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OXYGEN UPTAKE OF RAINBOW TROUT *ONCORHYNCHUS MYKISS* PHAGOCYTES FOLLOWING STIMULATION OF THE RESPIRATORY BURST

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Summary

The *in vitro* oxygen uptake of rainbow trout phagocyte-enriched head kidney leucocyte and head kidney macrophage suspensions was monitored. Stimulation of these cells with zymosan or phorbol myristate acetate induced a two- to 10-fold increase in oxygen uptake, the so-called respiratory burst. This respiratory burst activity was markedly enhanced in the presence of the calcium ionophore A23187 and inhibited in the presence of the NADPH oxidase inhibitor diphenyl iodonium or when glucose was absent from the buffer. The presence of sodium azide also inhibited the response of phagocyte-enriched suspensions by approximately 36 %, but only by 16 % for macrophage suspensions. The possible pathways responsible for the respiratory burst in fish phagocytes and its biological significance are discussed.

Introduction

Following membrane stimulation, mammalian phagocytes show an increased oxygen uptake called the respiratory burst (Karnovsky, 1962; Miller *et al.* 1972; Roos, 1980). The respiratory burst is not due to an increased demand by the mitochondrial cytochrome system, since inhibitors of this system do not prevent it occurring, but is thought to be primarily involved in the production of reactive oxygen species, important in the bactericidal activity of phagocytes (Roos, 1980; Babior, 1978, 1984; Baggiolini, 1984). The primary reaction that occurs is the reduction of atmospheric oxygen (O_2) into superoxide anion (O_2^-), catalysed by an NADPH oxidase unique to phagocytes (Babior, 1978; Baggiolini, 1984) *via* the reaction:

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2O_2^- + NADP^+ + H^+ .$$

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NADPH is generated from the hexose monophosphate (HMP) shunt in which glucose is oxidized to carbon dioxide and a five-carbon sugar, using $NADP^+$ as an electron receptor. The use of NADPH during the respiratory burst causes the HMP shunt to be accelerated (Babior, 1978, 1984; Baggiolini, 1984).

Most superoxide produced is immediately converted into H_2O_2, either spontaneously or catalyzed by superoxide dismutase:

$$2O_2^- + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2 .$$

H_2O_2 has a higher bactericidal potency than superoxide, because its activity is greatly enhanced by myeloperoxidase, an enzyme present in most polymorphonuclear phagocytes and monocytes (Babior, 1984), which catalyzes the oxidation of halide ions to hypohalite ions by H_2O_2:

$$X^- + H_2O_2 \xrightarrow{\text{Myeloperoxidase}} XO^- + H_2O .$$

Other oxygen species, such as hydroxyl radicals and singlet oxygen, are also produced and have a role in microbial killing, although their presence and actions are not as well documented.

Few studies concerning the respiratory burst have been performed on non-mammalian phagocytes. Even so, it is clear that lower vertebrate and even invertebrate phagocytes can produce reactive oxygen species after appropriate stimulation (Dikkeboom *et al.* 1987, 1988; Plytycz and Bayne, 1987; Chung and Secombes, 1988). For instance, in fish the respiratory burst activity of isolated phagocytes has been studied by means of chemiluminescence and by detecting the presence of H_2O_2 and O_2^- in neutrophil or macrophage cultures (Nash *et al.* 1987; Chung and Secombes, 1988; Secombes *et al.* 1988; Plytycz *et al.* 1989). It has been established that in fish, as in mammals, the production of such oxygen species is not sensitive to mitochondrial inhibition (Secombes *et al.* 1988). However, it has not been demonstrated whether the production of O_2^- and H_2O_2 is associated with an increased O_2 uptake.

The aim of this study was to determine whether the stimuli that increase the production of superoxide and hydrogen peroxide in rainbow trout macrophages also cause an increased oxygen uptake and to investigate whether it is dependent upon the HMP shunt and NADPH oxidase.

Materials and methods

Phagocyte isolation

Rainbow trout *Oncorhynchus mykiss* (Walbaum) phagocyte suspensions enriched for macrophages were obtained as previously described (Chung and Secombes, 1988; Secombes *et al.* 1988). The head kidney of 300–500 g trout was removed and pushed through a nylon mesh with L-15 (Leibovitz, Gibco) medium containing 2 % foetal calf serum (FCS, Gibco), 1 % Penicillin/Streptomycin (P/S,

Gibco) and 10 i.u. ml^{-1} heparin (Sigma). The obtained cell suspension was layered on a 34–51 % Percoll (Sigma) gradient and, after centrifugation at 400 *g* for 25 min, the band of cells lying at the 34–51 % interface was collected.

