Cell surface oxygen consumption by mitochondrial gene knockout cells

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Abstract

Mitochondrial gene knockout (\( \rho^0 \)) cells that depend on glycolysis for their energy requirements show an increased ability to reduce cell-impermeable tetrazolium dyes by electron transport across the plasma membrane. In this report, we show for the first time, that oxygen functions as a terminal electron acceptor for trans-plasma membrane electron transport (tPMET) in HL60\( \rho^0 \) cells, and that this cell surface oxygen consumption is associated with oxygen-dependent cell growth in the absence of mitochondrial electron transport function. Non-mitochondrial oxygen consumption by HL60\( \rho^0 \) cells was extensively inhibited by extracellular NADH and NADPH, but not by NAD\(^+\), localizing this process at the cell surface. Mitochondrial electron transport inhibitors and the uncoupler, FCCP, did not affect oxygen consumption by HL60\( \rho^0 \) cells. Inhibitors of glucose uptake and glycolysis, the ubiquinone redox cycle inhibitors, capsaicin and resiniferatoxin, the flavin centre inhibitor, diphenyleneiodonium, and the NQO1 inhibitor, dicoumarol, all inhibited oxygen consumption by HL60\( \rho^0 \) cells. Similarities in inhibition profiles between non-mitochondrial oxygen consumption and reduction of the cell-impermeable tetrazolium dye, WST-1, suggest that both systems may share a common tPMET pathway. This is supported by the finding that terminal electron acceptors from both pathways compete for electrons from intracellular NADH.

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1. Introduction

The energy requirements of most mammalian cells are met by aerobic respiration involving mitochondrial electron transport coupled to oxidative phosphorylation. Non-mitochondrial oxygen consumption has been observed at low levels in a variety of cells and tissues, but this has usually been attributed to inefficient mitochondrial electron transport, or to other cellular oxidative reactions not linked to energy metabolism. With \( \rho^0 \) cells that lack mitochondrial DNA, and therefore, are incapable of mitochondrial oxidative phosphorylation, oxygen consumption rates ranging from 10\% to 34\% that of mitochondrial competent parental cells have been observed in some [1–3] but not all studies [4–6]. The nature of this non-mitochondrial oxygen consumption is unknown and is the subject of this investigation.

The phenomenon of aerobic glycolysis was first described by Warburg [7–9] and has more recently been investigated by other authors [1,2,10–13]. It has been hypothesised that by avoiding reactive oxygen species (ROS) production, aerobic glycolysis may be advantageous to developing tumours [12]. Nevertheless, many rapidly proliferating tumour cell lines seem to have perfected the art of controlling ROS damage without resorting to aerobic glycolysis for their energy requirements, and the Warburg effect remains enigmatic [14,15].

Cells that use glycolytic metabolism produce fewer ROS [16,17] but more cytoplasmic NADH than cells dependent on mitochondrial respiration, and this NADH must be re-oxidised to support continued glycolysis for their energy requirements, and the Warburg effect remains enigmatic [14,15].
tion. Nevertheless, not all tumour acidification can be accounted for in terms of lactic acid production [18,19] suggesting the involvement of other redox mechanisms in recycling intracellular NADH [20]. One of the mechanisms that may contribute to maintaining a predominantly glycolytic metabolism involves electron transport across the plasma membrane. This was first demonstrated with cell-impermeable redox reagents like ferricyanide and indophenol dyes in the 1970s [21], although in vivo reduction of sulfonated vital dyes injected into rats had been observed 50 years earlier. Indeed, Larm et al. [1] showed that ferricyanide and dichloroindophenol support the survival and growth of \( \rho^0 \) Namalwa cells for several days in the absence of pyruvate and uridine, which are normally mandatory for their growth. This was attributed to an up-regulation of the trans-plasma membrane electron transport (tPMET) system in \( \rho^0 \) cells. We have shown that the cell-impermeable tetratolium salt, WST-1, in conjunction with the intermediate electron acceptor, 1-methoxyphenazine methosulfate (PMS), is efficiently reduced by rapidly dividing cells, but not by quiescent cells [22–24]. Both ferricyanide and WST-1/PMS reduction depend on cytosolic NADH flux but they differ in their sensitivity to the cell-impermeable sulfhydryl-blocking agent, \( p \)-hydroxymercuriphenylsulfonic acid (pCMBS).

