Glucose- and phorbol myristate acetate-stimulated oxygen consumption and superoxide production in rat peritoneal macrophages is inhibited by dexamethasone

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1. Rat peritoneal macrophages stimulated with phorbol 12-myristate 13-acetate (PMA) (40 nM) show an increase in the rate of oxygen consumption (measured with an O_2 electrode) and the production of superoxide (measured by cytochrome *c* reduction), which are both dependent on the presence of exogenous glucose. There is a 1:1 correlation between the oxygen consumed and the superoxide produced over a range of glucose concentrations (0–10 mM). 2. Preincubation of macrophages with dexamethasone (1 μ M) for 3 h significantly decreased the V_{max} for PMA-induced glucose-dependent oxygen consumption ($P < 0.001$) and glucose-dependent superoxide production ($P < 0.001$). However, dexamethasone did not significantly change the K_m for glucose in either PMA-induced oxygen

consumption or superoxide production. Dexamethasone is therefore a non-competitive inhibitor of PMA-stimulated glucose-dependent oxygen consumption ($K_i = 0.83 \pm 0.09 \mu$ M) and superoxide generation ($K_i = 0.87 \pm 0.09 \mu$ M). 3. The PMA-induced rate of oxygen consumption by macrophages was decreased at oxygen concentrations below approx. 15 μ M. The K_m of oxygen for PMA-induced oxygen consumption was $1.28 \pm 0.13 \mu$ M ($n = 12$), and this was not significantly different in the presence of dexamethasone; $K_m = 1.61 \pm 0.31 \mu$ M ($n = 12$). It is therefore concluded that *in vivo* macrophage superoxide production is not limited by external oxygen or glucose concentrations, even in hypoxic joints.

INTRODUCTION

Macrophages are phagocytic cells of the immune system, which have a vital role in killing infecting micro-organisms. Activation of these cells by a variety of chemotactic and phagocytic agents leads to the secretion of cytotoxic granular proteins, including lysozyme, proteases, phospholipases and cationic proteins, into the phagocytic vacuole, and this contributes to the destruction of the pathogen.

The granule proteins are produced largely via oxygen-independent processes, but, in aerobic conditions, macrophages will also generate the superoxide anion radical ($O_2^{\cdot-}$) from oxygen via a membrane-bound complex containing NADPH oxidase (for review see Rossi, 1986). Activation of NADPH oxidase is characterized by an increase in metabolism known as the 'respiratory burst', which involves the partial reduction of oxygen via a flavoprotein and a *b*-type cytochrome (Ellis et al., 1989) to superoxide at the expense of NADPH, produced from glucose 6-phosphate by the hexose monophosphate shunt (HMPS) (Babior, 1984).

Superoxide and its highly reactive products are directly toxic to micro-organisms, and in patients with chronic granulomatous disease, where there is a defective component of the NADPH oxidase, there is a much decreased ability to prevent many infections (Borregaard, 1988). In addition to their importance in pathogen destruction, reactive oxidants have also been suggested to lead to host-tissue damage following their inappropriate release in a number of disease conditions, such as rheumatoid arthritis. Phagocytic cells infiltrate inflamed tissues, such as wounds, and in the process are 'primed' for enhanced superoxide release (Paty et al., 1990).

However, it has been proposed that the availability of oxygen may limit superoxide production by phagocytes in inflamed tissues (Edwards et al., 1984; Edwards and Lloyd, 1988). Similarly, since it has been demonstrated that the generation of superoxide is totally dependent on the uptake of extracellular glucose in peritoneal macrophages (Rist et al., 1991) and a macrophage cell line (Kiyotaki et al., 1984), any decrease in the levels of glucose in inflamed tissues will inhibit the production of superoxide.

The aim of the present study was to measure the rate of sugar-dependent and oxygen-concentration-dependent oxygen consumption during the phorbol 12-myristate 13-acetate (PMA)-induced respiratory burst in rat peritoneal macrophages, in order to establish whether these substrates would be rate-limiting for superoxide production *in vivo*.