The cell suspension, enriched for macrophages, but also containing neutrophils (20 % and 55 %, respectively), was washed once in L-15 medium and centrifuged at 400 *g* for 5 min. After washing, the cell suspension was either adjusted to 1×10^7–3×10^7 cells ml^{-1} in an appropriate medium (usually the same medium as above) or buffer required for the experiment and used immediately in the respirometer, or adjusted to 1×10^7–2×10^7 cells ml^{-1} in L-15 medium containing 0.1 % FCS and 1 % P/S to purify macrophages. 5–8 ml of the latter suspension (approximately 10^8 cells) was added to 10 cm diameter culture dishes (Nunc) and left for 2 h at 18 °C before removal of unattached cells by washing twice with phosphate-buffered saline (PBS, pH 7.2, 0.15 mol l^{-1}). The monolayers were then incubated in L-15 medium supplemented with 5 % FCS and 1 % P/S and cultured for 48 h before use.

Just prior to use, residual non-adherent cells were removed by washing twice with PBS. The macrophage monolayers were then incubated with PBS containing 2 mmol l^{-1} EDTA (BDH) for 3–5 min. The cells were scraped from the dish surface, centrifuged for 5 min at 400 *g* and resuspended at 0.7×10^7–1×10^7 cells ml^{-1} in L-15 medium, supplemented with 2 % FCS, 1 % P/S and 10 i.u. ml^{-1} heparin for use in the respirometer.

Bacteria

Two strains of the fish pathogen *Aeromonas salmonicida* were used to stimulate the phagocytes: an A-layer-lacking, relatively non-virulent strain (004) and an A-layer-possessing, virulent strain (423) (Graham *et al.* 1988). Both strains were cultured in tryptic soy broth (TSB, Difco) and kept in logarithmic growth phase by passaging daily.

Prior to use bacteria were killed, using ultraviolet irradiation or 1 % formalin. Some bacterial suspensions were opsonized in PBS containing 2 % normal trout serum for 30 min at room temperature and concentrated in PBS to 0.5×10^{10}–3.7×10^{10} cells ml^{-1}. In two experiments live bacteria of both strains were used.

Stimulation and inhibition of the respiratory burst

Several agents were used to stimulate or inhibit the respiratory burst.

Phorbol myristate acetate (PMA, Sigma), a known soluble membrane stimulant, was applied at a final concentration of 2 μg ml^{-1}; this is a concentration known to induce O_2^- and H_2O_2 production by rainbow trout macrophages (Chung and Secombes, 1988).

Zymosan A (Sigma) was also used, to establish whether particulate stimulation of the respiratory burst could occur. Zymosan was opsonized before addition to the cells using a modification of the method of Scott and Klesius (1981), which involved boiling the zymosan in PBS for 30 min followed by incubation in Hank's

balanced salt solution (HBSS, Gibco) with 20% normal trout serum for 1 h at room temperature. The final concentration of the stock zymosan suspension was 20 mg ml^{-1}.

Stimulation by bacterial suspensions was similarly attempted. Bacteria of strains 004 and 423 were used, either live or after killing (and opsonization in a number of cases) at bacteria/phagocyte ratios varying between 10 and 300 (mostly between 40 and 80).

Since many intracellular pathways are known to be influenced by calcium fluxes, the Ca^{2+} ionophore A23187 (Sigma) was used (20 μmol l^{-1}) simultaneously with PMA to establish if there was a synergism between these two agents.

In a number of cases sodium azide (NaN_3, BDH) at a concentration of 15 mmol l^{-1} (0.1%) was added prior to, simultaneously with or after PMA to establish if the measured increase in oxygen utilization was actually due to the respiratory burst and independent of metabolic pathways.

The importance of the HMP shunt for the process was investigated by depleting cells of glucose. After isolation, cells were washed in PBS and centrifuged for 5 min at 400 *g*, prior to resuspension in PBS without or with glucose (5.5 mmol l^{-1}). As cell clumping occurred in the first experiments, 1% bovine albumin (Sigma) was added to the PBS to prevent this.

To investigate whether the respiratory burst was dependent on an NADPH oxidase similar to that described for mammals (Segal *et al.* 1986; Rossi *et al.* 1986), a specific inhibitor for this oxidase, diphenyl iodonium (DPI) (Cross and Jones, 1986), was used at a concentration of 10 μmol l^{-1} together with PMA to trigger the respiratory burst. The DPI was a generous gift from Dr A. R. Cross (Department of Biochemistry, University of Bristol).

