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The relationship between superoxide generation, cytochrome *b* and oxygen in activated neutrophils

Steven W. Edwards and David Lloyd [+]

Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX, England and [+] *Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, Wales*

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The O_2 affinity for $O_2^{\cdot-}$ generation (i.e. O_2 tension at which 50% of the O_2-saturated activity was observed) by rat neutrophils activated by the chemotactic peptide (*N*-formyl-methionyl-leucyl-phenylalanine) plus cytochalasin B was measured as 5.4 μM ($\pm$1.4, $n=5$). Similarly, the O_2 tension at which cytochrome *b* was oxidized to 50% of its aerobic steady-state level in activated cells was 4.7 μM ($\pm$1.0, $n=3$): in non-activated cells the corresponding value for cytochrome *b* oxidation was 11.4 μM ($\pm$2.8, $n=3$). It is proposed that O_2 depletion at inflammatory sites may limit oxidant generation by activated neutrophils and thus reduce oxidant damage to surrounding tissues.

Oxygen affinity; Cytochrome *b*; Superoxide generation; Neutrophil

1. INTRODUCTION

The production of a series of reactive oxygen metabolites during a respiratory burst of non-mitochondrial O_2 uptake by activated polymorphonuclear leukocytes (neutrophils) is recognized to be a crucial event necessary for efficient microbial killing during infections [1–4]. The initial product of the respiratory burst is $O_2^{\cdot-}$ [5] produced by the one electron reduction of O_2 in a reaction catalysed by an NADPH complex [6], the terminal components of which are yet to be fully defined. A key component of this complex appears to be cytochrome *b*-245 [7] characterized by having (for a *b*-type cytochrome) an unusually low redox potential of -245 mV [8] and some properties to suggest that it may indeed react with O_2 to function as a terminal oxidase [9,10].

In spite of the appreciation of the importance of oxidant generation in microbial killing and other inflammatory conditions [11,12], there have been surprisingly few studies examining the relationship between the pathway(s) responsible for the generation of reactive oxidants and the primary substrate, molecular O_2. Establishing this relationship is essential if we are to predict the function of this pathway in vivo since during an acute inflammatory response neutrophils will be exposed to wide variations in local O_2 tensions.

We have previously shown that the apparent K_m for O_2 during respiration and reactive oxidant generation (as assayed by luminol-dependent chemiluminescence) decreases 2–3-fold after neutrophil stimulation [13,14] and that the O_2 affinity for activated respiratory activity is within the range whereby local O_2 tensions at inflammatory sites may limit oxidant generation. The aim of the present study was to determine the O_2 affinity of $O_2^{\cdot-}$ generation by activated neutrophils and to correlate this with changes in the redox state of cytochrome *b*-245 in order to establish the role of this cytochrome during the respiratory burst.

2. MATERIALS AND METHODS

Rat neutrophils were prepared from peritoneal fluid after peritoneal injection of sodium caseinate, exactly as described in [15]. After purification they were suspended in a buffer containing (mM): 120 NaCl; 4.8 KCl; 1.2 KH_2PO_4; 1.3 $CaCl_2$; 1.2 $MgSO_4$; 25 Hepes (pH 7.4); 0.1% bovine serum albumin, and used within 6 h of preparation.

Correspondence address: S.W. Edwards, Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX, England

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Simultaneous spectrophotometric and O_2 measurements were made in a specially-constructed 'open' reaction chamber (maintained at 37°C) fitted within the light path of a Hitachi (Perkin-Elmer) 557 spectrophotometer operating in the dual-wavelength mode. O_2 tensions in the gas phase were regulated using a digital gas mixer and O_2 tensions in the suspensions were measured using a radiometer E5046 O_2 electrode [16]. For measurements of the rate of $O_2^{\cdot-}$ generation at defined O_2 tensions, suspensions (total volume of 6 ml) contained 2×10^5 cells/ml and 75 μM cytochrome *c*: the measuring wavelength was 550 nm and the reference wavelength was 540 nm. For measurements of the effects of O_2 tension on the steady-state oxidation level of cytochrome *b*, the cell density was 5×10^7 cells/ml, the measuring wavelength 426 nm and the reference wavelength was 403 nm. In all instances (unless stated otherwise) cell activation was achieved by the addition of 1 μM FMLP (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine) plus 1 μg/ml cytochalasin B (final concs).

