet-Leu-Phe, where biphasic oxygen consumption was stimulated, the first transient rate occurring after a lag of approx. 20 s with a maximum of approx. 2 nmol of O_2/min per 10^6 cells for only 60 s. This was followed by a slower prolonged phase lasting for at least 30 min, having a maximum rate of only 0.5 nmol of O_2/min per 10^6 cells. Although it was not possible to determine accurately the oxygen affinity of the first transient phase, the K_m for the second phase has been accurately measured at 3.7 μM-O_2 (Edwards *et al.*, 1983). It was therefore concluded that the characteristics of stimulation of the neutrophil oxidase by non-opsonized latex beads, but not by the chemotactic peptide, could be mimicked by the agents PMA, mezerein and OAG. The relative potencies of these three agents in activating

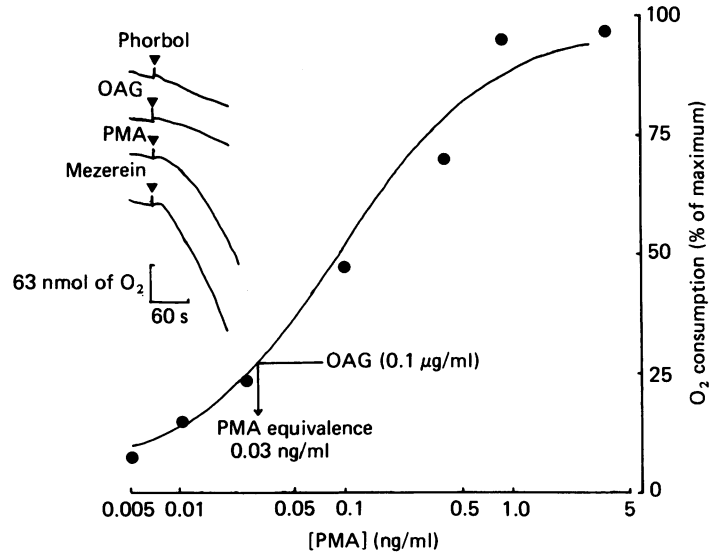

Fig. 1. Effect of C-kinase activators on neutrophil oxidase activity

The traces show the change in oxygen content in 3 ml of solution containing 2×10^7 rat neutrophils on addition of (*a*) phorbol (2 μg/ml), (*b*) OAG (0.1 μg/ml), (*c*) PMA (0.1 μg/ml) and (*d*) mezerein (1μg/ml), at the point indicated on the trace by the upward deflection of the event marker. The graph shows the relationship between PMA concentration and response, with the method used for determining the relative potency of the agent in terms of PMA equivalence as illustrated.

the neturophil oxidase was determined by relating the concentration required for stimulation to PMA (as shown in Fig. 1). The rank order of potency was PMA > mezerein > OAG, which was the same rank order as for C-kinase activation *in vitro* (Miyake *et al.*, 1984; Mori *et al.*, 1982). The possibility therefore existed that C-kinase activation was a common step in activation of the neutrophil oxidase by both these agents and latex beads.

Interaction of C-kinase activation with physiological stimulation

In order to test the possibility that C-kinase activation plays a role in physiological stimulation, the effect of activation of C-kinase artificially on subsequent physiological stimulation was determined. It was found that when C-kinase was activated submaximally with PMA, subsequent addition of latex beads produced no further activation [Fig. 2*a*(i)]. This would be predicted if C-kinase activation were a common step in the activation by both stimuli. When C-kinase was activated maximally, decreased responses to latex beads were also observed to an extent that was predictable from simple common step competition. (Fig. 2*b*). In contrast, after maximal stimulation with PMA, fMet-Leu-Phe was able to produce further stimulation [Fig. 2*a*(ii)] that was not abolished by the absence of extracellular Ca^{2+} [Fig. 2*a*(iii)]. At submaximal activations of C-kinase, the effect of fMet-Leu-Phe was slightly enhanced (Fig. 2*b*). After PMA treatment, the lag time for chemotactic peptide was decreased and the response was monophasic, with a K_m of 3.7 μM-O_2. These data were consistent with C-kinase

