The generation of hydroxyl radicals following superoxide production by neutrophil NADPH oxidase

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Stimulated neutrophils generate superoxide and hydroxyl radicals. A membrane-bound NADPH oxidase, inactive in the resting state, is responsible for superoxide production. The production of hydroxyl radicals is through a secondary reaction. A Fenton-catalysed Haber–Weiss reaction is proposed. Transferrin was used as the catalyst in this investigation.

Superoxide *Hydroxyl radical* *Neutrophils* *NADPH oxidase* *Transferrin* *EPR*

1. INTRODUCTION

Stimulation of neutrophils with membrane perturbing agents causes an increase in oxygen consumption concomitant with the production of superoxide and hydroxyl radicals and hydrogen peroxide (H_2O_2). A plasma membrane-bound NADPH oxidase which is inactive in resting neutrophils has been implicated in the production of these radicals [1,2]. Purified NADPH oxidase has recently been shown to produce only superoxide radicals [3].

Phagocytosing neutrophils are known to produce hypochlorite through the action of myeloperoxidase/H_2O_2/chloride. In [4] myeloperoxidase was implicated in the formation of hydroxyl radicals by stimulated neutrophils. Superoxide radicals have been shown to react with hypochlorite [5]. The products of this reaction have not been identified, however, it has been suggested [6] that hydroxyl radicals may be produced as a result of the reaction between superoxide radicals and hypochlorite.

Another reaction leading to the formation of hydroxyl radicals is the Haber-Weiss reaction [7]. Although this reaction is considered to be too slow to have any biological significance [8] a Fenton-type catalysed Haber-Weiss reaction has been proposed with the non-haem iron-containing proteins, transferrin [8] and lactoferrin [9,10] as the catalysts. Here, we provide the first demonstration, using electron paramagnetic resonance (EPR) spin trapping techniques, that hydroxyl radicals are generated in the presence of transferrin following superoxide production by purified guinea-pig neutrophil NADPH oxidase.

2. MATERIALS AND METHODS

NADPH (type III) was obtained from Sigma Chemical Co. (Poole, Dorset). The metal chelating agent diethylenetriamine pentaacetic acid was obtained from Aldrich Chemical Co. (Gillingham). The spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was prepared and purified as in [11]. All other reagents were of the highest grade available.

NADPH oxidase was isolated and purified from neutrophils as in [12]. Neutrophils were obtained from guinea-pigs injected intraperitoneally with 1% sodium caseinate. Cells were stimulated with 0.2 μg/ml phorbol myristate acetate (Sigma Chemical Co.) prior to enzyme isolation. The activity of purified NADPH oxidase from various preparations measured by the cytochrome *c* assay ranged from 61–172 nmol superoxide $\cdot$ min^{-1} $\cdot$ mg protein^{-1}. Purified enzymes were stored at 77 K in

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the presence of 20% glycerol. Human serum apotransferrin reconstituted with ferric chloride was a kind gift from Dr J.K. Heath (Dept. of Zoology, University of Oxford). Reconstituted protein conc. was measured using an extinction $E_{280}^{1\%} = 14.1$ [13]. Myeloperoxidase was prepared as in [14].

EPR was recorded on a VARIAN E104 X-band EPR spectrometer with an E900-3 data acquisition system. EPR spectra were recorded with a field set 3385G, modulation frequency 100 kHz, modulation amplitude 1.0 G, microwave power 10 mW and 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 spin adduct concentrations. Spin adduct concentrations were calculated from a calibrated double integral. Spin trapping experiments were performed on the superoxide generating NADPH oxidase in the presence of transferrin and myeloperoxidase. H_2O_2 was also added, since in the presence of 100 mM DMPO very little superoxide (< 1%) dismutes to H_2O_2 and oxygen [3].

3. RESULTS AND DISCUSSION

Purified neutrophil NADPH oxidase has been demonstrated [3] to form a superoxide spin adduct (DMPO-OOH) in the presence of NADPH and DMPO (fig. 1a). The production of DMPO-OOH was found to be inhibited by copper/zinc superoxide dismutase. When the reaction is carried out in the presence of 100 μM transferrin no other spin adduct is observed (fig. 1b). The superoxide spin adduct is, however, reduced by about 40%, indicating that transferrin is competing with the DMPO for superoxide radicals. Iron(III) transferrin reduced to iron(II) transferrin by superoxide radicals is unable to react further because of the absence of H_2O_2. When the situation is remedied by the addition of H_2O_2 to the transferrin/NADPH/NADPH oxidase reaction, a new component, in addition to the superoxide spin adduct DMPO-OOH, is observed in the EPR spectrum (fig. 1c). Computer-simulated spectra of the spin adduct formed, gave the following parameters: $g = 2.0050$, $a_N = a_H = 14.9$ G. These parameters are identical with those previously reported for the hydroxyl spin adduct of DMPO; 5,5-dimethyl-2-hydroxylpyrrolidine-1-oxyl (DMPO-OH). An increase in [DMPO-OH] is observed with an increase in [transferrin] (table 1).

