Studies on the electron-transfer mechanism of the human neutrophil NADPH oxidase

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A superoxide-generating NADPH oxidase was solubilized from phorbol 12-myristate 13-acetate-activated human neutrophils with a mixture of sodium deoxycholate (0.125%, w/v) and Lubrol-PX (0.125%, v/v). The solubilized preparation contained FAD (577 pmol/mg of protein) and cytochrome *b*₂₄₅ (479 pmol/mg of protein) and produced 11.61 mol of O₂^{•−}/s per mol of cytochrome *b* (340 nmol of O₂^{•−}/min per mg of protein). On addition of NADPH, the cytochrome *b*₂₄₅ was reduced by 7.9% and the FAD by 38% in the aerobic steady state; NADH addition caused little steady-state reduction of cytochrome *b* and FAD. In this preparation, and several others, the measured rate of O₂^{•−} production correlated with the turnover of cytochrome *b* calculated from the extent of cytochrome *b*₂₄₅ reduction under aerobic conditions. Addition of diphenyleneiodonium abolished the reduction of both the FAD and cytochrome *b*₂₄₅ components and inhibited O₂^{•−} production. The haem ligand imidazole inhibited O₂^{•−} generation and cytochrome *b* reduction while permitting FAD reduction. These results support the suggestion that the human neutrophil NADPH oxidase has the electron-transport sequence: NADPH → FAD → cytochrome *b*₂₄₅ → O₂.

INTRODUCTION

Neutrophils have an important role in the mammalian host defence system against microbial infection. During phagocytosis of bacteria, a neutrophil plasma-membrane-bound enzyme complex (the NADPH oxidase) is stimulated, leading to the production of superoxide radicals (O₂^{•−}). The oxygen radicals produced and their subsequent reaction products are thought to participate in the killing of invading micro-organisms and parasites and to be involved in tumour-cell killing (reviewed by Bellavite, 1988). In addition to the beneficial effects of these oxygen radicals, it is now thought that inappropriate stimulation of oxidase activity contributes to the tissue damage exhibited in certain inflammatory complaints such as rheumatoid arthritis (Dieppe & Doherty, 1982), reperfusion injury (Bulkley, 1983) and acute respiratory distress syndrome (Cochrane *et al.*, 1983).

The solubilized NADPH oxidase from pig neutrophils (Cross *et al.*, 1984) contains a flavoprotein, with non-covalently bound FAD as its prosthetic group (Cross *et al.*, 1982b), and a cytochrome *b* with a low oxidation-reduction potential ($E_{m,7} = -245$ mV; Cross *et al.*, 1981). NADPH reduces both components under anaerobic conditions, but the rate of cytochrome *b* reduction is slower than that required by the known rate of O₂^{•−} production (Cross *et al.*, 1984). This inconsistency disappears under steady-state aerobic conditions (Cross *et al.*, 1985), implying that oxygen is a requirement for efficient electron flow within the oxidase complex.

Previous studies in this laboratory have shown that diphenyleneiodonium (DPI) inhibits the NADPH oxidase of pig neutrophils (Cross & Jones, 1986; Cross, 1987), human neutrophils (Ellis *et al.*, 1988) and rat peritoneal macrophages (Hancock & Jones, 1987). DPI inhibits the reduction of both the cytochrome and

flavoprotein components, which strongly suggests that the site of action of DPI is at the level of (or before) the flavoprotein. The haem ligand imidazole has been shown to bind to cytochrome *b* and to inhibit O₂^{•−} production in pig and human neutrophils (Iizuka *et al.*, 1985).

On account of its involvement in inflammatory disorders, it is important to define the composition and mechanism of the human NADPH oxidase, but studies on human neutrophil oxidase have been hampered by the extreme instability of the enzyme activity after detergent extraction. In the present study we describe the solubilization of an active NADPH oxidase complex from human neutrophils and show that its mechanism appears similar to that of the pig neutrophil oxidase.

