Biochem. J. (1984) **217**, 851–854
Printed in Great Britain

Oxygen-radical production during inflammation may be limited by oxygen concentration

Steven W. EDWARDS, Maurice B. HALLETT and Anthony K. CAMPBELL
Department of Medical Biochemistry, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XN, Wales, U.K.

(*Received 10 October 1983/Accepted 8 December 1983*)

1. The relationship between oxygen-radical production by rat polymorphonuclear leucocytes and O_2 concentration was established by the measurement of luminol-dependent chemiluminescence at defined O_2 concentrations. 2. The O_2 concentration that gave 50% of the maximum stimulated oxygen-radical production was $31 \pm 9\,\mu M$ for non-opsonized latex beads and $22 \pm 9\,\mu M$ for chemotactic peptide. 3. The O_2 concentration in rheumatoid synovial fluid was approx. $30\,\mu M$. It is therefore proposed that radical production at an inflammatory site may be limited by O_2 concentration.

Acute inflammation is characterized by large infiltrations of polymorphonuclear leucocytes whose prime function is the killing of micro-organisms (Klebanoff & Clark, 1978). Activation of these cells by chemotactic and phagocytic stimuli results in a 'respiratory burst' and the production of oxygen radicals (Karnovsky & Bolis, 1982), which include O_2^- and $OH^\cdot$, as well as the related species OCl^-, H_2O_2 and $^1O_2$ (singlet oxygen) (Babior, 1978; Halliwell, 1982). These radicals play a key role in the killing mechanism, and inappropriate release may be crucial in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (Campbell *et al.*, 1984). Many workers have studied the mechanisms underlying the activation of the pathway responsible for radical production (Segal & Jones, 1978; Cross *et al.*, 1982; Green *et al.*, 1983; Hallett & Campbell, 1983). However, the relationship between the affinity of the substrate, O_2, in this system, and the concentration of O_2 surrounding the cells *in vivo* has never been established. This relationship is critical for the validity of the oxygen-radical hypothesis. Here we report the correlation between oxygen-radical production monitored by chemiluminescence and O_2 concentration. The high apparent K_m for O_2 suggests that O_2 may be a limiting factor *in vivo* during oxygen-radical-mediated tissue injury.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Experimental

Rat polymorphonuclear leucocytes were isolated from peritoneal fluid after intraperitoneal injection of sodium caseinate (Hallett *et al.*, 1981).

After purification they were suspended in a buffer containing (mM): NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; $CaCl_2$, 1.3; $MgSO_4$, 1.2; Hepes, 25 (pH 7.4); and 0.1% bovine serum albumin. Simultaneous O_2 and luminescence measurements were made at 37°C with a specially constructed 'open' oxygen-electrode system (Degn & Wohlrab, 1971) which was placed in a light-tight box fitted with a photomultiplier tube (Fig. 1). Mixtures of air and N_2 in the gas phase were provided by a digital gas mixture and thus luminescence measurements were made at precisely defined O_2 concentrations. Calibration of the oxygen electrode was with air-saturated water at 37°C, the oxygen concentration of which was taken to be $210\,\mu M$ (Seidell, 1940).

There was a linear relationship between luminol-dependent chemiluminescence and cell concentration when stimulated by both latex beads and chemotactic peptide in both normal ($210\,\mu M$) and low O_2 ($10\,\mu M$) concentrations.

Results and discussion

A number of stimuli have been found to initiate a respiratory 'burst' and oxygen-radical production in polymorphonuclear leucocytes (Klebanoff & Clark, 1978). We chose to examine oxygen-radical formation in response to phagocytic stimulus (latex