In this study, we have used the \( \rho^0 \) model of glycolytic metabolism to investigate the role of tPMET in cell growth and energy metabolism. We found that HL60\( \rho^0 \) cells showed oxygen-dependent growth and that non-mitochondrial oxygen consumption was inhibited strongly by the cell-impermeable pyridine dinucleotides NADH and NADPH. Oxygen consumption by \( \rho^0 \) cells is compared with mitochondrial oxygen consumption and with WST-1/PMS reduction, and oxygen and PMS were shown to compete for intracellular reducing equivalents that are responsible for WST-1 formazan production. Our results indicate that non-mitochondrial oxygen consumption plays a vital role in recycling cytosolic NADH to maintain glycolysis and therefore proliferation of \( \rho^0 \) cells.

2. Experimental procedures

2.1. Cells and cell culture

The mitochondrial gene-knockout cell line, HL60\( \rho^0 \), was derived from its parental cell line HL60 by long-term culture with ethidium bromide, and lack of mitochondrial DNA was verified by PCR. All cells were grown in RPMI-

2.2. Materials

Superoxide dismutase (SOD) was obtained from Boehringer Mannheim (Mannheim, Federal Republic of Germany), 2-deoxy-D-glucose from Fluka (Buchs, Switzerland), and WST-1 and PMS from Dojindo Laboratories (Kumamoto, Japan). Unless otherwise stated all other reagents were from Sigma Chemical Company (St. Louis, MO, USA).

2.3. Inhibitor studies

Maximum inhibitory concentrations of potassium cyanide (KCN, 1 mM); myxothiazol (1 μM), capsaicin (240 μM) and resiniferatoxin (15 μM) were determined by titration. Other inhibitors were used at concentrations previously reported in the literature: diphenyleneiodonium (DPI), 20 μM; dicoumarol, 20 μM; iodoacetamide (IA), 2 mM; 2-deoxy-D-glucose (2DOG), 5 mM; and carbonyl cyanide \( p \)-trifluoromethoxyphenylhydrazone (FCCP), 2 μM [22,26–28].

2.4. Oxygen consumption

Exponentially growing cultures were centrifuged at 130 \( \times g \) at room temperature for 5 min and resuspended in fresh medium to a density of 2.5 \( \times 10^7 \) cells/ml. Each experiment measured oxygen consumption by 600 μl of resuspended cells in a 1 ml chamber, surrounded by a water-filled chamber maintained at 37 °C. Oxygen consumption was measured with a Clark-type dissolved oxygen electrode, coupled to a Powerlab processor and a Macintosh computer using Powerlab software. Trace slopes were obtained using Powerlab software. In general, the effects of inhibitors on oxygen consumption were determined by spiking cells with the specified concentration of inhibitor after stable oxygen consumption had been recorded. Inhibition by 2DOG and IA was measured by incubating cells with either 2DOG or IA for 30 min. Cells were centrifuged and resuspended in fresh medium containing 2DOG or IA. Results, expressed as a percentage of control, were obtained by dividing slopes in the presence of inhibitor by control slopes within the same trace. All values were corrected for background readings obtained from fresh medium without cells.

2.5. Dye reduction assays

For inhibitor studies, WST-1/PMS reduction rates were measured in a microplate format as described previously [22,25], using 1 \( \times 10^6 \) cells/ml for each assay unless otherwise stated. WST-1/PMS reduction by cells grown under different percentage oxygen conditions were carried out as follows. Exponentially growing cells were centrifuged at 130 \( \times g \) for 5 min, and resuspended in fresh medium to a density of 1 \( \times 10^6 \) cells/ml, and 2 ml aliquots were measured in a microplate format as described previously [22,25], using 1 \( \times 10^6 \) cells/ml for each assay unless otherwise stated.
were pipetted into 20 ml universals. The headspace was continually flushed with a predetermined gas mixture, which was delivered through a purpose-built gas manifold (at a flow rate of 100 cc/min). After pre-incubation of cells in suspension for 1 h, PMS (different concentrations, depending on the experiment), and WST-1 (500 µM) were added. To maintain anaerobic conditions, small concentrated volumes of nitrogen-flushed liquids were added from nitrogen-flushed syringes. Samples (250 µl) were taken at different times and dye reduction was measured as the absorbance at 450 nm in a Biorad ELISA plate reader. All values were corrected for background readings of cells in the presence of WST-1 and inhibitor (where appropriate) but without PMS.