Oxygen measurements

Measurement of oxygen uptake

Oxygen uptake of resting and stimulated trout phagocytes was measured using a Ranks oxygen electrode (System model 10, Cambridge, England) connected to a Linseis pen recorder (LS-5, FRG). The Ranks oxygen electrode consisted of two respiration chambers equipped with variable-speed magnetic stirrers, providing optimal mixing throughout the experiment without any indication of damaging the cells in the chambers. Chamber volumes could be adjusted from less than 1 ml to 7.5 ml, using an air-tight stopper sliding into the chamber. The stopper contained a micropore to allow the injection of agents into the chamber without breaking the air-tight seal. The chamber was surrounded by a glass jacket in which water of a constant temperature was circulated. Oxygen uptake was measured at 18°C in a volume of 2–4 ml. Before use, the oxygen electrode was calibrated using air- and 100% N_2-saturated media at 18°C. Phagocytes in air-saturated medium were added to the respiration chamber, the chamber was sealed with the air-tight stopper and the decrease of P_{O_2} with time was monitored on the pen recorder. Within 1 min after sealing the chamber, a linear decline in P_{O_2} was achieved. After

measuring the oxygen uptake of resting cells for 5–10 min the agent to activate or inhibit the respiratory burst was injected into the chamber at a P_{O_2} of approximately 16–19 kPa (120–140 mmHg). The experiment was terminated when the P_{O_2} reached 2 kPa. Between experiments the stopper was removed and the chamber was rinsed at least three times with distilled water.

Oxygen uptake was calculated from the regression of the decline of P_{O_2} on the pen recorder trace. The maximum oxygen uptake was calculated over a 3- to 4-min interval during the peak of the respiratory burst.

Absolute solubility of oxygen in medium

To express the absolute rate of oxygen uptake in nmol min^{-1} 10^7 $cells^{-1}$, the oxygen content of air-saturated medium was determined for each type of medium used by the method of Tucker (1967). A 50 µl sample of air-saturated medium at 18°C was injected using a calibrated Hamilton micro-syringe (Bonaduz, Switzerland) into a water-jacketed glass chamber, constructed to fit over the end of a vertical oxygen electrode (Beckman), which was connected to an oxygen meter (Strathkelvin Instruments). The chamber contained a magnetic stainless-steel stirring rod and had a 1 mm capillary opening to allow the addition of the sample. This capillary was normally closed with a stainless-steel stopper during the measurements to prevent contamination of the chamber volume with atmospheric oxygen. The chamber contained approximately 0.5 ml of a degassed solution of 0.6% potassium ferricyanide [$K_3Fe(CN)_6$] (18 mmol l^{-1}) and 0.3% saponin in distilled water. This solution was used to release the oxygen from the medium to be measured by the electrode. The amount of oxygen released from the medium was then compared with the amount of oxygen released from air-saturated water at 0°C, with a known oxygen content.

Results

Oxygen uptake of resting and stimulated cells

The rates of oxygen uptake were calculated by measuring the maximum decrease in P_{O_2} of the medium in the respiration chamber after stimulation and expressing this as nmol O_2 min^{-1} 10^7 $cells^{-1}$. The oxygen uptake could be split into the oxygen uptake of resting cells, dependent on the mitochondrial cytochrome system, and uptake caused by the respiratory burst (RB uptake). The following three terms will be used: total oxygen uptake=RB oxygen uptake+oxygen uptake of resting cells (before stimulation, since during the course of an average experiment the rate of oxygen consumption from resting cells remained constant). The RB oxygen uptake was calculated by subtracting the oxygen uptake of resting cells from the total oxygen uptake after stimulation. If sodium azide (an inhibitor of mitochondrial respiration) was present, this subtraction was not performed. For each treatment, the mean effect and the standard deviation were calculated, using the values from each fish (Table 1). Where several experiments (maximum of three) were performed with cells from one fish the mean of the results of these

experiments was used. The within-fish variation (standard deviation) was on average 0.35 nmol O_2 min^{-1} 10^7 $cells^{-1}$ (N=6). Unless otherwise stated, values are for phagocyte suspensions enriched for macrophages.

Oxygen uptake of resting phagocyte suspensions varied from 0.65 to 2.22 nmol O_2 min^{-1} 10^7 $cells^{-1}$ (mean: 1.31), while macrophage suspensions gave values between 0.51 and 2.28 nmol O_2 min^{-1} 10^7 $cells^{-1}$ (mean: 1.51).

