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ENERGY METABOLISM BY TROUT RED BLOOD CELLS: SUBSTRATE UTILISATION

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Summary

The present study investigates energy metabolism by trout red blood cells. It is shown that they are able to use pyruvate at physiological concentrations as an aerobic source of energy. Moreover, microcalorimetric data suggest that trout erythrocytes are also able to use internal substrates, at least when maintained *in vitro*. Although the actual nature of these substrates has not been elucidated, glycogen appears to be the most probable.

The relationship between heat dissipation and oxygen consumption suggests that most of the oxygen is used to oxidize substrates, and the inhibition of respiratory activity by antimycin A indicates that there is no substantial utilisation of non-respiratory oxygen. However, the oxygen uptake by these cells does not appear to be related to substrate utilisation, measured from transformation of labelled molecules (either pyruvate or glucose); this may be due to mixing of labelled compounds with non-labelled molecules in the intracellular pools, because of the low metabolic rate of these cells.

Introduction

The metabolism of red cells has attracted considerable attention, mostly because of the ease with which these cells can be isolated, which allows satisfactory characterisation of the metabolic pathways (Agar and Board, 1983; Rapoport, 1986). Most of the current knowledge about red blood cell metabolism has been learned from non-nucleated mammalian erythrocytes in which other cell organelles, such as mitochondria, are also lacking and energy metabolism is restricted to anaerobic pathways. In contrast, non-mammalian red cells are nucleated and they also possess more or less well-formed mitochondria, allowing aerobic metabolism.

The metabolism of salmonid red cells has recently received widespread attention. From these studies, glucose appears to be the main exogenous substrate used to obtain energy (Walsh *et al.* 1990; Pesquero *et al.* 1992) once it has entered the cell without a specific carrier (Bolis *et al.* 1971; Tse and Young, 1990; Pesquero *et al.* 1992). In the presence of

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oxygen, external glucose is mostly converted to lactate, through the Embden–Meyerhoff and hexose-monophosphate shunt pathways (Pesquero *et al.* 1992).

Although red blood cells from most species use glucose as an external source of energy, there are some significant exceptions, such as erythrocytes from ruminants and pigs (Agar and Board, 1983). In a recent study, Tiihonen and Nikinmaa (1991) showed that carp erythrocytes used lactate and pyruvate in preference to glucose as a source of aerobic energy. Although Walsh *et al.* (1990) reported little utilisation of lactate by trout red cells, in the present study we considered the possibility that they may use pyruvate to obtain energy through its oxidation to CO_2.

There is no clear stoichiometry between the amount of CO_2 formed from labelled substrates by trout red blood cells and their rate of O_2 uptake. Walsh *et al.* (1990) argued that this discrepancy may arise from the use of whole blood which, in addition to the labelled substrates, also contained non-labelled molecules that could be used as fuel. Pesquero *et al.* (1992) used washed red cells resuspended in a modified Cortland buffer (Houston *et al.* 1985) rather than whole blood, and in these conditions, where the sole external source of energy was labelled glucose, a CO_2/O_2 ratio of only 0.01 was obtained. However, Tufts *et al.* (1991) obtained a CO_2/O_2 ratio of 0.8 for *Salmo salar* red blood cells (whole blood) under resting conditions, by measuring CO_2 directly instead of calculating CO_2 production from labelled molecules. Sephton *et al.* (1991) suggested that the low metabolic rate of fish red cells may impair the oxidation of external labelled substrates, both because long periods are required before their metabolic intermediates are incorporated into the citric acid cycle and because labelled compounds will mix with non-labelled intermediates, producing a drop in their specific activity.

The present study suggests that an internal substrate may be the source of some of these non-labelled metabolic intermediates. To evaluate the hypothesis of an endogenous substrate, metabolic activity of cells in the presence of different substrates was monitored by microcalorimetry and measurement of oxygen consumption (Daut and Elzinga, 1989; Gnaiger and Kemp, 1990; Hardewig *et al.* 1991; Lamprecht *et al.* 1991).

Materials and methods

Animals and chemicals

Brown trout (*Salmo trutta* L.) (250 and 400 g) were obtained from fish farms (Departament Medi Ambient, Generalitat de Catalunya) in the Pyrenees, where they were maintained in open-water circuits, directly connected to a river.

Sodium [1-^{14}C]pyruvate was from Amersham (UK). D-[U-^{14}C]glucose was from New England Nuclear (Germany). All other reagents used were of analytical grade.