3. RESULTS AND DISCUSSION

3.1. *Effect of O_2 tension on superoxide generation by activated neutrophils*

Suspensions of neutrophils in buffer containing cytochrome *c* were incubated in a gas phase of air (210 μM): no detectable increase in the rate of reduction of cytochrome *c* was observed, indicating that $O_2^{\cdot-}$ production by non-activated cells was not measurable under these conditions. Suspensions were then activated by the addition of 1 μM FMLP plus 1 μg/ml cytochalasin B (final concs) and the rate of $O_2^{\cdot-}$ generation followed by measuring the rate of cytochrome *c* reduction. The maximal rate of $O_2^{\cdot-}$ generation was found to be 13 $\pm$ 4 (n = 8) nmol/min per 10^6 cells. O_2 tensions in suspensions were then decreased in stages to 60 μM prior to addition of stimulant, but no detectable change in the activated rate of $O_2^{\cdot-}$ generation was observed. Thus, the rate of $O_2^{\cdot-}$ generation by activated neutrophils was independent of O_2 tension in the range 60–210 μM O_2. However, when the O_2 tension of the suspension was maintained at <60 μM O_2, the rate of $O_2^{\cdot-}$ generation after addition of stimulus was markedly O_2-dependent (fig.1) and the O_2 tension at which the rate of $O_2^{\cdot-}$ generation was decreased to 50% was 5.4 μM ($\pm$ 1.4, n = 5). In a similar series of experiments in which 0.1 μg/ml PMA was used to activate $O_2^{\cdot-}$ generation, a 50% maximal response was observed at a similar O_2 tension (not shown). When added to neutrophil suspensions incubated at 210, 50 and 5 μM O_2, superoxide dismutase (10 μg/ml) inhibited the rate of activated cytochrome *c* reduction by >97%, confirming that the cytochrome *c* reduction at all O_2 tensions was attributable to $O_2^{\cdot-}$ generation.

3.2. *Effect of O_2 tension on steady-state level of cytochrome* b *oxidation*

A similar series of experiments were then performed examining the O_2 dependence of the steady-state oxidation level of cytochrome *b*. With the gas phase consisting of air (210 μM O_2), a constant absorption at 426 nm indicated the aerobic steady-state oxidation of cytochrome *b*. When the gas phase was changed to Ar, the O_2 in the suspension was rapidly displaced and anaerobiosis was indicated by measurement with the O_2 electrode. As the suspension became anaerobic, cytochrome *b* became reduced and subsequently re-oxidized when the gas phase was returned to air. The O_2 tension at which cytochrome *b* was oxidized to 50%

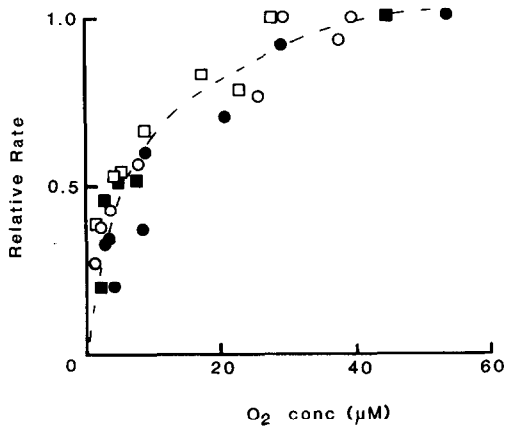

Fig.1. Effect of O_2 tension on $O_2^{\cdot-}$ generation. Neutrophils (2×10^5 /ml, total volume 6 ml) were incubated in buffer containing 75 μM cytochrome *c* at 37°C in a reaction chamber housed within a dual-wavelength spectrophotometer and fitted with an O_2 electrode [16]. The measuring wavelength was 550 nm, the reference wavelength 540 nm and the slit width 2 nm. The O_2 tension of the suspension (measured using an O_2 electrode) was regulated by controlling the gas phase above it by means of a gas mixer supplying mixtures of air and Ar; a stirrer driven by a synchronous motor ensured rapid equilibration of gases with the liquid phase. When a pre-selected O_2 tension was maintained in the suspension, neutrophils were activated by the addition of 1 μM FMLP plus 1 μg/ml cytochalasin B (final concs) and the rate of cytochrome *c* reduction continuously monitored. Confirmation that this reduction represented $O_2^{\cdot-}$ generation was achieved by inhibition by superoxide dismutase. Data are presented from 4 separate experiments, as indicated by different symbols.

of its aerobic steady-state level in non-activated cells was 11.4 μM ($\pm$ 2.8, n = 3) as shown in fig.2. When cells were activated by the addition of FMLP plus cytochalasin B, similar O_2-dependent changes in the redox state of cytochrome b were observed but the changes occurred at lower O_2 tensions, i.e. the O_2 affinity had increased (fig.2). Thus, in activated cells, the O_2 tension at which cytochrome b was oxidized to 50% of its aerobic steady-state level was 4.7 μM ($\pm$ 1.0, n = 3).