Stimulation of the respiratory burst (RB)

Following addition of the stimulants the kinetics of the RB oxygen uptake was monitored. The respiratory burst was deemed to have started when the oxygen uptake of resting cells before stimulation was exceeded. The maximum RB oxygen uptake was noted and the degree to which the respiratory burst was stimulated was expressed as a stimulation coefficient (SC) by dividing the maximum RB oxygen uptake by the oxygen uptake of resting cells before stimulation (Table 1).

Table 1. *Oxygen uptake and stimulation coefficients*

Treatment	Oxygen uptake	N	Stimulation coefficient
Phagocytes			
Resting cells (before stimulation/inhibition)	1.31±0.44 (0.65–2.22)	13	
PMA	4.43±1.76 (1.75–7.87)	6	4.57±2.13 (1.86–9.89)
PMA, azide	2.68±1.63 (1.02–4.40)	4	3.06±1.73 (1.51–5.35)
Zymosan A (1 mg ml^{-1})	8.60	1	7.32
Zymosan A (2 mg ml^{-1})	12.18	1	10.59
PMA–glucose	9.46±5.37 (5.66–13.25)	2	5.29±1.02 (4.57–6.01)
PMA+glucose	15.04±5.36 (11.25–18.83)	2	8.18±0.44 (7.87–8.49)
PMA, DPI	2.86±0.32 (2.63–3.08)	2	2.03±0.49 (1.68–2.37)
PMA, azide, DPI	1.38	1	0.87
Macrophages			
Resting cells (before stimulation/inhibition)	1.51±0.87 (0.51–2.28)	4	
PMA	5.46±3.85 (3.12–9.90)	3	4.57±1.44 (3.25–6.11)
PMA, azide	6.26	1	2.85

Oxygen uptake was expressed as nmol O_2 min^{-1} 10^7 $cells^{-1}$.

N, number of fish.

Values are mean±S.D. Values in parentheses show the minimum and maximum measurements.

PMA, phorbol myristate acetate; DPI, diphenyl iodonium.

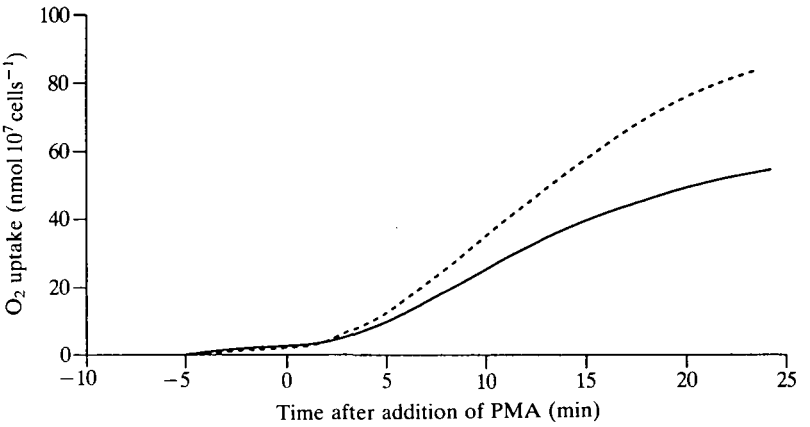

Fig. 1. Oxygen uptake of phagocyte-enriched head kidney leucocyte suspensions prior to and following stimulation with 2 μg ml^{-1} phorbol myristate acetate (PMA) in the absence (---) or presence (——) of 15 mmol l^{-1} sodium azide, added simultaneously with the PMA.

Phorbol myristate acetate

Using PMA at a final concentration of 2 μg ml^{-1}, the respiratory burst was triggered within 2 min in all but one case (lag time 3 min) (Fig. 1). The maximum oxygen uptake was reached between 7 and 11 min after PMA addition in phagocyte suspensions enriched for macrophages and between 6 and 9 min for suspensions of purified macrophages obtained after release from culture dishes. In both cases the respiratory burst continued for at least 45 min. The RB oxygen uptake for phagocyte suspensions varied from 1.75 to 7.87 nmol O_2 min^{-1} 10^7 cells^{-1} (mean: 4.43), with the SC ranging from 1.86 to 9.89 (mean: 4.57). Macrophage suspensions gave values ranging from 3.12 to 9.90 nmol O_2 min^{-1} 10^7 cells^{-1} (mean: 5.46) and an SC between 3.25 and 6.11 (mean:4.57) (Table 1).

