An EPR study of the production of superoxide radicals by neutrophil NADPH oxidase

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1. INTRODUCTION

The production of superoxide and hydroxyl radicals by neutrophils has been shown to occur following stimulation with membrane-perturbing agents [1]. The production of superoxide radicals appears to be due to a specific activation of a plasma membrane-bound NADPH oxidase which is inactive in the resting state [1–4]. The enzyme catalyses the reaction:

$$NADPH + 2\,O_2 \rightarrow 2\,O_2^- + NADP^+ + H^+ \text{ [3,4]}$$

Superoxide production by stimulated neutrophils and by preparations of NADPH oxidase has consistently been measured by an indirect method utilising the reduction of cytochrome *c* by superoxide radicals [4]. This reduction is inhibited by superoxide dismutase. EPR spectroscopy using the technique of spin trapping has been used to identify superoxide radicals formed by photo-irradiated spinach chloroplasts [5] and human neutrophils following stimulation [6]. This technique appears to be the least unambiguous method of detecting superoxide production by biological systems in their normal physiological state. Here, we provide the first direct demonstration of superoxide production by guinea-pig neutrophil NADPH oxidase. The results indicate that as seen by spin trapping, the superoxide radical is the only free radical produced by the enzyme in the presence of NADPH.

2. MATERIALS AND METHODS

NADPH (type III) and NADH (type III) were obtained from Sigma Chemicals (Dorset). The spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was prepared and purified as in [7]. Human copper/zinc superoxide dismutase was prepared from outdated blood [8]. Trifluoperazine was a gift from Smith, Kline and French (Herts). All other reagents were the highest grade available.

Neutrophils were obtained from guinea pig injected intraperitoneally with 1% sodium caseinate. Harvested cells were washed and suspended in Krebs–Ringer phosphate buffer (KRP, pH 7.4) containing 5 mM glucose and 0.5 mM $CaCl_2$. Stimulation was carried out by incubating 2×10^7 cells/ml in the presence of 0.2 μg/ml phorbol myristate acetate (Sigma) for 90 s at 37°C. Cells were homogenised with a Potter-type homogeniser at 4°C and the homogenate was centrifuged at $250 \times g$ for 8 min to sediment nuclei and unbroken cells. The supernatant obtained was centrifuged at 100 000 × g for 30 min. NADPH oxidase was solubilised from the particulate fraction with sodium deoxycholate and further purification was achieved by column chromatography as in [9]. The activity of purified NADPH oxidase, from various preparations, measured by the cytochrome assay ranged from 61–172 nmol superoxide · min^{-1} · mg $protein^{-1}$.

Electron paramagnetic resonance (EPR) spectra were recorded on a Varian E104 X-band EPR spectrometer with an E900-3 data acquisition system. EPR spectra were recorded with a field set

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3385 G, modulation frequency 100 kHz, modulation amplitude 1.0 G, microwave power 10 mW, microwave frequency 9., 478 kHz. Computer averaging of 4 scans from t = 0.5–4.5 min gave spectra whose intensities represent an estimate of the superoxide spin adduct. A quantitative measure of spin adduct production was obtained by a field set for the top of a peak in the superoxide spin adduct EPR spectrum and following the signal intensity with time. Spin adduct concentrations were calculated from a calibrated second integral.

Oxygen consumption during superoxide production by the NADPH oxidase was monitored using a Clark-type oxygen electrode.

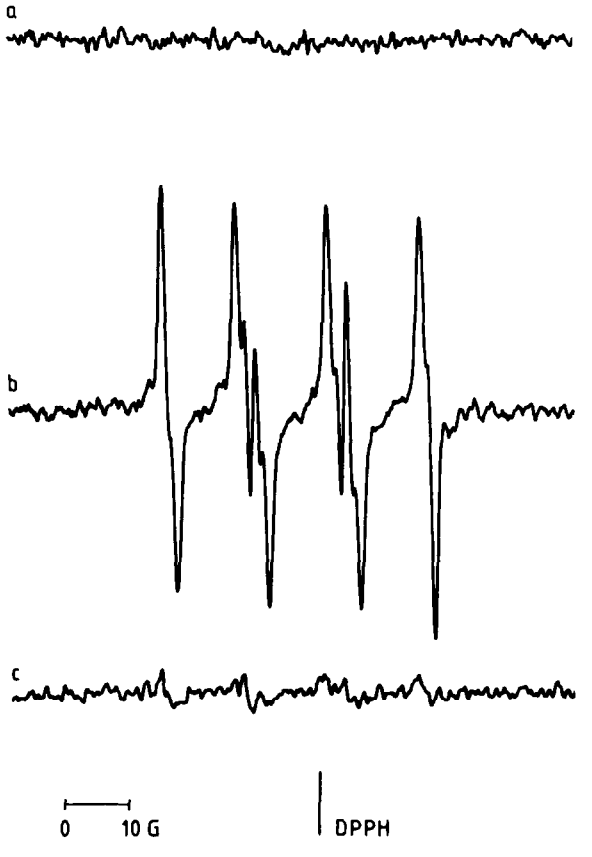

Fig.1. Spin trapping of superoxide produced by NADPH oxidase: (a) 100 mM DMPO + NADPH oxidase, 70 μg/ml; (b) DMPO + NADPH oxidase + NADPH, 100 μM; (c) DMPO + NADPH oxidase prepared from unstimulated neutrophils, i.e., resting enzyme.