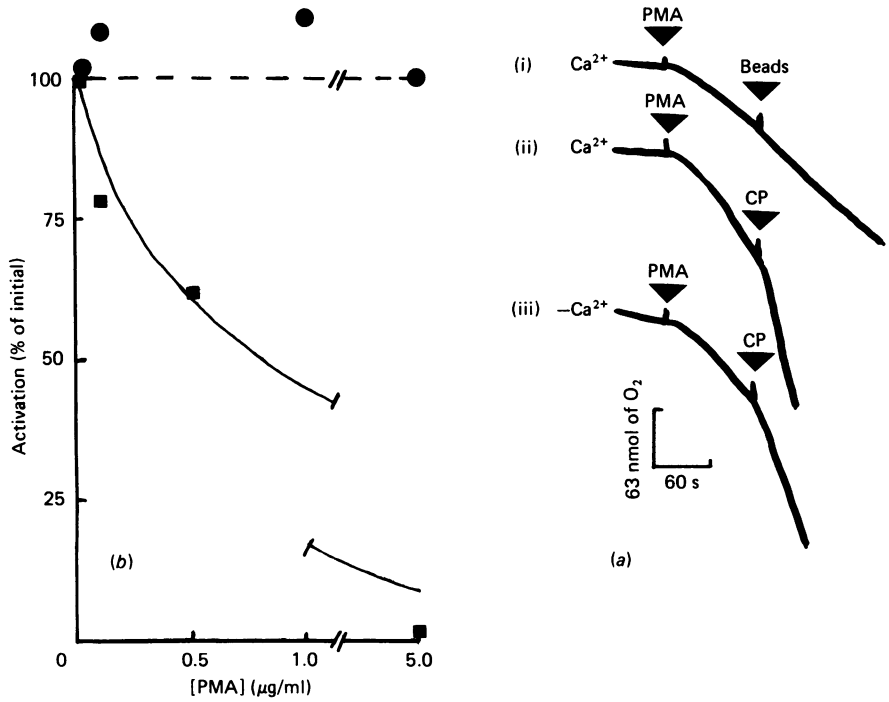

Fig. 2. Interaction between C-kinase activators and physiological stimulation

(*a*) The traces show the change in oxygen content in 3 ml of suspension containing 2×10^9 rat neutrophils. At the first arrow, PMA (5 ng/ml) was added, and at the second arrow, latex beads (4×10^9/ml), or fMet-Leu-Phe (1 μM) were added. The upper two traces were in the presence of Ca^{2+} (1.3 mM), whereas the lowest trace was in the absence of Ca^{2+} and the presence of EGTA (1 mM). (*b*) The experiment illustrated in (*a*) was repeated at various concentrations of PMA. The graph shows the relationship between the additional effect of the physiological stimulation at each PMA concentration. The stimuli used were beads (4×10^9/ml (■) or fMet-Leu-Phe (1 μM) (●). The continuous line is drawn to show the expected relationship between the two parameters if both agents competed for a common target from the equation:

$$\text{Response} = (KA + X)/(1 + KA + X)$$

where K is the affinity constant for PMA [1/(0.1 ng/ml)] determined from Fig. 1, A is the PMA concentration and X is the product of the affinity constant for latex beads and the concentration (taken as 2). The broken line shows the relationship expected if the two agents acted in complete independence.

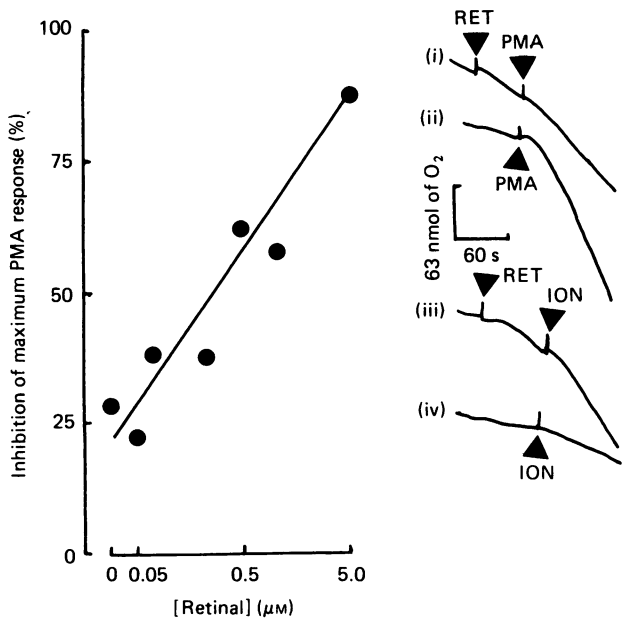

Fig. 3. Effect of retinal on PMA stimulation of neutrophil oxidase

The graph shows the relationship between retinal concentration and the inhibition of PMA activation calculated as:

$$\frac{1-(\text{PMA}_R - \text{RET})}{\text{PMA}} \times 100\%$$

where PMA_R was the response to PMA observed after retinal, RET the response after retinal, and PMA the response after PMA alone. This was genuine inhibition as opposed to competition between agonists, since the inhibitions could not be accounted for by approach of the cell response to its maximum level. This is illustrated in the upper two traces, which show the change in oxygen content in 3 ml of suspension containing 2×10^7 rat neutrophils on addition of (i) retinal (RET); PMA (0.1 μg/ml) failed to produce the maximum stimulation observable by PMA alone (ii). (iii) In contrast, after retinal (RET), ionophore A23187 (ION) (0.1 μM) produced a marked response (third trace) bigger than that observed with ionophore alone (iv).