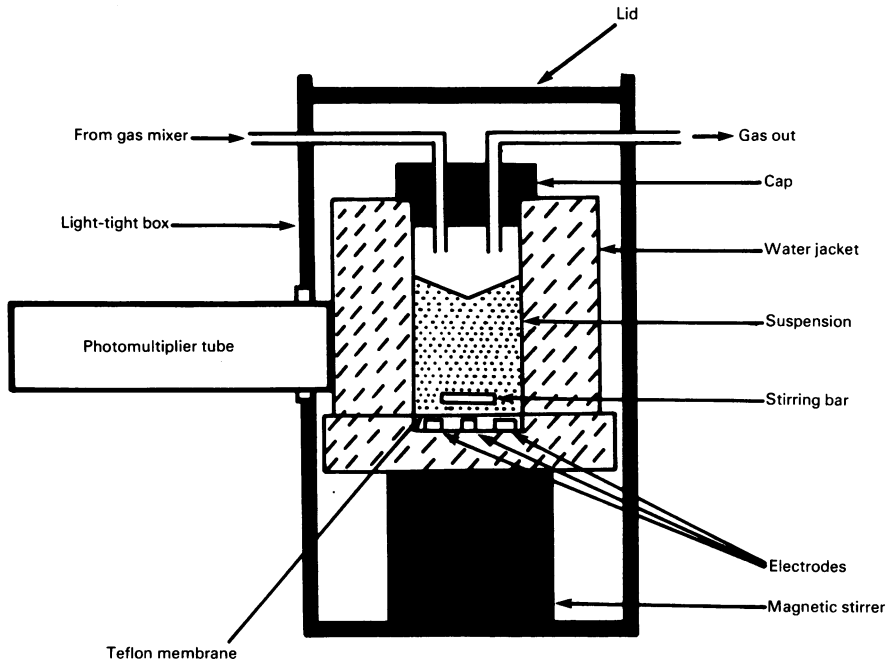

Fig. 1. *Apparatus for simultaneous O_2 and luminescence measurement*
A water-jacketted Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.) was fitted with a cap which allowed inflow and outflow of gases. The stirred working volume was 3.0ml. Concentrations of O_2 in the gas phase were varied by means of a digital gas mixer providing mixtures of air and N_2. Concentrations of O_2 in the liquid phase were thus dependent on O_2 concentration in the gas phase, the diffusion coefficient and the respiration of the cell suspension. The system is based on that described previously in more detail (Degn & Wohlrab, 1971). The electrode was placed in a light-tight box fitted with a photomultiplier tube. All measurements were made at 37°C.

beads) and chemotactic stimulus (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine). It has previously been shown that the addition of cytochalasin B potentiates both the respiratory burst (Edwards *et al.*, 1983) and luminol-dependent luminescence in these cells (Hallett & Campbell, 1983; Dahlgren & Stendahl, 1983). The addition of chemotactic peptide plus cytochalasin B to suspensions of rat polymorphonuclear leucocytes in the presence of luminol caused a rapid increase in luminescence that was maximal 0.4min after the addition. Lowering the O_2 concentration in the suspension by means of decreasing the ratio of air to N_2 in the gas mixture did not affect the magnitude of the luminescence response down to 120μM-O_2 (Fig. 2). Below this concentration, however, the luminescence was O_2-dependent and its intensity decreased as the O_2 concentration was lowered. In the experiment shown in Fig. 2 the K_m (50% decrease in the luminescence response) was 15μM-O_2. The mean result for five experiments gave a K_m of $22 \pm 9\mu$M ($n = 5$). Since low amounts of O_2 were introduced during the addition of stimulus, and the sensitivity of the electrode was about 1μM, the lowest O_2 concentration at which a response was accurately measured under these conditions was 2μM. At this concentration the response was decreased by 85%.

A similar O_2-dependence of the luminescence response resulting from the addition of a phagocytic stimulus (latex beads) was also found (Fig. 3) No affect of O_2 concentration on the magnitude of the response was observed at concentrations of 120μM or above. Below this value, decreasing the O_2 concentration decreased the response and the K_m obtained in the experiment shown was 22μM-O_2. The mean result for four experiments gave a value of $31 \pm 9\mu$M. The lowest O_2 concentration at which a response was measured was 5μM, when luminescence was inhibited by 85%. There was no significant difference between the mean K_m values for cells activated by either stimulus.

We have shown that the apparent K_m for O_2 of respiration of these cells is decreased after stimulation (Edwards *et al.*, 1983). Similarly, the O_2 affinities of the individual redox components of the electron-transport chain involved in the respiratory burst increase after the addition of chemotactic peptide and cytochalasin B (S. W. Edwards, M. B. Hallett, D. Lloyd & A. K. Campbell,