Zymosan A

The particulate stimulant zymosan A used at a final concentration of 1 or 2 mg ml^{-1} triggered the respiratory burst after a lag period of approximately 2.5 min (Fig. 2). The maximum oxygen uptake was established between 10 and 18 min after zymosan addition, but the burst continued at the same rate for at least 30 min (Fig. 2). The RB oxygen uptakes were 8.60 and 12.18 nmol O_2 min^{-1} 10^7 cells^{-1} for zymosan concentrations of 1 and 2 mg ml^{-1}, respectively, with SCs of 7.32 and 10.59 (Table 1). This latter value was the highest SC recorded.

Bacteria

In a series of 14 experiments (using cells from eight different fish), in which bacteria were added to phagocyte suspensions, the respiratory burst was activated only once by ultraviolet-killed strain 004 and three times by ultraviolet-killed strain

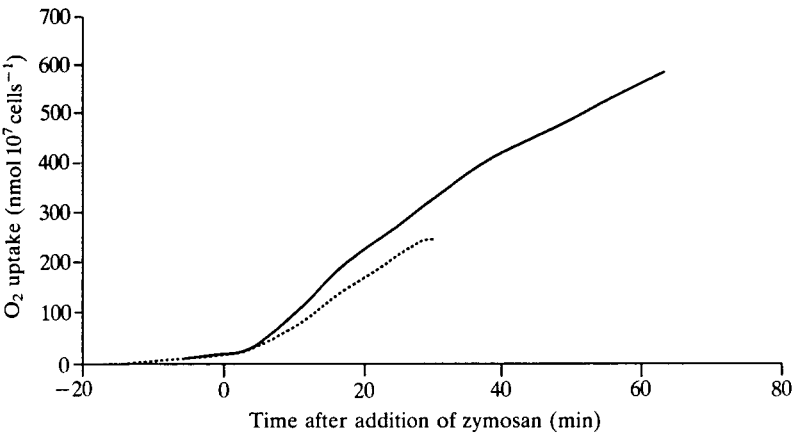

Fig. 2. Oxygen uptake of phagocyte-enriched head kidney leucocyte suspensions prior to and following stimulation with 1 mg ml^{-1} (---) or 2 mg ml^{-1} (——) zymosan.

423. With strain 423 both opsonised and nonopsonised bacteria triggered the respiratory burst. Thus, opsonisation was not essential for a response. Similarly, no correlation could be established between the phagocyte/bacterial ratio and the occurrence of the respiratory burst. Where a response was seen there was no lag time, and the maximum oxygen uptake was reached between 10 and 18 min after bacteria were added and continued for at least 30 min. A mean (±S.D.) maximum oxygen uptake of 6.16±1.38 nmol O_2 min^{-1} 10^7 cells^{-1} was recorded, with a mean stimulation coefficient (±S.D.) of 3.71±2.40. In a number of cases, when bacteria failed to trigger a respiratory burst, PMA was subsequently added; in all such cases the respiratory burst was triggered.

Ca^{2+} ionophore A23187

When the Ca^{2+} ionophore A23187 was added simultaneously with PMA the maximum oxygen uptake in the presence of the ionophore was 67% higher than the control value (only PMA added) (13.37 *vs* 8.03 nmol O_2 min^{-1} 10^7 cells^{-1}). The stimulation coefficient was similarly 67% higher (10.21 *vs* 6.13) and was the highest SC found in this study. No differences in duration were noted, which was comparable with the above PMA experiments. [The mean oxygen uptake of resting phagocytes (1.31 nmol O_2 min^{-1} 10^7 cells^{-1}) was used to calculate the above values, since in this experiment the resting oxygen uptake could not be reliably calculated.]

Sodium azide

Sodium azide was used to confirm that the occurrence of the respiratory burst was independent of mitochondrial pathways. A typical result is shown in Fig. 3. The oxygen uptake of resting cells was inhibited when azide was added to a phagocyte suspension prior to addition of PMA, but PMA was still able to trigger a

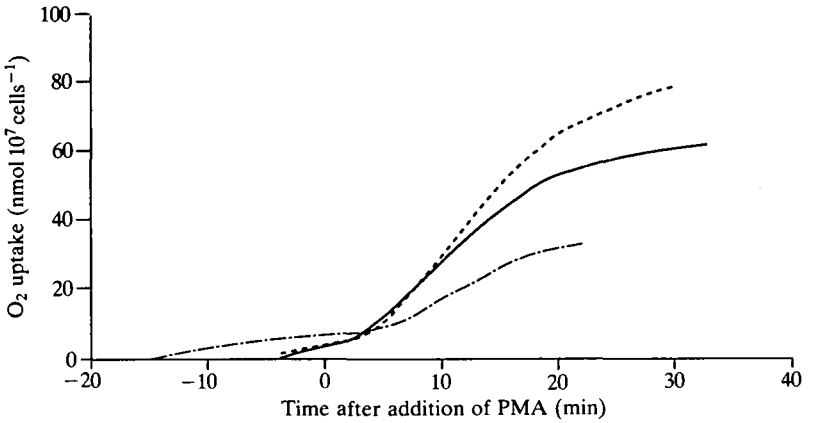

Fig. 3. Oxygen uptake of phagocyte-enriched head kidney leucocyte suspensions prior to and following stimulation with 2 μg ml^{-1} phorbol myristate acetate (PMA) (---). In some cases sodium azide (15 mmol l^{-1}) was added 11 min before (−·−) or 11 min after (——) addition of PMA.