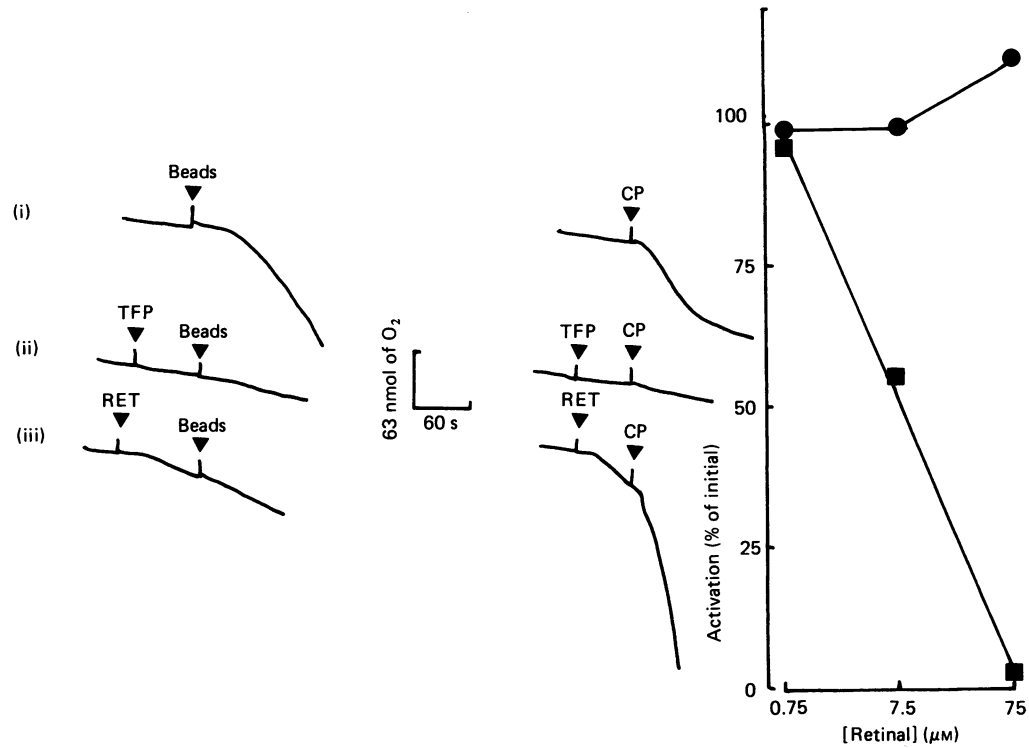

Fig. 4. Effect of C-kinase inhibitor on physiological stimulation

The traces show the change in oxygen content in 3 ml of suspension containing 2×10^7 rat neutrophils on addition of (left-hand side) beads (4×10^9/ml) (i) alone, (ii) after trifluoperazine (TFP) (50 μM) and (iii) after retinal (75 μM), and (right-hand side) to fMet-Leu-Phe (1 μM) (i) alone, (ii) after trifluoperazine (50 μM) and (iii) after retinal (75 μM). The graph shows the relationship between the percentage of uninhibited activation and retinal concentration. As before, these were calculated after subtraction of the small stimulatory effect of retinal, and again the inhibitions were not explainable by the stimulatory effect of the first (iii) agent [compare traces (i) and (ii), left hand side].

activation being the crucial step in stimulus–response coupling with latex beads as the stimulus. However, it was also concluded that fMet-Leu-Phe generated an intracellular messenger that acted in addition to C-kinase activation.

Inhibition of the neutrophil oxidase by C-kinase inhibitors

Inhibitors of C-kinase activation were required to test further the possible role of C-kinase activation in stimulus–oxidase-activation coupling. Trifluoperazine was not suitable, since it inhibited both C-kinase and calmodulin. Retinal (retinaldehyde) has been reported to inhibit C-kinase activation *in vitro* (Taffet *et al.*, 1983). In order to determine the usefulness of the latter agent for cellular experiments, it was necessary to show that it could inhibit neutrophil stimulation by a known C-kinase activator acting independently of raised intracellular Ca^{2+}. Retinal produced a marked inhibition of neutrophil activation by PMA when added before the phorbol ester (Fig. 3), with a K_i of approx. 0.4 μM. In contrast, retinal did not inhibit activation by the Ca^{2+} ionophore A23187, which was enhanced. It was concluded that retinal provided a useful tool for investigating the role of C-kinase.