Glucocorticoids suppress the inflammatory response and tumoricidal activity of macrophages (Grand Perret and Lemaire, 1986; Hogan and Vogel, 1988) and it has been proposed that this is at least partly due to their ability to inhibit superoxide production (Schultz et al., 1985; Maridonneau-Parini et al., 1989). Dexamethasone, a synthetic glucocorticoid, has been shown to inhibit both PMA-induced sugar transport and HMPS activity in rat peritoneal macrophages (Rist and Naftalin, 1991), leading to a decrease in HMPS-produced NADPH, the substrate required by NADPH oxidase for superoxide generation. Here, we describe the effects of dexamethasone on PMA-stimulated glucose-dependent superoxide generation (measured by superoxide dismutase-inhibitable cytochrome *c* reduction) and the rate of oxygen consumption in peritoneal macrophages measured directly with an O_2 electrode equipped with a direct-reading rate-meter.

MATERIALS AND METHODS

Macrophages were isolated from anaesthetized Wistar rats, as previously described (Rist et al., 1990), and diluted in Hepes-buffered saline (Hepes, 5 mM; NaCl, 140 mM; KCl, 5 mM; MgCl₂, 1.2 mM) at pH 7.3 to a final cell concentration of approx. $(1-2) \times 10^6$ macrophages/ml. The number of cells was estimated with an Elzone 280PC particle analyser (Particle Data Ltd., Kingstone, Hereford, U.K.), and differential staining of the cell preparation showed > 90% intact monocytes and macrophages. All chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K., unless otherwise stated.

PMA-induced superoxide production by the macrophages was measured by superoxide dismutase (SOD)-inhibitable cytochrome *c* (horse heart) reduction (Rist et al., 1991); 20 μ M cytochrome *c* was present in the Hepes buffer during the incubation, and subsequently the absorbance spectrum of the samples was measured between 540 and 550 nm in a Cecil CE2020 spectrophotometer. The reduced cytochrome, and hence the superoxide generated, was calculated from the absorption coefficient of 21.1 mM⁻¹·cm⁻¹, and the results were expressed as μ mol/min per 3×10^9 cells after subtraction of the SOD- (10 μ g/ml) uninhibitable component.

Oxygen consumption was measured with an oxygen-meter (Strathkelvin Instruments, Bearsden, Glasgow, Scotland, U.K.; model 781, with a 1302 electrode fitted to a MC 100 microcell). Suspensions of macrophages in Hepes-buffered saline were injected into a 70 μ l sample chamber with the oxygen electrode forming the base section, and the temperature was maintained at 37 ± 0.05 °C throughout the experiment by a glass water jacket connected to a Haake E3 water bath and circulator. The electrode was calibrated with air-saturated water, which was assumed to have an oxygen concentration of 210 μ M (Seidell, 1940), and the zero setting was obtained by using a borate (0.01 mM)/sulphite solution.

The output from the oxygen-meter was plotted on-line throughout the experiment on an Apple Macintosh IIsi with maths co-processor, by using the analog connection WorkBench (Strawberry Tree Computers Inc., Sunnyvale, CA, U.S.A.), which converts the computer desktop into a virtual instrument. The oxygen-meter signals were sampled at 100 Hz. The oxygen concentration was obtained from the scaled rolling average of the input signal over 5 s intervals, and the rate of oxygen consumption from the first derivative of the oxygen-concentration level with respect to time. The average O₂ concentration and rolling average of the rate of O₂ consumption (over 20 s intervals) were then logged at 1–10 Hz on to a hard disk, and the data were subsequently processed off-line (Microsoft Excel 3 and Kaleidagraph 2.1, Abelbeck Software). The rates of oxygen consumption were normalized to μ mol/min per 3×10^9 cells; 3×10^9 cells is equivalent to 1 ml of cell water. The data were normally collected over 20 min periods, which was the time required to exhaust the O₂ content of the measuring cell.

The cells were stimulated with PMA before injection of the sample into the oxygen-electrode microcell. The delay between activation and the start of the experiment was < 10 s. The level of oxygen in the bathing medium was adjusted by bubbling either oxygen or nitrogen (BOC Medical Gases, Guildford, Surrey, U.K.) through it for various periods of time before use. The rate of oxygen consumption in PMA-stimulated macrophages is high, and it is therefore possible to monitor the rate of change of oxygen consumption as a function of changing dissolved oxygen concentration during the respiratory burst in these cells.