MATERIALS AND METHODS

Isolation of human neutrophils

Neutrophils were isolated from buffy coats (kindly supplied by South West Regional Blood Transfusion Centre, Southmead Hospital, Bristol, U.K.; used 16 h after collection) by dextran sedimentation and centrifugation through Ficoll (Histopaque-1077; Sigma) and haemolysis of residual erythrocytes as described previously (Ellis *et al.*, 1988). Cells were finally suspended in phosphate-buffered saline (PBS), pH 7.4, supplemented with 5 mM-glucose, at approx. 5×10^7 cells/ml.

Determination of superoxide production

NADPH-dependent superoxide production was determined by the superoxide dismutase-sensitive rate of reduction of cytochrome *c* as described by Cross *et al.* (1982a). Samples of neutrophil post-nuclear supernatant plasma membranes or solubilized oxidase were added to

Abbreviations used: DPI, diphenyleneiodonium; PMA, phorbol 12-myristate 13-acetate; MPO, myeloperoxidase; PBS, phosphate-buffered saline (Ellis *et al.*, 1988).

a cuvette containing 1 ml of 100 μM horse heart cytochrome *c* (Sigma, type III) and 2 mM-NADPH, in Krebs-Ringer buffer, pH 7.4, at 25 °C.

Spectroscopy

Absorbance spectra in 0.4 ml samples were recorded in a rapid-scanning spectrophotometer with facilities for averaging spectra, as described by Cross *et al.* (1984). Spectra of the aerobic steady-state level of reduction of the redox components were recorded in the presence of 0.5 mM-NADPH at 25 °C. Aerobiosis was maintained by stirring the cuvette contents while the stepping motor was driving down to the starting wavelength (400 nm) before each scan (Cross *et al.*, 1985). Spectra were scanned four times for averaging; the scan speed was approx. 20 nm/s (total scan time 80 s).

Determination of cytochrome *b*₋₂₄₅ and flavin contents

The cytochrome *b*₋₂₄₅ content was assayed by dithionite-reduced-minus-air-oxidized difference spectroscopy, by using $A_{559-540} = 21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Cross *et al.*, 1982a). An estimation of the flavin content was made from reduced-minus-oxidized difference spectra, by using $A_{450-500} = 11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Dawson *et al.*, 1969). A more accurate value for flavin content was obtained from separate experiments where total flavins were extracted by heating samples for 3 min in a water bath at 100 °C, before assay of fluorescence (Faeder & Seigel, 1973). Agreement between the two methods was good.

Activation of neutrophils and preparation of soluble oxidase

The method for isolating neutrophil plasma membranes was a modification of that of Gabig & Lefker (1986). Neutrophil suspension (15 ml) was prewarmed to 37 °C in a water bath. An equal volume of PBS, pH 7.4, containing 2 mM- NaN_3 and 2 μg of PMA/ml was also prewarmed to 37 °C. The two solutions were mixed, and incubation was continued for 7 min at 37 °C, after which the temperature was rapidly lowered to < 4 °C by transferring the suspension to a ice/water bath. All subsequent operations were carried out at 4 °C or on ice. The neutrophils were collected by centrifugation at 670 *g* for 10 min and resuspended in 8.5 ml of unbuffered 0.25 M-sucrose (AnalaR) containing 4 mM- MgCl_2 , 6H₂O, 1 mM- NaN_3 and 1 mM- CaCl_2 , 2H₂O. The cell suspension was disrupted by sonication for three 15 s bursts with 1 min cooling between each burst. The disrupted cell suspension was centrifuged at 600 *g* for 7 min, and the post-nuclear supernatant was collected and adjusted to 40 % (w/v) sucrose by the addition of approx. 3.32 g of ground sucrose (AnalaR). The post-nuclear supernatant was stirred on ice until all the sucrose had dissolved, and was then centrifuged at 67000 *g* for 30 min at 2 °C. The supernatant containing the plasma membranes was diluted 5-fold with ice-cold double-distilled water and re-centrifuged at 117000 *g* for 1.5 h at 2 °C. The membrane pellet was resuspended in unbuffered 0.25 M-sucrose at a protein concentration of 2–3 mg/ml, and mixed with the extraction buffer and glycerol in the proportions 2 vol. of membranes suspension:1 vol. of extraction buffer:1 vol. of glycerol. The extraction buffer (adapted from Glass *et al.* 1986) consisted of 20 mM-sodium glycinate, pH 8, containing 1.7 μM - CaCl_2 , 2H₂O, 1 mM- MgSO_4 , 7H₂O, 0.5 % (w/v) sodium deoxycholate (Sigma) and