Isolation of red blood cells

Blood was obtained by caudal puncture and diluted (1:4) with heparinized RPMI 1640 (Sigma Co., USA). Red blood cells were separated from lymphocytes and other mononuclear cells by centrifugation with Histopaque-1077 (Sigma Co., USA), following the procedure suggested by the supplier, slightly modified to overcome the high viscosity

of trout blood (0.75 vols blood:4 vols Histopaque). The cells were rinsed four times in Cortland buffer (pH 7.4) (Houston *et al.* 1985), slightly modified (NaCl, 141 mmol l^{-1}; KCl, 3.5 mmol l^{-1}; $MgSO_4$, 1 mmol l^{-1}; NaH_2PO_4, 3 mmol l^{-1}; $CaCl_2$, 1 mmol l^{-1}; Hepes, 10 mmol l^{-1}; bovine serum albumin, 0.3 %). This buffer will be referred to as MCB. The osmolality was adjusted to 305 mosmol kg^{-1} with NaCl. Red blood cells were finally resuspended in MCB at a haematocrit of 10 %.

Microcalorimetric measurements

The rate of heat production of trout red blood cells was measured at 15 °C in a thermal activity monitor (Thermometrics AB/LKB Järfalla, Sweden), which consists of three twin heat conduction microcalorimeters; the design and principle are explained in detail by Suurkusk and Wadsö (1982) and by Görman *et al.* (1984). The calorimetric vessels were filled with the cell suspension (2.7 ml). A turbine stirrer (100 revs min^{-1}) ensured efficient mixing of the cell suspension. Aerobic conditions in the culture were tested by miniaturized polarographic oxygen sensors incorporated into the sample compartment (Bäckman and Wadsö, 1991). When assembled, the unit was lowered to the measurement position of the calorimeter in four 10 min steps. Further equilibration of the calorimeter took 1 h before a steady heat reading was obtained. Injections were performed with a gas-tight Hamilton syringe driven by a stepper motor. Data were recorded every second and the mean values for 180 s were stored on an IBM PS-30 hard disk.

Measurement of O_2 consumption

The rate of oxygen consumption was measured in a Clark-type oxygen electrode thermostatically controlled to 15.0±0.1 °C (Rank Brothers, UK), filled with 2.7 ml of cell suspension. Measurements were performed within the linear range (P_{O_2} between 14.5 and 20.8 kPa).

Measurement of CO_2 production

Experiments measuring CO_2 production from labelled glucose or pyruvate were performed in closed vials, containing the red blood cell suspension in MCB plus the desired substrate, fitted with a well containing a piece of dry filter paper. At the end of the experiment, this was fully soaked in 2-methoxyethylamine (Merck, Germany), to trap CO_2, by injecting the liquid through the rubber cap without opening the vial. A total release of the CO_2 produced by the cells was achieved by acidifying the cell suspension with sufficient $HClO_4$ to obtain a final concentration of 6 %, followed by vigorous shaking for 2 h.

$^{14}CO_2$ was converted to micromoles of substrate oxidized on the basis of their specific activities.

Analytical procedures

Pyruvate and lactate in plasma and whole blood were measured using the methods of Beutler (1985). Red cell concentrations of these metabolites were calculated using:

$$CE = [WB - P(1 - \mathrm{Hct})]/\mathrm{Hct}\,,$$

where *CE* is the concentration of the metabolite in red cells, *WB* is its concentration in

whole blood and *P* is its plasma concentration. Hct is the haematocrit of whole blood. Haemoglobin concentration was determined using Drabkin's reagent.

Expression of results

Results are expressed as mean ± S.E.M.; the number of experiments is given in parentheses.

Results and discussion

Physiological blood and plasma pyruvate concentrations for trout were 30.9±4.0 μmol l^{-1} (*N*=8) and 59.1±9.1 μmol l^{-1} (*N*=7) respectively, suggesting that there was no pyruvate within erythrocytes (whole-blood haematocrit 35.8±0.6, *N*=14). These values did not change following stressful exercise (in fact, there was a marginally significant drop in plasma values), while plasma lactate concentration rose from 0.6±0.1 to 1.3±0.1 mmol l^{-1} (*N*=7; *P*<0.01). Since membranes are highly permeable to pyruvate, once it has entered the cell it is rapidly converted to lactate or CO_2. Fig. 1 shows that the rate of CO_2 production from 50 μmol l^{-1} pyruvate was several times higher than that from 1 mmol l^{-1} glucose and that CO_2 production from 500 μmol l^{-1} pyruvate was 80 times higher, suggesting that pyruvate was a good aerobic source of energy for these cells. The presence of non-labelled glucose did not interfere with pyruvate oxidation (data not shown). Houston *et al.* (1985) suggested that trout red cells need pyruvate to maintain their nucleotide triphosphate levels during long periods of incubation. Moreover, they used a concentration of 4 mmol l^{-1}, which is well above physiological values. Walsh *et al.* (1990) reported that the rate of CO_2 production from lactate was only one-third of that from glucose under resting conditions although, following exhaustive exercise, the rate of CO_2 production from lactate was twice that from glucose under the same conditions (Wood *et al.* 1990). Thus, lactate cannot be ruled out as a source of energy, but its role under resting conditions seems to be limited in trout red blood cells. The ability of