In order to evaluate the experimental system used in this study, the kinetic parameters of the previously defined oxygen-utilizing

enzyme cytochrome oxidase (EC 1.9.3.1; Sigma) were determined. The rate of O₂ consumption of cytochrome oxidase (10–25 units/ml) was examined in Hepes buffer containing cytochrome *c* (40 μ M), ascorbate (1 mM) and *NNN'*-tetramethyl-*p*-phenylenediamine (200 μ M), pH 7.0, 37 °C (Jiang et al., 1992). The change in rate was monitored as the O₂ level within the microcell fell, and the rates of O₂ consumption were replotted as a function of O₂ concentration. The observed K_m for O₂ was 0.69 ± 0.09 μ M ($n = 6$) and was not significantly different from the value reported previously for this enzyme, i.e. < 1 μ M (Vanderkooi et al., 1987; Wilson et al., 1988; Jiang et al., 1992).

The effect of endogenous SOD on the rate of oxygen consumption in these experiments was investigated by addition of the SOD inhibitor diethyldithiocarbamic acid (DDC) to PMA-stimulated cells. In the concentration range 0.5–100 μ M DDC (Mashiba et al., 1991), for 1–3 h (results not shown) there was no significant change in the apparent rate of oxygen consumption by the PMA-stimulated macrophages, suggesting that there is little effect of endogenous SOD on the apparent oxygen consumption.

RESULTS

Effects of dexamethasone on PMA-induced glucose-dependent oxygen consumption and superoxide production

Oxygen consumption

Figure 1(a) shows the effects of dexamethasone (1 μ M) on the rate of oxygen consumption over a range of glucose concentrations (0–10 mM) in PMA-stimulated rat peritoneal macrophages. In the absence of any external glucose, PMA-stimulated cells showed almost negligible oxygen consumption, but, as the concentration of sugar was raised, oxygen consumption increased. The glucose-dependent increase in oxygen consumption was saturable above 1 mM D-glucose. Non-linear least-squares regression to the Michaelis–Menten equation gave a K_m value of 0.37 ± 0.11 mM glucose for oxygen consumption and a V_{max} of 2.82 ± 0.18 μ mol/min per 3×10^9 cells ($n = 7$). Pre-incubation with dexamethasone for 3 h caused a decrease in the rate of oxygen consumption over a range of concentrations of external glucose; the largest effects were observed at the higher glucose concentrations. As a result, the V_{max} for oxygen consumption is significantly decreased by dexamethasone to 1.28 ± 0.09 μ mol/min per 3×10^9 cells ($P < 0.001$; $n = 7$; Student's *t* test); there was no significant change in the K_m , 0.21 ± 0.08 mM. These data indicate that dexamethasone acts as a non-competitive inhibitor of glucose-dependent oxygen consumption in activated macrophages. The K_i for dexamethasone on glucose-dependent oxygen consumption can be calculated from the formula $K_i = [\text{inhibitor}] / [(V_{max}/V_p) - 1]$ where V_p is the V_{max} in the presence of the inhibitor (Dixon and Webb, 1964). The K_i for dexamethasone is therefore 0.83 ± 0.09 μ M, and is not significantly different from the K_i for dexamethasone (1 μ M; 2 h exposure) on PMA-stimulated zero-*trans* 2-deoxyglucose uptake by these cells (Rist and Naftalin, 1991).

Superoxide production

The PMA-stimulated production of superoxide was measured by SOD-inhibitable cytochrome *c* reduction over the same time course and with the same experimental protocol as for the sugar-dependent oxygen consumption (Figure 1b). The data from control and dexamethasone-treated macrophages are similar to the data shown for oxygen consumption. The Michaelis–Menten parameters for PMA-induced glucose-dependent superoxide production in control cells ($K_m = 0.28 \pm 0.08$ mM; $V_{max} =$

Figure 1 Effects of dexamethasone (1 μM) on PMA-stimulated glucose-dependent oxygen consumption and superoxide production by rat peritoneal macrophages