3. RESULTS AND DISCUSSION

The reaction of NADPH oxidase isolated from guinea pig neutrophils with NADPH in the presence of DMPO results in the production of a spin adduct (fig.1b). No spin adduct formation is observed in the absence of NADPH indicating that the enzyme does not interact with DMPO (fig.1a). A very small quantity of spin adduct is formed when the reaction is carried out using a NADPH oxidase production from unstimulated cells (fig.1c). The enzyme isolated from resting cells was found to have a NADPH oxidase activity of 2 nmol superoxide · min^{-1} · mg $protein^{-1}$ measured by the reduction of cytochrome c. Computer simulated spectra of the spin adduct formed (fig.1b) gave the following parameters: g = 2.0061, a_N = 14.3 G and a_H^{β} = 11.7 G, a_H^{γ} = 1.25 G. These parameters are identical with those previously reported for the superoxide spin adduct of DMPO; 5,5-dimethyl-2-hydroxyperoxypyrrolidino-1-oxyl (DMPO-OOH) [10]. The signal formed

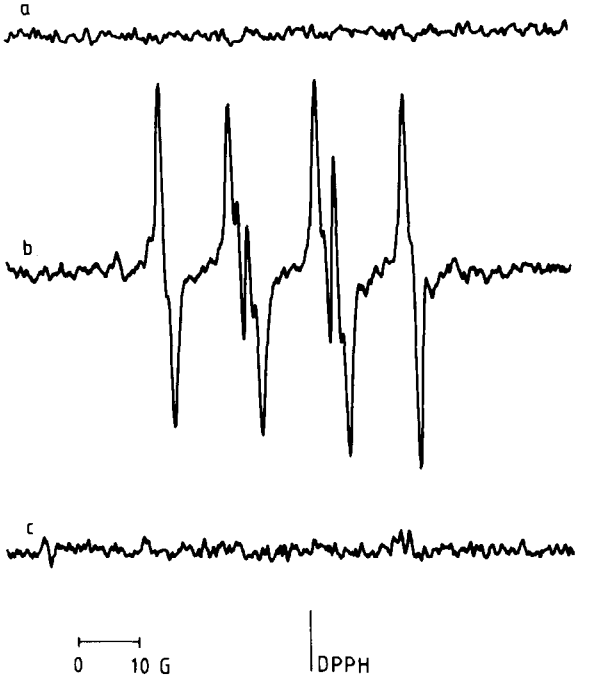

Fig.2. Spin trapping of superoxide produced by NADPH oxidase in the presence of: (a) copper/zinc superoxide dismutase 100 μg/ml; (b) catalase 500 U/ml; (c) trifluoperazine 40 μM.

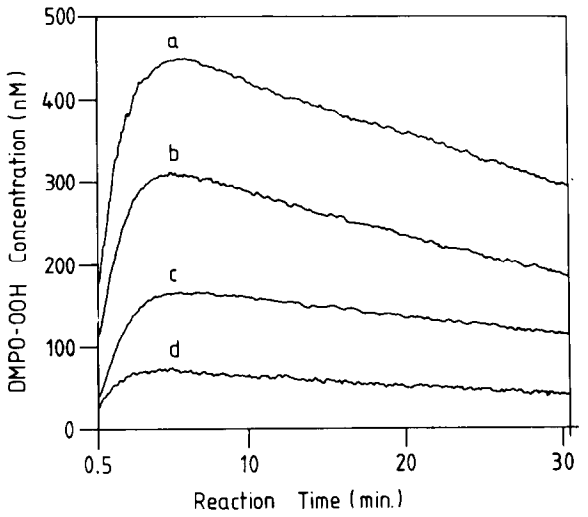

Fig.3. Production of superoxide spin adduct by NADPH oxidase in the presence of various [NADPH]: (a) 50 μM; (b) 22.4 μM; (c) 5.6 μM; (d) 1 μM.

was confirmed to be mainly DMPO-OOH because it disappeared in the presence of copper/zinc superoxide dismutase (fig.2a) and further confirmation was obtained because catalase had no effect on the signal (fig.2b).