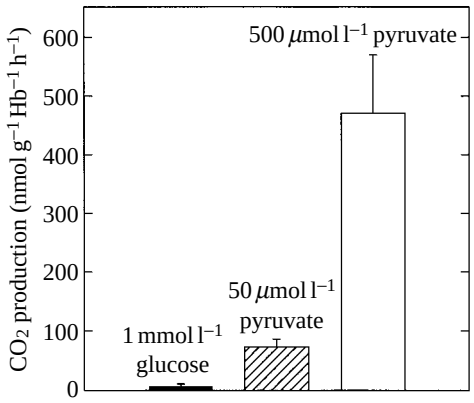

Fig. 1. CO_2 production by trout red blood cells incubated aerobically for 3 h in the presence of 1 mmol l^{-1} glucose and 50 μmol l^{-1} or 500 μmol l^{-1} pyruvate. Results are expressed as the mean + S.E.M. (*N*=6).

these cells to obtain energy from either lactate or pyruvate is not easily understood because of the similarity of the molecules. Perhaps the activity of lactate dehydrogenase in converting lactate to pyruvate *in vivo* is impaired, despite the high activity measured *in vitro* (data not show).

The much higher rate of CO_2 production from pyruvate compared with that from glucose was not reflected by the amount of oxygen taken up by these cells, which was $37\pm3\,\text{nmol}\,O_2\,g^{-1}\,\text{Hb}\,\text{min}^{-1}$ (N=6) in the presence of $1\,\text{mmol}\,l^{-1}$ glucose or $50\,\mu\text{mol}\,l^{-1}$ pyruvate. Although plasma pyruvate may yield more CO_2 than plasma glucose, raising the CO_2/O_2 ratio, there is still a large discrepancy between the utilisation of labelled substrate and total oxygen consumption by salmonid red blood cells. There are several possible explanations for this difference. Red cells could be using non-labelled substrates at the same time as exogenous labelled substrates (Walsh *et al.* 1990; Ferguson and Storey, 1991). However, Pesquero *et al.* (1992) used a 'defined' medium, in which there was no unknown exogenous energy source, and the results obtained were similar. Alternatively, Sephton *et al.* (1991) showed that, in cells with a relatively low metabolic rate, such as fish red blood cells, external labelled substrates may be diluted or mixed into the intracellular pools before they are oxidized, artificially lowering the oxidation rate. This may be more marked for glucose, because trout red cells are only slightly permeable to it (Tse and Young, 1990; Pesquero *et al.* 1992).

For cells living in a very rich environment, such as plasma, it is to be expected that the source of non-labelled intermediates would be substrates taken up previously. The possibility that these cells could use a non-labelled endogenous substrate, at least when maintained *in vitro*, has not been considered previously. This hypothesis was tested by comparing the metabolic activities of two samples maintained in the presence, or absence, of $1\,\text{mmol}\,l^{-1}$ glucose as the only exogenous substrate. Fig. 2 shows the microcalorimetric traces from one such measurement. Heat production was maintained when there was no external substrate, suggesting the existence of an internal energy

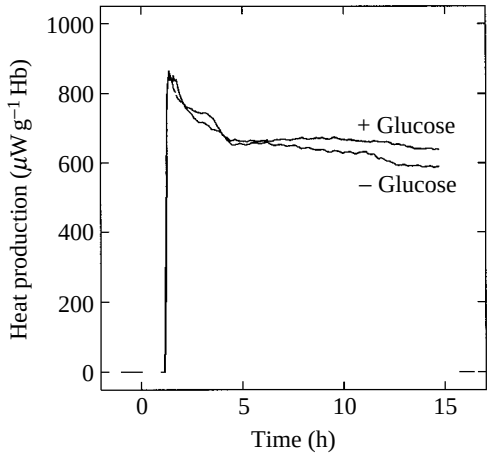

Fig. 2. Aerobic heat production by trout erythrocytes at 15 ˚C in the presence of $1\,\text{mmol}\,l^{-1}$ glucose as the only external substrate or without external substrate.