(a) Freshly isolated macrophages were preincubated with or without dexamethasone (1 μM) in Hepes buffer (sugar-free) for 3 h at 37 °C, and then transferred into buffer containing PMA (40 nM), with various concentrations of glucose (0–10 mM). The consumption of oxygen by the cells over the subsequent 20 min was then measured by use of an oxygen-meter (see the Materials and methods section). Results are expressed as μmol/20 min per 3×10^9 cells, with each data point representing the mean $\pm$ S.E.M. of seven separate determinations, and lines were fitted by non-linear least-squares regression to the Michaelis–Menten equation. In control macrophages (○), the $V_{\max}$ for PMA-stimulated glucose-dependent oxygen consumption is 2.82 ± 0.18 μmol/min per 3×10^9 cells, and this is significantly decreased by dexamethasone (□) to 1.28 ± 0.09 μmol/min per 3×10^9 cells ($P < 0.001$; Student's *t* test). However, the K_m of glucose for PMA-dependent oxygen consumption in control cells is 0.37 ± 0.11 mM, and is not significantly different from the K_m for cells preincubated with dexamethasone (0.21 ± 0.08 mM). (b) Macrophages were preincubated as described for (a), and then transferred into buffer containing 20 μM cytochrome *c* and PMA (40 nM), with various concentrations of glucose (0–10 mM). The incubation was continued for 20 min, and the reduced cytochrome *c*, and thus the superoxide produced, was measured by spectrophotometry (see the Materials and methods section). Results are expressed as SOD (10 μg/ml)-inhibitable superoxide production, and the units are μmol/20 min per 3×10^9 cells. Each data point is the mean $\pm$ S.E.M. of seven separate determinations, and the lines were again fitted by non-linear least-squares regression. In control cells (■), the $V_{\max}$ for PMA-stimulated glucose-dependent superoxide production is 3.35 ± 0.18 μmol/min per 3×10^9 cells, and this is significantly decreased by dexamethasone (●) to 1.56 ± 0.11 μmol/min per 3×10^9 cells ($P < 0.001$; Student's *t* test). However, the K_m of glucose for PMA-dependent superoxide production is not significantly different between control cells (0.28 ± 0.08 mM) and dexamethasone-treated cells (0.34 ± 0.08 mM).

3.35 ± 0.18 μmol/min per 3×10^9 cells; $n = 7$) are not significantly different from those for the PMA-induced glucose-dependent oxygen consumption in identically treated cells ($K_m = 0.37 \pm 0.11$ mM; $V_{\max} = 2.82 \pm 0.18$ μmol/min per 3×10^9 cells; $n = 7$).

Dexamethasone (1 μM) decreased PMA-stimulated glucose-dependent superoxide production, significantly decreasing the $V_{\max}$ to 1.56 ± 0.11 μmol/min per 3×10^9 cells ($P < 0.001$; $n = 7$), but without significantly changing the K_m for glucose, 0.34 ± 0.08 mM. Once again, the K_m and $V_{\max}$ for dexamethasone-inhibited superoxide production were not significantly different from the same values for dexamethasone-inhibited oxygen consumption ($K_m = 0.21 \pm 0.08$ mM; $V_{\max} =$

Figure 2 Time course of PMA-stimulated oxygen consumption by rat peritoneal macrophages

Macrophages, preincubated for 1 h in sugar-free medium at 37 °C, were simultaneously stimulated with PMA (40 nM) and exposed to 0.5 mM glucose. The cells were then immediately injected into the 70 μl MC 100 microcell attached to a Strathkelvin model 781 oxygen meter (see the Materials and methods section), and the oxygen concentration (○) and the rate of oxygen consumption (□) were measured on-line throughout the experiment.

1.28 ± 0.09 μmol/min per 3×10^9 cells; $n = 7$). The K_i for dexamethasone on glucose-dependent superoxide production was 0.87 ± 0.09 μM, and was not significantly different from the K_i for glucose-dependent oxygen consumption.

These data indicate that dexamethasone pre-treatment of macrophages causes an approx. 2-fold decrease in the maximal rate of glucose-dependent oxygen consumption and the generation of superoxide. Also, it is clear that there is a direct 1:1 correlation between the oxygen consumed during the PMA-stimulated respiratory burst in rat peritoneal macrophages and the superoxide anion produced over the same period. This conclusion was verified by determining the proportion of the increased O_2 consumption by PMA-stimulated macrophages which could be eliminated by addition of high concentrations of SOD (200 μg/ml) and catalase (100 units/ml), which return the superoxide and H_2O_2 produced to O_2 . It was found that approx. 85% of the O_2 consumption could be inhibited by these enzymes (results not shown).