respiratory burst. If azide was applied to a cell suspension that was already undergoing a respiratory burst, the burst continued, although the oxygen uptake was less than in the absence of azide (Fig. 3). A decrease in total oxygen uptake in the presence of azide, compared to a control (only PMA added), was also seen when PMA and azide were added simultaneously (Fig. 1). This decrease was only partly caused by the inhibition of the mitochondrial oxygen uptake, since comparison of the RB oxygen uptake of phagocytes in the presence of azide with that in the absence of azide showed that the former was between 22 and 46 % smaller (mean: 36.5 %). The SCs were between 29 and 44 % lower (mean: 39 %) in the presence of azide (Table 2).

For macrophage suspensions the maximum RB oxygen uptake in the presence of PMA and azide was 16 % lower than in the absence of azide, while the SC was 13 % lower.

Inhibition of the respiratory burst

Glucose depletion

To examine the importance of the HMP shunt, the respiratory burst was compared in the absence and presence of glucose.

Stimulation with PMA caused a respiratory burst after a normal lag time in both circumstances. In the absence of glucose, however, the oxygen uptake decreased after approximately 15 min to values near to the resting oxygen uptake (Fig. 4). In the presence of glucose the respiratory burst was significantly longer, although the maximum activity occurred within 20 min.

The maximum RB oxygen uptake was calculated to be 40 % less in the absence

of glucose and the SC was 35.5 % smaller. The total oxygen uptake during the first 40 min of the respiratory burst was on average 65 % less in the absence of glucose.

Inhibition of NADPH oxidase with diphenyl iodonium

DPI is known to inhibit triggering of NADPH oxidase completely, thus

Table 2. *Comparison of paired treatments*

Treatment	Difference between treatments: Respiratory burst oxygen uptake	Difference between treatments: Stimulation coefficient	*N*
Phagocytes			
PMA *vs* PMA, azide	−36.50±12.66 (22.03–45.52)	−39.07±9.09 (28.57–44.35)	3
PMA *vs* PMA, ionophore	+57.23		1
PMA+glucose *vs* PMA−glucose	−39.68±14.22 (29.63–49.74)	−35.53±8.98 (29.18–41.88)	2
PMA *vs* PMA, DPI	−44.17±1.20 (43.32–45.02)	−40.10±4.21 (37.12–43.08)	2
PMA, azide *vs* PMA, azide, DPI	−58.52	−60.08	1
Macrophages			
PMA *vs* PMA, azide	−15.91	−13.04	1

Except for the value in the presence of the Ca^{2+} ionophore, all values of oxygen uptake have been corrected for oxygen uptake of resting cells before stimulation (subtraction of resting oxygen uptake if no azide is present). *N*, number of fish.

Values are given as a percentage±S.D. Values in parentheses are minimum and maximum measurements.

PMA, phorbol myristate acetate; DPI, diphenyl iodonium.

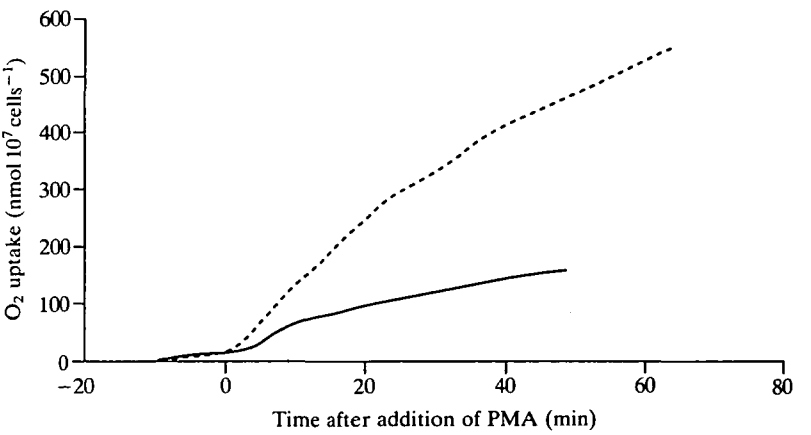

Fig. 4. Oxygen uptake of phagocyte-enriched head kidney leucocyte suspensions prior to and following stimulation with 2 μg ml^{-1} phorbol myristate acetate (PMA) in the absence (——) or presence (---) of 5.5 mmol l^{-1} glucose.