0.5 % (v/v) Lubrol-PX (Sigma). The final detergent concentration was 0.125 % of each, and the final glycerol concentration 25 % (v/v). The membrane-detergent suspension was sonicated for two 15 s bursts, with 1 min cooling between the bursts, stirred on ice for 30 min and then centrifuged at 119000 *g* for 1 h at 2 °C. The clear supernatant contained NADPH-dependent $\text{O}_2^{\cdot-}$ generating activity.

Inhibitors

DPI was synthesized by the method of Collette *et al.* (1956), and dissolved in 50 % (v/v) ethanol and standardized by using an A_{284} value of $15.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Ragan & Bloxham, 1977). Imidazole (BDH, Poole, Dorset, U.K.) was dissolved in 50 mM-Hepes, pH 7.4.

Protein assay

The method of Bradford (1976) was used.

Myeloperoxidase (MPO) assay

This was done by the method of Baggiolini (1974).

RESULTS AND DISCUSSION

Oxidase activity was routinely solubilized from human neutrophil plasma membranes by the method described above, although there was a variation in the $\text{O}_2^{\cdot-}$ -generating activity between preparations. The detergent-solubilized fractions were optically clear and possessed little or no MPO. Recovery of oxidase activity from the post-nuclear supernatant was approx. 30–35 %.

Some properties of a typical solubilized preparation are shown in Table 1. There was no requirement for addition of FAD for maximal $\text{O}_2^{\cdot-}$ -generating activity. No $\text{O}_2^{\cdot-}$ -generating activity was solubilized from unstimulated neutrophils. Oxidase activity in the starting

Table 1. Properties of one preparation of the detergent-solubilized active human neutrophil superoxide-generating NADPH oxidase

Human neutrophils were isolated from buffy coats, stimulated with PMA, and an active NADPH oxidase was detergent-solubilized from neutrophil plasma membranes, as described in the Materials and methods section. The most active preparation had a specific activity of 400 nmol of $\text{O}_2^{\cdot-}$ /min per mg of protein, but extracts with very low activity (below 20 nmol/min per mg) were obtained in about 5 % of experiments, and an average activity was about 200 nmol/min per mg.

Property	Measured value
NADPH-dependent $\text{O}_2^{\cdot-}$ production	340 nmol of $\text{O}_2^{\cdot-}$ /min per mg of protein
Protein content	1.3 mg/ml
K_m (NADPH) for $\text{O}_2^{\cdot-}$ production	57 μM
K_m (NADH) for $\text{O}_2^{\cdot-}$	495 μM
Cytochrome <i>b</i> ₋₂₄₅ content	479 pmol/mg of protein
Flavin content	577 pmol/mg of protein
Stability in solubilization buffer:	
half-life at 4 °C	12 h
half-life at 25 °C	20 min
Stability in Krebs-Ringer assay buffer:	
half-life at 25 °C	1.2 min

Fig. 1. Effect of DPI on the aerobic steady-state absorption spectra of a detergent-solubilized preparation of the NADPH oxidase prepared from stimulated human neutrophils

The oxidized spectrum of the undiluted solubilized oxidase was recorded, then NADPH was added (final concn. 0.5 mM) and the spectrum recorded within 80 s. The activity of undiluted enzyme was found to be stable over this time. The difference absorption spectrum was calculated, and is shown in (a). DPI (final concn. 10 μ M) was then added to the sample and the spectrum recorded (b). The absorbance scale of (a) and (b) is expanded 5-fold. Sodium dithionite (a few crystals) was finally added to give complete reduction of the flavin and cytochrome b (c). The flavin and cytochrome b contents were calculated to be 577 and 479 pmol/mg of protein respectively.

stimulated whole neutrophils was approx. 100 nmol of $O_2^{\cdot-}$ /min per mg of protein; 10 μ M-DPI caused 98% inhibition and 40 mM-imidazole caused 65% inhibition of $O_2^{\cdot-}$ production from detergent-solubilized oxidase, results very similar to those obtained with the pig enzyme (Cross & Jones, 1986).