Effects of oxygen concentration and dexamethasone on PMA-stimulated oxygen consumption by peritoneal macrophages

Macrophages stimulated with PMA (40 nM) showed a characteristic activation of oxygen consumption (Figure 2). After the addition of PMA there was an initial lag period, followed by a rapid acceleration of oxygen consumption, which reached a maximum between 2 and 5 min. The maximum rate of oxygen consumption was then maintained for approx. 15–20 min (in conditions where oxygen was not limiting), before falling slowly to resting levels (results not shown). A similar time course for the activation of PMA-stimulated superoxide generation has been reported in human neutrophils (Christiansen, 1988). The addition of a high concentration of the sugar-transport inhibitor phloretin (100 μM) or cytochalasin B (10 μM), together with PMA, totally inhibited the increased oxygen consumption, such that it was not significantly different from unstimulated levels (results not shown).

Oxygen consumption in unstimulated macrophages was found to be $< 10\%$ of the stimulated levels, with no lag phase, plateau or decline (results not shown).

In PMA-activated cells, after the initial activation, the maximum rate of oxygen consumption could be maintained over

Figure 3 Effects of oxygen concentration on PMA-stimulated oxygen consumption by rat peritoneal macrophages

(a) Macrophages were preincubated for 1 h in glucose-free medium at 37 °C, and then stimulated with PMA (40 nM) in buffer containing 1 mM glucose and 80 μ M oxygen. The cells were in the maximal plateau phase of the respiratory burst at the start of the experiment, and there was no decrease in oxygen consumption due to the cessation of the respiratory burst during the time course of the experiment. The data are presented on a double-y-axis plot, with the rate of oxygen consumption ($\bullet$) and the oxygen concentration ($\square$) of the medium plotted against time. (b) Macrophages were treated as described in the legend to (a) and then transferred to buffer containing PMA (40 nM), 1 mM glucose and 30 μ M oxygen. The data are presented on a double-y-axis plot, with the rate of oxygen consumption ($\circ$) and the oxygen concentration of the medium (—) plotted against time.

15–20 min, but this was dependent on the availability of oxygen in the bathing solution. Figure 3(a) shows the data from a representative experiment, with the rate of oxygen consumption of PMA-treated macrophages in buffer containing 1 mM glucose and the oxygen concentration of the medium plotted against time. The initial oxygen concentration of the bathing solution was approx. 80 μ M and fell over a 14 min period to approx. 25 μ M, whereas the rate of cellular oxygen consumption by the cells remained fairly constant over the same time course. In contrast, when the initial oxygen concentration was decreased to 30 μ M (Figure 3b), the maximal rate of oxygen consumption was maintained until the oxygen concentration reached approx. 15 μ M and then fell rapidly over the subsequent 5 min, as the oxygen concentration fell until both the concentration and rate of oxygen consumption reached zero. These results demonstrate that rat peritoneal macrophages can consume oxygen at high rates even when its concentration is extremely low.

Figure 4 shows the data from a representative experiment on the effects of dexamethasone (1 μ M) on the relationship between the oxygen concentration in the bathing medium and the oxygen

Figure 4 Effects of dexamethasone (1 μ M) on the PMA-stimulated oxygen-concentration-dependent rate of oxygen consumption by rat peritoneal macrophages

Macrophages were preincubated with or without dexamethasone (1 μ M) in HEPES buffer (sugar-free) for 3 h at 37 °C, and then transferred into buffer containing PMA (40 nM) and 5 mM glucose. The cells were then injected into a MC 100 microcell linked to a Strathkelvin model 781 oxygen-meter (see the Materials and methods section) and the oxygen concentration and the rate of oxygen consumption were measured on-line throughout the experiment. The cells were in the maximal plateau phase of the respiratory burst at the start of the experiment (see Figure 2), and there was no decrease in oxygen consumption due to the cessation of the respiratory burst during the time course of the experiment. Lines were fitted to the data by non-linear least-squares regression to the Michaelis–Menten equation. In control cells ($\circ$), the $V_{\max}$ for the oxygen-concentration-dependent rate of oxygen consumption was 3.30 ± 0.40 μ mol/min per 3×10^9 cells and the K_m was 2.66 ± 0.15 μ M oxygen. Dexamethasone ($\bullet$) (1 μ M) significantly inhibited the maximum rate of oxygen consumption in PMA-stimulated cells compared with control ($V_{\max} = 1.80 \pm 0.04$ μ mol/min per 3×10^9 cells; $P < 0.01$; Student's t test), but did not significantly alter the K_m (2.97 ± 0.20 μ M oxygen).

consumption by rat peritoneal macrophages. In control cells, the rate of oxygen consumption was constant over a range of external oxygen concentrations from 20 μ M down to approx. 10 μ M, and then, below this level, the rate of oxygen consumption was inhibited, reaching zero when the oxygen concentration of the bathing medium reached zero. The data were plotted by non-linear least-squares regression to the Michaelis–Menten equation, to give a $V_{\max}$ of 3.30 ± 0.40 μ mol/min per 3×10^9 cells, and a K_m of 2.66 ± 0.15 μ M oxygen for the oxygen-dependent rate of oxygen consumption.