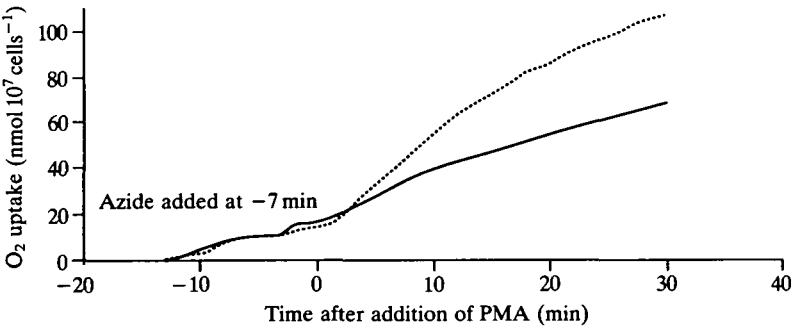

Fig. 5. Oxygen uptake of phagocyte-enriched head kidney leucocyte suspensions prior to and following stimulation with 2 μg ml^{-1} phorbol myristate acetate (PMA) plus 15 mmol l^{-1} sodium azide (added 7 min earlier), in the absence (---) or presence (——) of 10 μmol l^{-1} diphenyl iodonium (DPI) added simultaneously with the PMA.

preventing the respiratory burst, in mammalian neutrophils at concentrations of 5 μmol l^{-1} and higher (A. R. Cross, personal communication). Using DPI at this concentration it was found that the SCs were 40 and 60 % lower in the absence and presence of azide, respectively (Table 2, Fig. 5).

Discussion

The present results show that following membrane stimulation rainbow trout phagocytes have a markedly increased oxygen uptake, which is dependent upon the presence of glucose, but independent of mitochondrial pathways.

The soluble membrane stimulant PMA was a good inducer of this increased oxygen uptake, agreeing with earlier studies on its ability to induce/increase the production of O_2^- and H_2O_2 in trout macrophages (Chung and Secombes, 1988; Secombes *et al.* 1988). The respiratory burst in rainbow trout is similar to the increase in oxygen uptake found in mammalian neutrophils after stimulation with PMA (Babior, 1978, 1984; Lambeth, 1988), although the latter cells have been shown to react to PMA within 1 min (Lambeth, 1988), while in this study lag times of 2 min were normal. It is not clear what causes this difference, but a possible reason could be the lower temperature at which the fish phagocytes were used.

The great variation in the level of stimulation found using PMA may be partly caused by individual differences between trout. However, the cell suspension that is taken from the Percoll gradient contains a variable percentage of non-phagocytes in each experiment. If the number of non-phagocytes is relatively high, the stimulation of the respiratory burst will be expected to be relatively low, and *vice versa*. The observation that purified macrophage suspensions show a smaller, though still substantial, variation in stimulation rates supports this idea. Several of the phagocyte suspensions taken from the Percoll gradient showed a higher stimulation coefficient with PMA than any of the macrophage suspensions,

suggesting that the neutrophils in these suspensions could have a higher respiratory burst activity than the macrophages. It is also possible, however, that the macrophages were damaged when they were scraped from the culture dishes, and subsequently showed lower SCs than undamaged cells.

By stimulating the phagocytes in the presence of sodium azide it was shown that the occurrence of the respiratory burst is independent of the mitochondrial cytochrome system, agreeing with studies on mammalian phagocytes (Karnovsky, 1962; Babior, 1978, 1984). The amount of oxygen uptake in the presence of azide was, nevertheless, lower than predicted by subtracting the oxygen uptake of resting phagocytes from the oxygen uptake during the respiratory burst in the absence of azide. This discrepancy ranged from 22 to 46% in phagocyte suspensions, while macrophages only showed a 16% difference in the oxygen uptake (Table 2). As the production of superoxide anion by trout macrophages is not affected by the presence of azide (Secombes *et al.* 1988), possibly this difference was due to the stimulation by PMA of processes other than the respiratory burst that were dependent on the mitochondrial pathways. Indeed, since the macrophage-enriched cell suspensions, which contained a proportion of non-phagocytes, showed a greater discrepancy than the purified macrophage suspensions, it is possible that PMA was able to stimulate mitochondrial-dependent processes in these contaminating non-phagocytic cells. Protein kinase C, which is activated by PMA, has a complex function in cells (Tauber, 1987) and could account for this result. Further studies concerning these effects would be interesting.