3. Results

3.1. HL60ρ0 cells grow under aerobic but not anaerobic conditions

Cells that are devoid of mitochondrial electron transport function would be expected to grow equally well under anaerobic and aerobic conditions. To test this prediction, HL60ρ0 cells and parental HL60 cells were grown under aerobic (20% O2, 75% N2, 5% CO2) and anaerobic (95% N2, 5% CO2) conditions and viable cell numbers recorded over 5 days. Fig. 1 shows that HL60ρ0 cells grew under aerobic conditions (closed squares), albeit more slowly than HL60 cells (open squares) with doubling times in the exponential phase of approximately 37 and 24 h, respectively. When HL60ρ0 cells were grown under anaerobic conditions (closed circles), they remained viable for 48 h after which viable cell numbers declined. In contrast, under anaerobic conditions, HL60 cells remained viable for 24 h only (open circles).

3.2. Non-mitochondrial oxygen consumption by HL60ρ0 cells

To determine whether oxygen-dependent growth by HL60ρ0 cells was associated with oxygen consumption, a Clark-type dissolved oxygen electrode was used to measure oxygen utilization by HL60 and HL60ρ0 cells. Fig. 2A shows that parental HL60 cells (2.5 × 10^7 cell/ml) consumed all available oxygen within 17 min at an initial rate of 11.4 ± 0.4 pmol O2 s^{-1} 10^6 cells^{-1} (n = 4). Lineweaver–Burk analysis showed a K_M for oxygen of 10 nM. HL60ρ0 cells consumed oxygen at an initial rate of up to 30% that of parental HL60 cells (3.4 ± 0.3 pmol O2 s^{-1} 10^6 cells^{-1} (n = 4)). However, HL60ρ0 cells failed to consume all the oxygen in the medium and oxygen consumption rates declined with time to 0.27 ± 0.05 pmol O2 s^{-1} 10^6 cells^{-1} after 30 min and 0.12 ± 0.03 pmol O2 s^{-1} 10^6 cells^{-1} after 45 min. Non-mitochondrial oxygen consumption by HL60ρ0 cells did not exhibit classical Lineweaver–Burk kinetics and therefore a K_M value could not be determined (data not shown). Oxygen consumption rates of HL60ρ0 (closed circles) and HL60 cells (open circles) were cell concentration-dependent (Fig. 2B).

3.3. Non-mitochondrial and mitochondrial oxygen consumption show distinct properties

Non-mitochondrial oxygen consumption by HL60ρ0 cells was unaffected by the mitochondrial electron transport inhibitors, myxothiazol and KCN, or by the uncoupler, FCCP (Fig. 3B, Table 1). In contrast, the metabolic inhibitors 2DOG and IA, which inactivates the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, inhibited both mitochondrial and non-mitochondrial oxygen consumption to a similar extent (32–39% at 5 mM 2DOG and 53–54% at 2 mM IA). These results indicate that non-mitochondrial and mitochondrial oxygen consumption are both dependent on glycolysis. The vanilloids, capsaicin, which inhibits redox-cycling of membrane ubiquinones [30] and resiniferatoxin, a more potent fungal analogue, inhibited mitochondrial and non-mitochondrial oxygen consumption in distinctive ways (Table 1). The maximum inhibitory effect of both resiniferatoxin and capsaicin on non-mitochondrial oxygen consumption by HL60ρ0 cells was similar (57% and 54%, respectively) although resiniferatoxin was able to achieve this inhibition at a much lower concentration than capsaicin.