Macrophages preincubated in dexamethasone (1 μ M) also showed a constant rate of oxygen consumption down to approx. 10 μ M external oxygen, and this rate then rapidly decreased until both the oxygen concentration and the rate of oxygen consumption reached zero. Dexamethasone significantly inhibited the maximum rate of oxygen consumption in PMA-stimulated cells compared with control ($V_{\max} = 1.80 \pm 0.04$ μ mol/min per 3×10^9 cells; $P < 0.01$), but did not significantly alter the K_m (2.97 ± 0.20 μ M oxygen).

The data from a number of experiments identical with that shown in Figure 4 were processed by non-linear least-squares regression to the Michaelis–Menten equation (as described above), and averaged to give the cumulative K_m value for oxygen concentration on the rate of PMA-stimulated oxygen consumption. The averaged K_m for the control condition was 1.28 ± 0.13 μ M oxygen ($n = 12$), and this was not significantly different from the K_m for dexamethasone-treated macrophages, 1.61 ± 0.31 μ M oxygen ($n = 12$).

DISCUSSION

The results presented show the following: (a) that the stimulus to oxygen consumption occurs within 2–5 min after contact with PMA (40 nM) and leads to an elevated constant level of

consumption, lasting for at least 20 min, provided that the oxygen concentration of the bathing medium is above 15 μM ; (b) that the PMA-dependent increase in oxygen consumption in rat peritoneal macrophages is dependent on the presence of exogenous glucose; phloretin and cytochalasin B, inhibitors of glucose transport, abolished the glucose-stimulated oxygen consumption; (c) that dexamethasone acts as a non-competitive inhibitor of glucose-dependent oxygen consumption ($K_i = 0.83 \pm 0.09 \mu\text{M}$, after 3 h exposure) and superoxide production ($K_i = 0.87 \pm 0.09 \mu\text{M}$, after 3 h); (d) that the K_m of oxygen for the PMA-induced respiratory burst response is $1.28 \pm 0.13 \mu\text{M}$, and this is unaffected by dexamethasone.

These data are consistent with previous reports that show that PMA-stimulated superoxide production and HMPS activity in rat peritoneal macrophages are inhibited by cytochalasin B, and decreased by dexamethasone (Rist et al., 1991; Rist and Naftalin, 1991). The results are also consistent with previous reports showing that dexamethasone inhibits superoxide production in macrophages (Schultz et al., 1985; Maridonneau-Parini et al., 1989).

New findings in this paper are (i) the 1:1 stoichiometry of PMA-stimulated oxygen consumption and superoxide production, over a wide range of glucose concentrations, and (ii) the apparent non-competitive inhibition of PMA-stimulated glucose-dependent oxygen consumption and superoxide generation by dexamethasone. This contrasts with the competitive inhibition of the PMA-dependent stimulation of 2-deoxyglucose uptake by dexamethasone (Rist and Naftalin, 1991).

Superoxide is produced in PMA-treated macrophages when oxygen is reduced at the expense of NADPH by the NADPH oxidase complex:

Superoxide rapidly dismutates in aqueous solutions to produce H_2O_2 and oxygen in a reaction also catalysed by SOD:

Moreover, in the presence of catalase, H_2O_2 produced by the dismutation of superoxide will be converted into water and oxygen:

However, the contributions of endogenous SOD and catalase in this system have been assessed by using exogenous SOD, catalase and the SOD inhibitor DDC (see the Materials and Methods and Results sections). The finding that 85% of the observed oxygen consumption in PMA-stimulated macrophages could be eliminated by high concentrations of SOD and catalase indicates that in this system little of the superoxide produced is dismutating to form H_2O_2 and oxygen, and thus water and oxygen from the reactions shown above. It is clear that endogenous SOD and catalase do not significantly catalyse the formation of secondary products from superoxide, and so have only a small effect on the apparent stoichiometry of PMA-induced oxygen consumption and superoxide production. The lack of dismutation of superoxide observed in the absence of exogenous SOD and catalase suggests that superoxide may be reacting with other components within the system, such as membrane lipids, proteins and other biological molecules, and therefore avoids the dismutation reaction and the regeneration of oxygen. Lipid peroxidation, protein oxidation and oxidation of polysaccharides, proteoglycans etc. have been extensively studied and are known to be of importance in a variety of disease conditions (Merry et al., 1990).