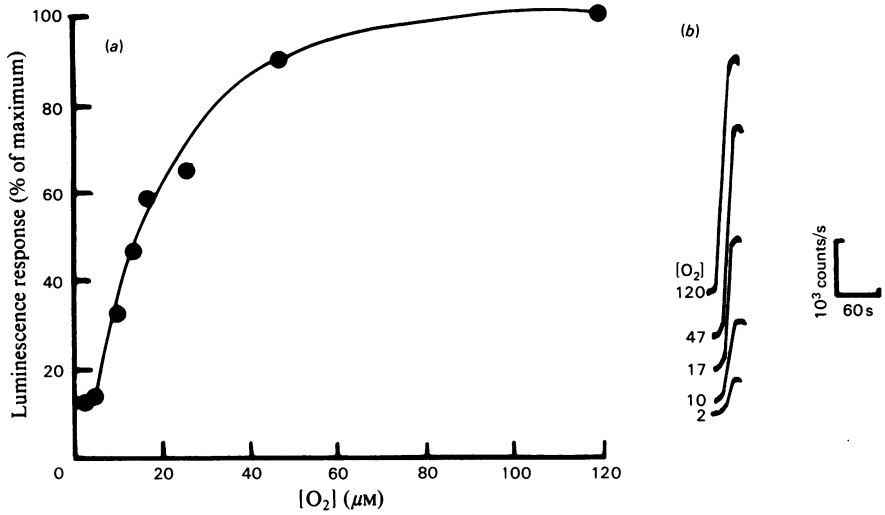

Fig. 2. *O_2-dependence of luminescence stimulated by chemotactic peptide and cytochalasin B*
Cells were diluted to between $(1-2) \times 10^6$ cells/ml in buffer and 3.0ml were placed in the chamber of the oxygen electrode, together with 10 μM(final concn.)-luminol. O_2 concentrations in the liquid phase were varied by changing the air/N_2 mixture in the gas phase by means of a digital gas mixer. When the required concentration was obtained, a background luminescence count was recorded, which was usually in the range of 20–70 counts/s. The chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (1 μM final concn.) and cytochalasin B (3 μg) were then injected into the suspension through a gas-tight membrane. A continuous trace of luminescence was recorded and maximum counts obtained (at $\geqslant 120$ μM-O_2) were 4400 counts/s (2×10^6 cells/ml). (*a*) Luminescence response (% of maximum) as a function of O_2 concentration, and (*b*) representative luminescence traces obtained at O_2 concentrations (μM) as indicated.

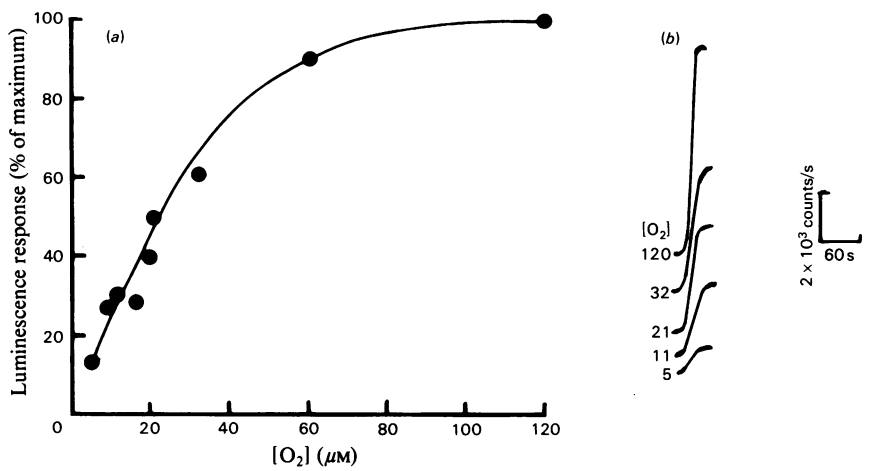

Fig. 3. *O_2-dependence of luminescence stimulated by latex beads*
Experimental details were essentially as described in the legend to Fig. 2, except that the stimulus was the addition of 4×10^9 latex beads (diameter 1.01 μm)/ml. Maximum counts ($\geqslant 120$ μM-O_2) were 8740 counts/s (10^6 cells/ml). (*a*) luminescence response (% of maximum) as a function of O_2 concentration, and (*b*) representative luminescence traces obtained at O_2 concentration (μM) as indicated.

unpublished work). The K_m for O_2 of oxygen-radical formation also decreased after stimulation, the K_m in resting cells being 45 μM (M. B. Hallett, S. W. Edwards & A. K. Campbell, unpublished work).

The affinities of reactions utilizing O_2 have not been well characterized. However, it has been established that several oxidases (Lloyd *et al.*, 1980, 1982) including cytochrome $a + a_3$ (Oshino *et al.*, 1972) have a high affinity for O_2, with K_m values in the region of 1 μM or less. Many bioluminescent reactions such as that catalysed by

bacterial luciferase also have high affinities for O_2 (Lloyd *et al.*, 1981).

Our results show the oxygen-radical-generating pathway in polymorphonuclear leucocytes involves an oxidase(s) with a relatively low affinity (high apparent K_m) for O_2. The K_m is sufficiently high for oxygen-radical production to be limited by O_2 concentrations found physiologically; for example, in venous blood the O_2 concentration is approx. 50 μM (Documenta Geigy, 1959), and in synovial fluid from rheumatoid joints we have measured O_2 concentrations as low as 20 μM (range 20–40 μM, $n = 5$). Human polymorphonuclear leucocytes show a respiratory burst and oxygen-radical production qualitatively similar to that of the rat cells used here. It is now necessary to determine the K_m value of O_2 for the oxygen-radical production by human polymorphonuclear leucocytes in order to determine whether oxygen radicals have a role in human inflammatory disease.

Polymorphonuclear-leucocyte infiltration has been implicated in the tissue damage that occurs in rheumatoid arthritis (Campbell *et al.*, 1984) and may also be involved in extending infarct size after myocardial infarction (Romson *et al.*, 1983). We now propose that O_2 availability to activated cells limits radical production at inflammatory sites *in vivo*. Thus limited O_2 supply at sites of infection will control inflammation, whereas uncontrolled radical release may occur under pathological conditions.

We thank Dr. M. E. Holt and Dr. J. Carey of the Department of Rheumatology of this School of Medicine for collection of synovial-fluid samples, Professor D. Lloyd for the loan of the gas mixer, and the Arthritis and Rheumatism Council and the Science and Engineering Research Council for financial support.

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