Stimulation with opsonized zymosan confirmed that the respiratory burst could also be triggered with particulate stimuli. This is in agreement with other studies on teleost (Scott and Klesius, 1981; Stave *et al.* 1984), snail (Dikkeboom *et al.* 1987) and mammalian (Babior, 1978; Lambeth, 1988) phagocytes, looking at the production of various reactive oxygen species in response to zymosan stimulation. In this study the lag time for zymosan to act was approximately 2.5 min, longer than that seen with PMA. This is in agreement with mammalian studies (Lambeth, 1988). As with PMA, the response was approximately 1 min slower than the corresponding mammalian response (Lambeth, 1988).

The observation that in most experiments bacteria did not stimulate the respiratory burst is surprising, especially since other studies in fish looking at the production of reactive oxygen species (Stave *et al.* 1984, 1985, 1987; Nash *et al.* 1987) and mammalian studies looking at oxygen uptake (Karnovsky, 1962; Miller *et al.* 1972; Root and Metcalf, 1977; Baggiolini, 1984; Babior, 1978, 1984) clearly show that bacteria can elicit the respiratory burst. As live bacteria were unable to elicit a respiratory burst in this study, it is unlikely that inactivation of the bacteria by killing them was a reason for the poor response. Even opsonization of the bacteria, which enhances their ability to trigger the above responses (Stave *et al.* 1984; Nash *et al.* 1987; Root and Metcalf, 1977), did not have any effect. Indeed, since nonopsonised killed bacteria did elicit a response in one instance it appears that opsonins are not essential to trigger respiratory burst activity in trout

phagocytes. Why a biologically important particle, such as *A. salmonicida*, should give these inconsistent responses is unclear. It could be argued that a bacterial pathogen of fish may in some way impair the triggering of the respiratory burst, as seen for chemiluminescence responses to *Yersinia ruskeri* in striped bass (Stave *et al.* 1987). This is difficult to reconcile with the ease with which strain 004 is killed *in vitro* by rainbow trout macrophages (Graham *et al.* 1988), or with the fact that on three occasions the respiratory burst was elicited by killed bacteria of the virulent strain 423 (without a corresponding response to strain 004 in two of these experiments). Indeed, bactericidal activity of trout macrophages for different strains of *A. salmonicida* appears to correlate with O_2^- and H_2O_2 production (Graham *et al.* 1988), suggesting an important role of the respiratory burst in fish anti-bacterial defences.

As reported by Lambeth (1988) for mammals, the Ca^{2+} ionophore has a synergistic effect on oxygen uptake by fish phagocytes when added simultaneously with PMA. Although in this study it could not be established what part of the increased oxygen uptake was caused by mitochondrial-dependent processes, it seems likely that at least part of the large increase (57%) is caused by the respiratory burst. Since the production of O_2^- by trout macrophages is not dependent on extracellular Ca^{2+} (Secombes *et al.* 1988), it is likely that Ca^{2+} and PMA trigger the respiratory burst independently of each other, as they do in mammals (Tauber, 1987; Lambeth, 1988).

In the absence of glucose, superoxide anion production by trout macrophages is completely inhibited (Secombes *et al.* 1988). This was not seen with oxygen uptake, but the respiratory burst was lower than in the presence of glucose and oxygen uptake decreased to the level of resting cells very quickly. The dependency on glucose is consistent with the view that the HMP shunt is required to provide NADPH for the reduction of molecular oxygen to superoxide anion during the respiratory burst (Karnovsky, 1962; Root and Metcalf, 1977; Babior, 1978, 1984; Tauber, 1987).

Since the respiratory burst in trout phagocytes seems to be very similar to that seen in mammalian cells, regarding stimuli, kinetics and possible pathways, it is interesting to investigate whether it is also dependent upon the unique membrane NADPH oxidase present in mammalian phagocytes. The experiments showed a significant (albeit incomplete) inhibition of the respiratory burst when DPI was added at a concentration of $10\,\mu mol\,l^{-1}$, indicating that the oxidase in fish phagocytes is at least partly similar to the NADPH oxidase in pig neutrophils, which is completely inhibited at this concentration (Cross and Jones, 1986). Further identification of the NADPH oxidase in fish neutrophils and macrophages and comparison with the mammalian oxidase could give valuable insights into the relationships between and the evolution of the non-specific defence mechanisms.

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