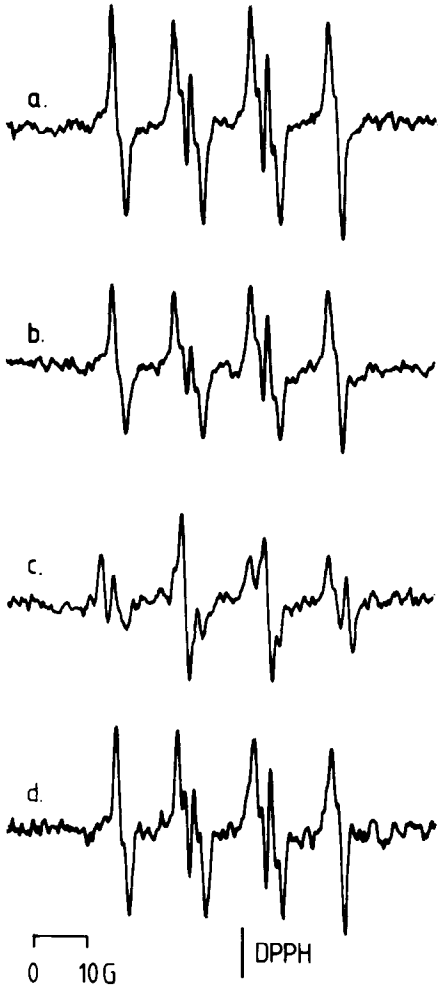

Fig. 1. Spin trapping of oxygen-centred radical, in the presence of 100 mM DMPO, produced by: (a) NADPH oxidase 20 μg/ml; (b) NADPH oxidase/50 μM transferrin; (c) NADPH oxidase/100 μM transferrin/178 μM H_2O_2; (d) NADPH oxidase/80 μM myeloperoxidase/178 μM H_2O_2. All reaction mixtures contained 1 mM NADPH.

No hydroxyl radical spin adduct is observed in the presence of NADPH/NADPH oxidase H_2O_2 and Cl^- when purified myeloperoxidase was added (fig. 1d; table 1). This is in contrast with the results obtained by [6] who found that endogenously added peroxidase increases the rate of hydroxyl radical formation by the stimulated leucocytes. However, hydroxyl radicals were estimated by the measurement of ethylene produc-

Table 1

Production of superoxide and hydroxyl radicals by NADPH oxidase, transferrin and myeloperoxidase

	DMPO-OOH (nM)	DMPO-OH (nM)
NADPH oxidase	105	–
+ 50 μM transferrin	69	–
+ 223 μM H_2O_2	67	16
+ 100 μM transferrin		
+ 223 μM H_2O_2	30	34
+ 40 μM myelo-peroxidase		
+ 223 μM H_2O_2	93	–

NADPH oxidase was 20 μg/ml and all reaction mixtures contained 1 mM NADPH

tion when hydroxyl radicals react with KMB (α-keto-γ-methiol-butyric acid). Ethylene production from KMB is not a reaction specific to hydroxyl radicals, whereas DMPO-OH formation from DMPO is a reaction specific to hydroxyl radicals.

The results obtained indicate that the formation of hydroxyl radicals by stimulated neutrophils is a secondary reaction taking place following superoxide production. A Fenton-type reduction of H_2O_2 catalysed by a non-haem iron protein such as transferrin or lactoferrin appears to play an important role in hydroxyl radical formation.

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REFERENCES

[1] Patriarca, P., Dri, P., Kakinuma, K., Tedesco, F. and Rossi, F. (1975) Arch. Biochem. Biophys. 385, 380–386.

[2] Babior, B.M., Curnette, J.T. and McMunsch, B.J. (1976) J. Clin. Invest. 58, 989–996.

[3] Bannister, J.V., Bellavite, P., Serra, M.C., Thornalley, P.J. and Rossi, F. (1982) FEBS Lett. 145, 323–326.

[4] Takanaka, K. and O'Brien, P.J. (1980) FEBS Lett. 110, 283–286.

[5] Long, C.A. and Bielski, B.H.J. (1980) J. Phys. Chem. 84, 555.

[6] Hill, H.A.O. and Okolow-Zubkowska, M.J. (1981) in: Oxygen and Life, pp. 98–106, spec. pub. Royal Society of Chemistry, London.

[7] Haber, F. and Weiss, J. (1934) Proc. Roy. Soc. Edin. 147, 332–351.

[8] McCord, J.M. and Day, E.D. (1978) FEBS Lett. 86, 139–142.

[9] Ambruso, D.R. and Johnston, R.B. (1981) J. Clin. Invest. 67, 352–360.

[10] Bannister, J.V., Bannister, W.H., Hill, H.A.O. and Thornalley, P.J. (1982) Biochim. Biophys. Acta 715, 116–120.

[11] Bonnet, R., Brown, R.F.C., Sutherland, I.O. and Todd, A. (1959) J. Chem. Soc. 2094.

[12] Bellavite, P., Serra, M.C., Davoli, A., Bannister, J.V. and Rossi, F. (1983) Mol. Cell. Biochem. in press.

[13] Aisen, P., Aasa, R., Malmström, B.G. and Vanngård, T. (1967) J. Biol. Chem. 242, 2484–2490.

[14] Bakkenist, A.R.J., Wever, R., Vulsma, T., Plat, H. and Van Gelder, B.F. (1978) Biochim. Biophys. Acta 524, 45–54.