The neutrophil NADPH oxidase appears to be a complex composed of a flavoprotein [11] and cytochrome *b* [12]. A role for calmodulin in the control of neutrophil NADPH oxidase has been suggested [13], isolated guinea-pig neutrophil NADPH oxidase is a high M_r complex composed of a flavoprotein, cytochrome *b* and possibly calmodulin [9]. The production of the superoxide spin adduct (DMPO-OOH) in the presence of trifluoperazine, a well-known inhibitor of calmodulin, is inhibited (fig.2c) could confirm a positive involvement by calmodulin.

Table 1

Effect of various concentrations of NADPH oxidase on the production of the superoxide spin adduct in the presence of 100 μM NADPH

NADPH oxidase (mg/ml)	DMPO-OOH (nM)
73.7	312
36.85	169
7.37	32

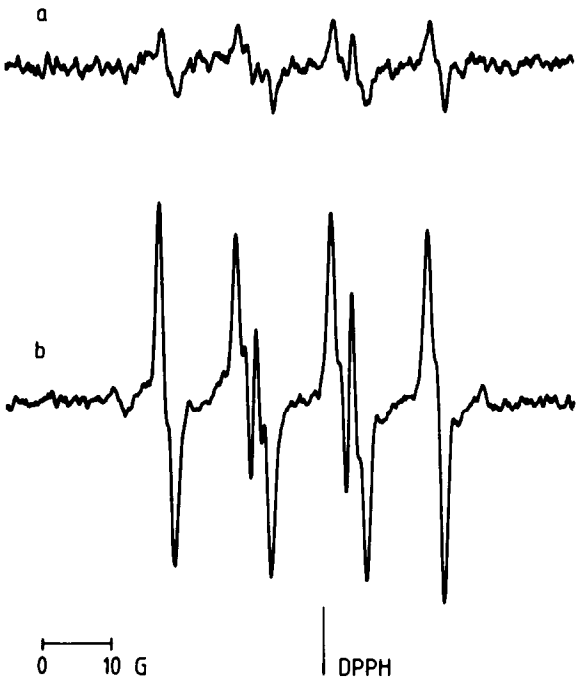

Fig.4. Spin trapping of superoxide produced by NADPH oxidase using NADH as substrate: (a) 100 μM NADH; (b) 1 mM NADH.

The dependence of superoxide production (DMPO-OOH) by NADPH oxidase on [NADPH] is shown in fig.3. An increase in superoxide production with increase in [NADPH] is clearly evident. At a fixed 22.4 μM NADPH, the formation of DMPO-OOH was also found to increase with increase of [NADPH oxidase] (Table 1).

The production of superoxide radicals in the presence of 100 μM NADPH is much greater than in the presence of a similar [NADH] (fig.1b,4a).

A clear DMPO-OOH signal could only be observed in the presence of 1 mM NADH. These results confirm that the neutrophil oxidase is specific for NADPH. The K_m-values of the reaction between the oxidase and NADPH and NADH have been reported to be 15.8 μM and 434 μM, respectively [9].

The results obtained clearly demonstrate that the main function of the neutrophil NADPH oxidase is to produce superoxide radicals as the terminal products of the reduction of oxygen and that the superoxide radicals formed are released to the surrounding medium. DMPO was found to double

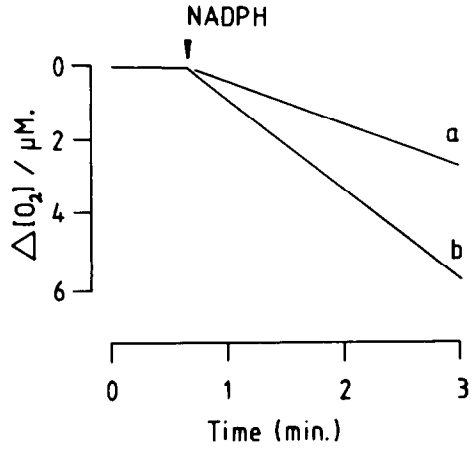

Fig.5. Oxygen uptake by NADPH oxidase (0.26 mg/ml) in the (a) absence and (b) presence of 100 mM DMPO; [NADPH] was 100 μM.

the rate of oxygen consumption indicating that no direct production of hydrogen peroxide takes place (fig.5). The spin trap was found not to consume oxygen and also the decomposition of the spin adduct formed does not consume oxygen. In the absence of DMPO, the superoxide produced dismutes to hydrogen peroxide and oxygen, however, in the presence of DMPO all the superoxide produced is trapped. This result indicates that only a one-electron reduction of oxygen takes place during the enzymatic reaction and that any hydrogen peroxide produced must therefore result from the spontaneous dismutation of superoxide radicals.

The direct formation of hydroxyl radicals by stimulated neutrophils must therefore be due to a secondary reaction taking place following superoxide production. A Fenton-type reduction of hydrogen peroxide catalysed by lactoferrin has been postulated [14].

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