The dithionite-reduced minus air-oxidized difference absorption spectrum (Fig. 1c) of the detergent-solubilized preparation of the human neutrophil oxidase had absorbance maxima at 428, 530 and 558 nm, which are characteristic of cytochrome b_{-245} (Cross *et al.*, 1981). A trough centred around 450 nm corresponds to the reduction of FAD (Cross *et al.*, 1985). The aerobic steady-state reduction of the oxidase by 0.5 mM-NADPH (Fig. 1a) reduced the cytochrome b_{-245} by 7.9% and the flavin by 38% (see Table 2). The low MPO content prevented spectral interference around 450 nm, allowing observation of the flavin trough. The low level of MPO present in this preparation was unlikely to contribute to the trough at 450 nm, since NADPH addition to human MPO did not cause such a spectroscopic change (results not shown).

Aerobic steady-state reduction of cytochrome b_{-245} was abolished when 10 μ M-DPI was added to the solubilized enzyme (Fig. 1b), and there was an increase in absorbance around 450 nm, presumed to be due to the oxidation of reduced flavin. Hence it seems probable that the site of action of DPI is the same as that described for the solubilized pig neutrophil oxidase (Cross & Jones, 1986),

Table 2. Rates of superoxide production and steady-state cytochrome b_{-245} reduction of different preparations of solubilized NADPH oxidase

The neutrophil NADPH oxidase was solubilized from PMA-stimulated neutrophils, and superoxide generation was measured as described in the Materials and methods section. The NADPH-dependent aerobic steady-state reduction was measured as described in the Materials and methods section. Oxidase preparation 4 was solubilized from unstimulated neutrophils. Abbreviation used: N.D., not detected.

Oxidase preparation	Specific activity (nmol of $O_2^{\cdot-}$ /min per mg of protein)	Cytochrome b_{-245} reduction by NADPH (%)	FAD reduction by NADPH (%)
1	340	7.9	38
2	172	4.2	20
3	134	3.0	15
4	2	N.D.	N.D.

where it was proposed to act on, or before, the flavoprotein in the electron-transport sequence shown in Scheme 1.

The cytochrome b_{-245} possesses an absorption spectrum typical of a hexa-co-ordinate low-spin haem, and the nitrogenous base imidazole is known to bind to haem iron and substitute internal ligand in hexa-co-ordinated cytochrome c (Sutin & Yandell, 1972). Imidazole inhibited the aerobic steady-state reduction of cytochrome b_{-245} by NADPH (Fig. 2b), with little effect on flavin reduction. This would be expected if cytochrome b_{-245} is the site of inhibition by imidazole and acting as a component of the human NADPH oxidase between flavin and oxygen. Since imidazole and DPI each inhibits $O_2^{\cdot-}$ -production, it suggests that both cytochrome b and flavin together are needed for oxidase activity.

Effect of varying the concentration and nature of the electron donor

Cytochrome b_{-245} was partly reduced after addition of 50 μ M-NADPH (approx. 38% of reduction given by 500 μ M-NADPH; results not shown). However, 100 μ M-NADH caused no measurable reduction of cytochrome b_{-245} (Fig. 3b). The solubilized human NADPH oxidase has a K_m for NADPH approx. one-tenth of that for NADH (Table 1), which is in agreement with the values reported for the solubilized pig neutrophil NADPH

Scheme 1. Proposed electron-transport sequence of the neutrophil NADPH oxidase, showing the possible sites of action of the inhibitors DPI and imidazole

Fig. 2. Effect of imidazole on the aerobic steady-state absorbance spectrum of a detergent-solubilized preparation of the NADPH oxidase prepared from stimulated human neutrophils

The methods were as described in Fig. 1. The NADPH difference absorption spectrum is shown in (a). Imidazole (final concn. 40 mM) was then added to the sample and the spectrum recorded (b). The absorbance scale of spectra (a) and (b) is expanded 5-fold. Spectrum (c) shows the complete reduction of the cytochrome *b* and flavin by sodium dithionite. The flavin and cytochrome *b* contents were calculated to be 577 and 479 pmol/mg of protein respectively.