A 1:1 correlation between oxygen consumption and superoxide production has been reported previously in phagocytosing human granulocytes in the presence of azide (Root and Metcalf, 1977). In those studies, oxygen consumption and superoxide production were measured simultaneously in cells treated with cytochalasin B, which inhibits phagosome formation and thus only allows the production of superoxide into the extracellular compartment.

A possible explanation for the non-competitive inhibition by dexamethasone is that it decreases hexokinase activity at the endofacial surface of the glucose transporter (Rist and Naftalin, 1991). Glucose 6-phosphate is a good substrate for glucose-6-phosphate dehydrogenase, and therefore its metabolism via the HMPS and superoxide generation is rate-limited by the rate of net sugar uptake via the membrane transport system, i.e. by the very low concentration of glucose 6-phosphate at the endofacial surface. Hence, a dexamethasone-dependent decrease in endofacial hexokinase activity at all external glucose concentrations will decrease the maximal rate of superoxide production, leading to non-competitive inhibition kinetics by dexamethasone. This contrasts with the situation when 2-deoxyglucose is the transported sugar, since this sugar is poorly metabolized by glucose-6-phosphate dehydrogenase but is rapidly metabolized by hexokinase. As a result, the HMPS pathway is not rate-limited by the endofacial concentration of 2-deoxyglucose 6-phosphate, and dexamethasone-dependent decrease in hexokinase activity affects HMPS activity and superoxide production only at low concentrations of 2-deoxyglucose when transport across the cell membrane is the rate-limiting step in the HMPS pathway.

The data reported here do not exclude the possibility that dexamethasone acts via a different mechanism other than directly decreasing endofacial hexokinase activity. Indeed, it has been reported that the inhibitory effects of dexamethasone on superoxide production by alveolar macrophages can be mimicked by lipocortin 1 (Maridonneau-Parini et al., 1989), suggesting that dexamethasone may act indirectly by inducing synthesis or release of a non-competitive inhibitor which may act at one or more points between sugar transport and superoxide production.

Oxygen affinity of macrophage superoxide production process

There have been few studies which have investigated the relationship between the concentration of molecular oxygen and the production of superoxide during the respiratory burst in phagocytic cells. It has been reported that the K_m for oxygen on superoxide production (measured by luminol chemiluminescence) in chemotactic-peptide-stimulated rat neutrophils is $22 \pm 9 \mu\text{M}$ (Edwards et al., 1984), and those authors concluded that this apparently high K_m would mean that *in vivo* oxygen would limit superoxide-mediated tissue injury. Subsequently, the same authors measured superoxide production by cytochrome *c* reduction and obtained a K_m of $5.4 \pm 1.4 \mu\text{M}$ for oxygen in cells stimulated with either chemotactic peptide or PMA (Edwards and Lloyd, 1988). The conclusion was that the oxygen affinity of superoxide generation was such that oxygen would limit superoxide production during an inflammatory response.

Our results show that the K_m of oxygen for the glucose-dependent respiratory burst, in the presence or absence of dexamethasone, is in the range 1–2 μM . This value is so low that, in practice, no circumstance could arise where oxygen concentration rate-limits superoxide production by rat peritoneal macrophages. The oxygen concentration in the synovial fluid of rheumatoid-arthritis patients is between 20 and 40 μM (Edwards et al., 1984), a level which would not significantly limit the PMA-stimulated respiratory-burst activity of these cells.

The glucose concentration in the synovial fluid of patients with rheumatoid arthritis has been reported to be 3.81 mM (range 0.77–4.48 mM) (Altman, 1961). The K_m of glucose for PMA-induced superoxide production or oxygen consumption is between 0.3 and 0.4 mM (Figure 1), and therefore, as with oxygen, the decreased concentration of glucose, at least in arthritic joints, cannot greatly inhibit the production of superoxide by rat peritoneal macrophages.

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