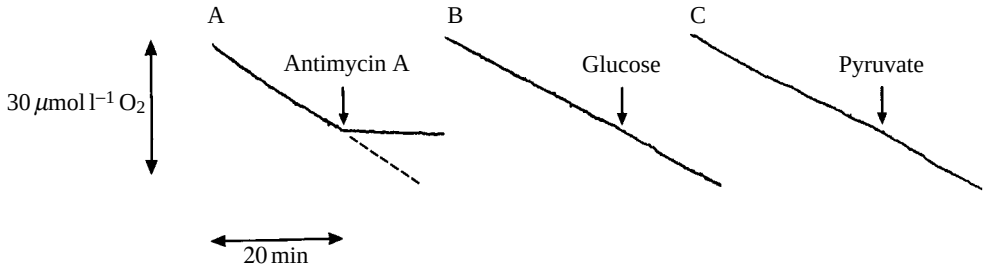

Fig. 3. Effects of 20 μmol l^{-1} antimycin A (A), 1 mmol l^{-1} glucose (B) and 2 mmol l^{-1} pyruvate (C) on the rate of oxygen consumption of trout red blood cells. The traces show the decline in O_2 concentration in the medium. Arrows show the addition of compounds. Cells in A were maintained with 1 mmol l^{-1} glucose from the start of incubation, while in B and C there were no external substrates before the addition of either glucose or pyruvate. These results are representative of three independent experiments.

source and only partial dependence on external substrates. The rate of oxygen consumption of these cells was also the same in the presence and in the absence of exogenous glucose (Fig. 3). The existence of an endogenous substrate may also explain why the addition of 1 mmol l^{-1} glucose did not change the rate of aerobic heat production, whereas the addition of 2 mmol l^{-1} pyruvate increased this rate by only 10 % (Fig. 4).

Although calorimetric data do not reveal the nature of the molecules used as the source of energy, the similarities between the traces in Fig. 2 indicate that in both situations trout red blood cells use the same (or very similar) substrate. Glycogen is the most probable internal source of energy. Walsh *et al.* (1990) showed that glycogen levels in rainbow

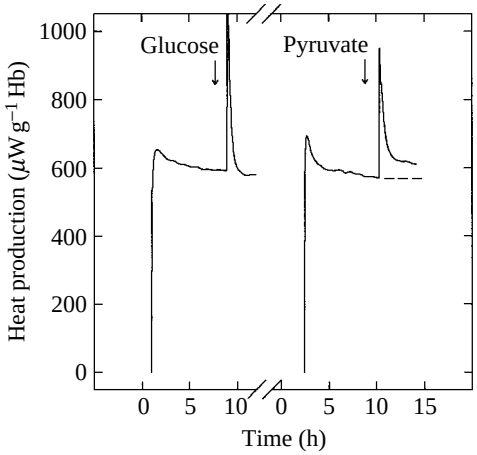

Fig. 4. Aerobic heat production by trout red cells incubated without external substrate and after addition of 1 mmol l^{-1} glucose or 2 mmol l^{-1} pyruvate. Arrows show when these substrates were added. The exothermic peaks are caused by the mechanical disturbance of injections and by the heat of dilution of the chemicals added. The discontinuous line indicates the heat production by the control cell suspension.

trout red blood cells are about 0.25 μmol g^{-1} cell wet mass, which is enough to sustain cells for 2 h, considering the metabolic rate (37±3 nmol O_2 g^{-1} Hb min^{-1}) measured in the current study. However, an attempt to label internal glycogen with [U-^{14}C]glucose was unsuccessful and we were therefore unable to investigate its utilisation as an energy source.

The possibility that some of the oxygen consumption could be accounted for by non-respiratory activity was ruled out by measuring the antimycin-resistant rate of oxygen uptake. Rapoport (1986) showed that about one-third of the total rate of oxygen consumption of reticulocytes can be accounted for by non-respiratory activity. This oxygen consumption is due to lipid oxidation, through the activity of lipo-oxygenase (Rapoport, 1986). Addition of 20 μmol l^{-1} antimycin A (Fig. 3A) resulted in full inhibition of the oxygen consumption of trout red blood cells, and heat dissipation fell to one-third of its initial value (corresponding to anaerobic metabolism; T. Roig, J. Pesquero, J. Bermúdez and J. Sánchez, in preparation).

The present study extends the overall picture of energy metabolism by trout red blood cells. The use of substrates other than glucose, as a source of energy, cannot be ruled out. Exogenous pyruvate appears to be a potent energy substrate for trout red cells *in vitro*. Other molecules, such as adenosine or some amino acids, have been suggested as important sources of energy for red cells of different species. For example, pig red cells, which are a good example of glucose-impervious mammalian erythrocytes (Wagner *et al.* 1984), use adenosine (Kim, 1983). However, the activity of adenosine deaminase in trout red cells is negligible (J. Sánchez, unpublished results), making the use of this substrate unlikely. Some amino acids, such as alanine (Walsh *et al.* 1990) or glutamic acid (Pesquero *et al.* 1992), can be ruled out as possible substrates, but others, such as glutamine, have not yet been tested.

Data obtained from these experiments also suggested that trout red cells *in vitro* do not use external substrates exclusively, but may also use endogenous energy sources. Glycogen, which is present in trout red cells in sufficient quantities, may be such an energy source.

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