Table 1 summarises the relative O_2 affinities of some neutrophil functions utilizing data presented in this or previous reports.

The data presented here show that, in common with O_2 uptake [13] and luminol-dependent chemiluminescence [14], the O_2 affinity (i.e. the O_2 tension resulting in oxidation to 50% of the aerobic steady-state value) of neutrophil cytochrome b oxidation increases 2–3-fold after cell activation by FMLP plus cytochalasin B (table 1). Since we did not detect any cytochrome c reduction by non-activated cells, it was not possible to establish a similar increase in $O_2^{\cdot-}$ affinity for $O_2^{\cdot-}$ generation. Furthermore, the finding that the O_2 affinity of this redox change in activated cells (4.7 μM) is similar to that of both activated O_2 uptake (3.7 μM) and $O_2^{\cdot-}$ generation (5.4 μM), strongly suggests that these processes share a common pathway. These data thus provide further evidence [9,10] that cytochrome b is involved in the pathway of activated O_2 uptake leading to $O_2^{\cdot-}$ generation during the respiratory burst of neutrophils.

These results also provide a further insight into the relationship between the oxidant-generating pathway of neutrophils and its substrate (molecular O_2), and thus extend our understanding of the regulation and activity of this process in pathological conditions. The vast majority of studies of neutrophil function have employed experimental systems operating at O_2 saturation or occasionally complete O_2 depletion (e.g. [17–19]), but few have attempted to design in vitro conditions approximating in vivo conditions with respect to physiological O_2 tensions. The O_2 tension of normal blood varies over the range 50–120 μM [20] and at some inflammatory sites has been measured as low as 12 μM [21]. Also, it is now appreciated that considerable O_2 gradients exist within tissues and we have measured O_2 tensions of synovial fluid from patients with

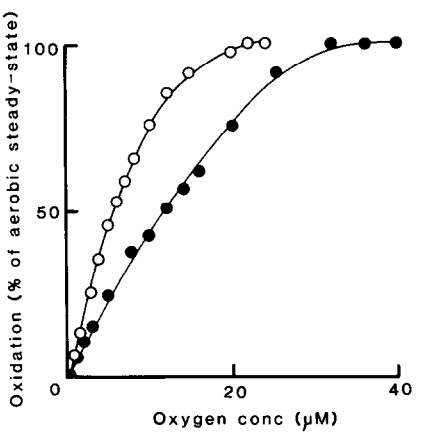

Fig.2. Effects of O_2 tension on cytochrome b oxidation. Neutrophils were incubated under conditions described in the legend to fig.1 except that the cell suspension contained 5×10^7 cells/ml, the measuring wavelength was 426 nm and the reference wavelength was 403 nm. Suspensions were incubated under a gas phase of air (or a 40% air:Ar mixture) and an aerobic steady-state level of oxidation was indicated by a constant absorption value. The gas phase above the suspension was then changed to Ar and the decreasing O_2 tension in the suspension was measured using an O_2 electrode until anaerobiosis was attained. The absorption of cytochrome b correspondingly increased during this period due to increased reduction of this component. The suspension was then re-aerated by changing the gas phase to 40% (or 10%) air and the O_2 dependence of oxidation of cytochrome b measured as the aerobic steady-state level of oxidation was restored. (●) Unstimulated cells; (○) cells stimulated by the addition of 1 μM FMLP plus 1 μg/ml cytochalasin B (final concs) 1 min prior to the aerobic/anaerobic transitions.

Table 1

O_2 affinities of neutrophil functions

	Unstimulated cells	Stimulated cells
Cytochrome b oxidation	11.4 ± 2.8 (n = 3)	4.7 ± 10 (n = 3)
O_2 uptake (reference 13)	9.6 ± 0.67 (n = 3)	3.7 ± 16 (n = 3)
Chemiluminescence	45	22 ± 9 (n = 5)
Superoxide generation	N.D.	5.4 ± 14 (n = 5)

Values presented are O_2 tensions (μM) giving 50% of aerobic activity. N.D., not detectable

rheumatoid arthritis as low as 20 μM [14]. Thus the O_2 affinity of $O_2^{\cdot-}$ generation is within the range whereby local variations of O_2 tensions may limit its production during an inflammatory response. In this way, therefore, O_2 utilization at an inflammatory site to form $O_2^{\cdot-}$ may result in an O_2 depleted environment and hence oxidant generation will be self-limited thus restricting damage to surrounding tissues. This mechanism together with the myeloperoxidase system [22], provides a novel means of regulating oxidant generation during inflammation, but also offers the possibility that in pathological conditions where O_2 supply is not restricted such self-regulation cannot operate; excessive oxidant production may then damage surrounding cells and tissues.

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