Fig. 1. Comparison of growth rates of HL60 and HL60ρ0. Viable cell numbers, as determined by trypan blue exclusion, were recorded at 24-h intervals over 5 days for HL60 (open symbols) and HL60ρ0 (closed symbols), grown under 20% (squares) and 0% (circles) O2. Results are the averages ± standard errors from two separate experiments, carried out in duplicate.
(15 and 240 µM, respectively). At these concentrations, mitochondrial oxygen consumption was poorly inhibited by resiniferatoxin but completely inhibited by capsaicin, perhaps reflecting differential accessibility of these drugs to their targets in the mitochondrial electron transport pathway. These results indicate that non-mitochondrial oxygen consumption involves membrane ubiquinone.

The flavin centre inhibitor, DPI, which has also been implicated in several tPMET systems, inhibited non-mitochondrial oxygen consumption by 41% at 20 µM, supporting flavin centre involvement. The NQO1 inhibitor and uncoupler, dicoumarol, inhibited oxygen consumption in HL60ρ⁰⁰ cells (Table 1) in a concentration-dependent manner (results not shown), but stimulated mitochondrial

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Fig. 2. Oxygen consumption by HL60 and HL60ρ⁰⁰ cells. (A) Oxygen consumption rates were measured using 2.5 × 10⁷ cells/ml in fresh medium. Results are representative of four separate experiments. O₂ consumption rates were 11.4 ± 0.4 and 3.4 ± 0.3 pmol O₂ s⁻¹ 10⁶ cells⁻¹ for HL60 and HL60ρ⁰⁰ cells, respectively. (B) Cell concentration-dependent oxygen consumption by HL60 cells (open circles) and HL60ρ⁰⁰ cells (closed circles). Results are averages and standard errors from three separate experiments.

Fig. 3. The effect of myxothiazol and NADH on oxygen consumption by (A) HL60 and (B) HL60ρ⁰⁰ cells. Oxygen consumption rates were measured using 2.5 × 10⁷ cells/ml in fresh medium. Myxothiazol and NADH were added at the specified concentrations after stable oxygen consumption traces had been established (arrows). Results are representative of four separate experiments.
Non-mitochondrial oxygen consumption by HL60 cells was compared with a simple tetrazolium dye reduction assay for measuring electron transport across the plasma membrane [22–25]. In this assay, the cell-impermeable tetrazolium salt, WST-1, is reduced to its soluble formazan, using reducing equivalents derived from intracellular NADH. Dye reduction is mediated by an intermediate electron acceptor, PMS, which picks up low potential electrons transported across the plasma membrane.

Inhibitor profiles of non-mitochondrial oxygen consumption and WST-1/PMS reduction by HL60 cells, were very similar (Table 1). Little inhibition was observed with the mitochondrial inhibitors, KCN (1 mM), myxothiazol (1 μg/ml) and the uncoupler, FCCP (2 μM). The uncoupler and NQO1 inhibitor, dicoumarol (20 μM), inhibited both non-mitochondrial oxygen consumption and WST-1/PMS reduction by HL60 cells to a similar extent (35% and 43%, respectively). Compounds that interfere with ubiquinone redox cycling, capsaicin and resiniferatoxin, showed similar inhibition of both non-mitochondrial oxygen consumption and WST-1/PMS reduction (54% and 66% for 240 μM capsaicin, and 57% and 69% for 15 μM resiniferatoxin). Likewise, inhibition of non-mitochondrial oxygen consumption and WST-1/PMS reduction by the flavin centre inhibitor, DPI (20 μM) were comparable (41% and 43%, respectively). Some minor differences were observed in that 2DOG (5 mM) inhibited non-mitochondrial oxygen consumption more strongly than WST-1/PMS reduction (39% and 30%, respectively), while the glycolytic inhibitor, IA (2 mM), preferentially inhibited WST-1/PMS reduction (75% compared with 54% for non-mitochondrial oxygen consumption). In contrast to the general similarities in inhibitor profiles between non-mitochondrial oxygen consumption and WST-1/PMS reduction, there was little correlation between inhibitor profiles of mitochondrial oxygen consumption and WST-1/PMS reduction by parental HL60 cells.