Fig. 3. Aerobic steady-state absorption spectra of the detergent-solubilized oxidase, with NADPH and NADH as electron donors

The methods were as described in Fig. 1. The difference absorption spectra shown are for (a) 100 μM -NADPH or (b) 100 μM -NADH used as the electron donor. The absorbance scale of spectra (a) and (b) is expanded 5-fold. Spectrum (c) shows the complete reduction of the cytochrome *b* and flavin by sodium dithionite. The flavin and cytochrome *b* contents were calculated to be 411 and 345 pmol/mg of protein respectively.

oxidase (Cross *et al.*, 1985). The cellular NADPH concentration of neutrophils was found to be $70 \pm 5.6 \mu\text{M}$ (Ellis, 1988) and the cellular NADH level approx. 200 μM (Heyneman & Bauwens-Monbaliu, 1981), which is consistent with NADPH being the preferred substrate for the NADPH oxidase. In addition, the steady-state levels of reduction of the cytochrome after addition of NAD(P)H are compatible with its suggested role in Scheme 1.

Measurement of the extent of steady-state reduction of cytochrome b_{-245} and FAD by NADPH in several solubilized oxidase preparations under aerobic conditions permits calculation of rates of turnover of cytochrome *b*, by using the pseudo-first-order rate constant for human cytochrome *b* oxidation of 147 s^{-1} (Cross *et al.*, 1982a). The calculated rates are almost identical with the measured rate of $\text{O}_2^{\cdot-}$ production (Fig. 4), in agreement with Scheme 1. This correlation held for oxidase preparations of widely varying activity. In addition, it can be deduced that every electron transferred from NADPH to O_2 passes through the cytochrome b_{-245} , and so the electron-transport chain must be unbranched.

In the aerobic steady state the flavin component is more reduced than the cytochrome b_{-245} (Table 2), in agreement with the proposed electron-transport sequence in Scheme 1, where FAD is placed between NADPH and cytochrome *b*.

The human neutrophil NADPH oxidase has been detergent-solubilized in an active form and shown to contain a flavoprotein and cytochrome b_{-245} as redox components. The arrangement of the components is best

Fig. 4. Relationship between the measured rate of $\text{O}_2^{\cdot-}$ production and turnover rate of cytochrome *b* calculated from the steady-state reduction of cytochrome *b*

The measured rate of $\text{O}_2^{\cdot-}$ production from four different preparations of the detergent-solubilized NADPH oxidase was compared with the calculated turnover rate of cytochrome *b*. This was obtained by using the relationship $v = k \times \% \text{ reduction of cytochrome } b \text{ in the steady state}$, where v is the turnover of cytochrome *b* (mol/s) and k is the pseudo-first-order rate constant for the oxidation of human cytochrome b_{-245} (147 s^{-1} ; Cross *et al.*, 1982a). The numbering of the preparations [(1)–(4)] refers to the oxidase preparations shown in Table 2.

explained as shown in Scheme 1. This evidence strongly suggests that the human neutrophil oxidase is very similar (if not identical) in composition and reactivity to the pig neutrophil NADPH oxidase. It is apparent, however, that our results are incompatible with those who have found that preparations of apparently purified NADPH oxidase contain FAD but not cytochrome *b* (Glass *et al.*, 1986), or cytochrome *b* but little FAD (Bellavite *et al.*, 1985). Studies on the reaction mechanisms of such oxidase preparations would obviously be of great interest.

This work has been supported by grants from the Medical Research Council; A.R.C. is supported by the Arthritis and Rheumatism Council, and J.A.E. by a S.E.R.C. Studentship.

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Received 6 February 1989/3 April 1989; accepted 10 April 1989