Retinal produced a marked inhibition on stimulation by latex beads (Fig. 4), whereas it had no effect, or gave a slight enhancement, on that by fMet-Leu-Phe (Fig. 4). The concentration required to inhibit the stimulation by latex beads to 50% was significantly higher than that observed with PMA (Fig. 3). It was concluded that retinal also provided evidence for a role for C-kinase activation during phagocytosis, but that stimulation by chemotactic peptide was more complex.

DISCUSSION

The results presented here have demonstrated that phagocytotic stimulation of neutrophil oxidase activity can (i) be accurately mimicked by three structurally dissimilar C-kinase activators (Fig. 1), (ii) compete with C-kinase activation in a simple common 'target' manner (Fig. 2), and (iii) be abolished by inhibitors of C-kinase (Fig. 4), including retinal, shown to be specifically antagonistic towards Ca^{2+} independent C-kinase activation (Fig. 3). These data provide strong evidence for a crucial role of C-kinase in neutrophil activation by this physiological route, and we therefore propose a simple hypothesis, namely that interaction of latex beads with the neutrophil membrane leads to oxidase activation via a step involving C-kinase activation (Fig. 5). Since it is well established that C-kinase activation occurs *in vitro* by the concerted action of Ca^{2+}, phosphatidylserine and diacylglycerol (Nishizuka, 1984), any of these three individually, or, in combination, could in theory act as a trigger for physiological C-kinase activation. We have previously demonstrated that elevated intracellular Ca^{2+} was not required for oxidase activation by phagocytotic

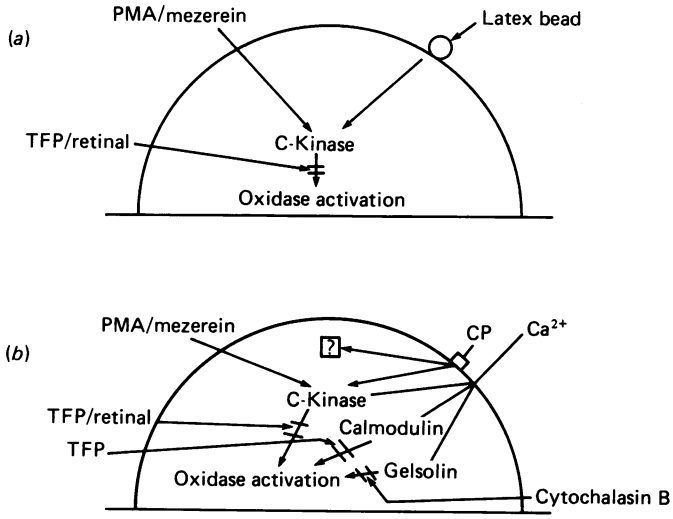

Fig. 5. Hypothesis of roles of C-kinase in activation by (*a*) latex beads and (*b*) fMet-Leu-Phe

Abbreviation used: TFP, trifluoperazine.

stimuli (Campbell & Hallett, 1983), and we must therefore conclude that either diacylglycerol, phosphatidylserine, or both, serve as the physiological activator.

In contrast with the simple role of C-kinase postulated for activation by latex beads, the results presented here show that activation by fMet-Leu-Phe: (i) cannot be accurately mimicked by C-kinase activation alone (Fig. 1), (ii) does not compete simply with C-kinase activation (Fig. 2), and (iii) was not inhibited by retinal, a C-kinase inhibitor (Fig. 4). Clear evidence that fMet-Leu-Phe produced stimulation by a route independent of C-kinase activation was provided by the demonstration that, after maximal stimulation of C-kinase by high concentrations of PMA, fMet-Leu-Phe was able to produce further stimulation (Fig. 2). Since the response to fMet-Leu-Phe was totally dependent on a trifluoperazine-sensitive step (Fig. 4) and was enhanced by the microfilament disrupter cytochalasin B (Cooke *et al.*, 1985), additional intracellular receptors for Ca^{2+} may be calmodulin and gelsolin (Fig. 5).

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The demonstration of two distinct molecular mechanisms for oxidase activation has two important implications. Firstly, characteristics of the oxidase isolated from activated neutrophils may depend upon the stimulus used. Secondly, the possibility exists that useful specific pharmacological interventions of pathological oxidase activation may be possible.

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