### 3.5. Non-mitochondrial oxygen consumption and WST-1/PMS reduction share similar properties

The above results show a close similarity between cell surface oxygen consumption by HL60 cells and WST-1/PMS reduction. If both systems use the same plasma membrane electron transport pathway, they would be expected to compete for electrons from intracellular NADH. In order to address this issue, cells were grown under carefully controlled gaseous conditions, using a multi-station magnetic stirrer, combined with a temperature controlled water bath. WST-1/PMS reduction, by cells grown under different percentage oxygen conditions was measured.
after 45 min. Fig. 4A shows that oxygen inhibited WST-1/PMS reduction by HL60 and HL60\(^0\) cells in a concentration-dependent manner. In contrast, under hypoxic conditions, WST-1/PMS reduction increased 450% for HL60\(^0\) and 350% for HL60 cells.

3.7. Oxygen and PMS compete for electrons from tPMET

We next looked to see whether the inhibitory effect of oxygen on dye reduction was a result of a direct competition between oxygen and the intermediate electron acceptor that mediated WST-1 reduction for intracellular electrons. HL60 and HL60\(^0\) cells were grown under different percentage oxygen conditions in the presence of increasing concentrations of PMS. Double reciprocal Lineweaver–Burk plots of PMS concentration against dye reduction show competitive inhibition of WST-1/PMS reduction by oxygen. \(V_{\text{max}}\) values for HL60 grown under 0%, 20% and 40% oxygen conditions were similar (5.6 ± 0.2, 5.7 ± 0.2 and 5.8 ± 0.1 milliA 450 min\(^{-1}\) 10^5 cells\(^{-1}\), respectively, \(n = 2\)), while \(K_M\) values were 60.9 ± 0.7, 45.2 ± 0.4, and 30.4 ± 0.1 μM PMS, respectively. Likewise, \(V_{\text{max}}\) values for HL60\(^0\) grown under different percentage oxygen conditions were similar, but higher than those for HL60 (12.8 ± 0.8, 11.6 ± 0.5, and 12.1 ± 0.6 milliA 450 min\(^{-1}\) 10^5 cells\(^{-1}\), respectively, \(n = 2\)), with \(K_M\) values being very similar to those for HL60 at corresponding percentage oxygen conditions (59.1 ± 1.0, 42.1 ± 0.1, and 28.9 ± 0.1 μM PMS, respectively).

4. Discussion

In this study, we show for the first time that non-mitochondrial oxygen consumption by a human leukaemic cell line with compromised mitochondrial respiratory function can be accounted for by electron transport across the plasma membrane. Although the existence of a cell surface oxygen-reducing system linked to intracellular NADH production and so to glycolytic energy metabolism was hypothesised many years ago, definitive evidence for this potential alternative bioenergetic pathway has remained elusive. A major difficulty concerned the problem of investigating alternative pathways of cellular oxygen consumption in the presence of an overwhelming capacity to employ mitochondrial respiration. The discovery of cells defective in mitochondrial oxidative phosphorylation, initially ‘petite’ mutants in yeasts and later human \(\rho^0\) cells devoid of mitochondrial DNA [4] provided a means of investigating non-mitochondrial oxygen consumption.

Although it is assumed that oxygen is essential for the survival and growth of all mammalian cells, the question of whether HL60\(^0\) cells require oxygen for growth has not been addressed. Our results show that HL60\(^0\) cells are unable to grow in the absence of oxygen, although cell viability was prolonged compared with parental cells. This suggests that \(\rho^0\) cells may be better able to tolerate anoxic conditions than their parental counterparts. The inability of HL60\(^0\) cells to grow under anaerobic conditions is significant because it demonstrates that anaerobic glycolysis is insufficient to sustain cell growth in the absence of mitochondrial respiration. The growth pattern of \(\rho^0\) cells under
aerobic conditions over 5 days was similar to that of parental cells, with cell numbers reaching plateau levels and declining within the same timeframe as parental cells, albeit at lower cell densities. This might be related to their increased ability to acidify the culture medium via lactic acid production and proton transport mechanisms.

Oxygen-dependent growth of HL60p0 cells implies that oxygen is utilized to support the bioenergetic requirements of cell growth by a mechanism that does not depend on mitochondrial cytochrome oxidase. We showed that HL60p0 cells consume oxygen with an initial rate of up to 30% that of parental cells, although this rate declined with time. This non-linear pattern of oxygen consumption could be explained by increased acidification of the culture medium that we have observed with p0 cells. Previously, oxygen consumption rates of 10–34% those of parental cells have been reported for some human p0 cell lines [1–3,29]. Fukuyama et al. [6] failed to detect oxygen consumption by human neuroblastoma p0 cells, while others have measured p0 oxygen consumption rates that were less than 5% those of parental cells with HepG2, 143B osteosarcoma, HeLa S3 cervical carcinoma and neuroblastoma-derived SILA cells [4,5]. In these latter cases, oxygen consumption appears to have been measured in the absence of glucose as an energy source, possibly explaining failure to detect significant rates of oxygen consumption. We have observed that glucose deprivation, in the absence of other energy sources, results in severe inhibition (>90%) of oxygen consumption in both HL60 and HL60p0 cells (results not shown).

Further investigation of the properties of non-mitochondrial oxygen consumption by HL60p0 cells support earlier findings of resistance to mitochondrial respiratory poisons. FCCP, an uncoupler of oxidative phosphorylation stimulated mitochondrial oxygen consumption but had no significant effect on oxygen consumption by p0 cells. These results are consistent with a previous study where CCCP caused a two-fold increase in mitochondrial oxygen consumption by MOLT-4 cells, but had no effect on its p0 counterpart [3]. Inhibitors that interfered with glucose uptake and glycolytic metabolism partially inhibited oxygen consumption by both p0 and parental cells indicating a common requirement for glycolysis in both pathways. A role for membrane ubiquinones in non-mitochondrial oxygen consumption was demonstrated by sensitivity of p0 cell oxygen consumption to capsaicin and resiniferatoxin, which are known to inhibit ubiquinone redox cycling [30]. The level of inhibition was similar to that observed for WST-1/PMS reduction by both p0 and wild-type cells. Vanillloid ubiquinone analogues have previously been shown to inhibit tPMT systems [20,22,27,31–39]. The NQO1 inhibitor and uncoupler, dicoumarol [40], inhibited p0 oxygen consumption and WST-1/PMS reduction in both p0 and parental cells to a similar extent, but increased mitochondrial oxygen consumption as expected. These results indicate the possible involvement of NQO1 in non-mitochondrial oxygen consumption and this is further supported by the increased sensitivity of non-mitochondrial oxygen consumption to the flavin-centre inhibitor, DPI.

Contrary to expectations that reduced pyridine dinucleotides would promote oxygen consumption by p0 cells via a surface oxidoreductase [23], NADH and NADPH extensively inhibited non-mitochondrial oxygen consumption, whereas mitochondrial oxygen consumption remained unaffected. Because reduced pyridine dinucleotides are not transported across the plasma membrane, non-mitochondrial oxygen consumption is therefore localized at the cell surface. NAD(P)H inhibition of oxygen consumption at the cell surface of p0 cells could be explained by their ability to bind to the surface NADH-oxidase responsible for WST-1 reduction, resulting in intracellular electrons being directed to membrane ubiquinones instead of oxygen.

Similar inhibition profiles of oxygen consumption and WST-1/PMS reduction by HL60p0 cells imply that both systems use the same tPMT pathway. Further support for this view came from the observation that oxygen and PMS compete for reducing electrons from the tPMT system.

Although oxygen is the physiological electron acceptor for tPMT, other extracellular electron sinks are likely, particularly in the hypoxic environment found in solid tumours, which is often associated with reduced pH. These conditions do not appear to favour cell surface oxygen consumption, raising the question of its physiological significance. Nevertheless, evidence that tPMT in p0 cells may support glycolytic metabolism has been presented previously in studies where ferricyanide promoted growth of p0 cells in the absence of pyruvate [1]. We postulate that cell surface oxygen consumption supports glycolytic metabolism in mammalian cells under normoxic conditions by oxidising excess cytosolic NADH, redressing the intracellular NADH/NAD+ imbalance. The extent to which this process occurs in health and disease has been difficult to assess. Most of the early literature employed animal ascites models but more recently, several cell culture models of aerobic glycolysis have emerged, including the p0 model, non-adherent HeLa S3 cells and activated rat thymocytes [12]. It is worth noting that the ability to reduce oxygen at the cell surface to support glycolytic metabolism may be a fingerprint of the early endosymbiotic origin of eukaryotic cells.

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