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The contributions of respiration and glycolysis to extracellular acid production

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ABSTRACT

Background: The rate at which cells acidify the extracellular medium is frequently used to report glycolytic rate, with the implicit assumption that conversion of uncharged glucose or glycogen to lactate$^-$ + H^+ is the only significant source of acidification. However, another potential source of extracellular protons is the production of CO_2 during substrate oxidation: CO_2 is hydrated to H_2CO_3, which then dissociates to HCO_3^- + H^+.
Methods: O_2 consumption and pH were monitored in a popular platform for measuring extracellular acidification (the Seahorse XF Analyzer).
Results: We found that CO_2 produced during respiration caused almost stoichiometric release of H^+ into the medium. With C2C12 myoblasts given glucose, respiration-derived CO_2 contributed 34% of the total extracellular acidification. When glucose was omitted or replaced by palmitate or pyruvate, this value was 67–100%. Analysis of primary cells, cancer cell lines, stem cell lines, and isolated synaptosomes revealed contributions of CO_2-produced acidification that were usually substantial, ranging from 3% to 100% of the total acidification rate.
Conclusion: Measurement of glycolytic rate using extracellular acidification requires differentiation between respiratory and glycolytic acid production.
General significance: The data presented here demonstrate the importance of this correction when extracellular acidification is used for quantitative measurement of glycolytic flux to lactate. We describe a simple way to correct the measured extracellular acidification rate for respiratory acid production, using simultaneous measurement of oxygen consumption rate.
Summary statement: Extracellular acidification is often assumed to result solely from glycolytic lactate production, but respiratory CO_2 also contributes. We demonstrate that extracellular acidification by myoblasts given glucose is 66% glycolytic and 34% respiratory and describe a method to differentiate these sources.

1. Introduction

Assessing metabolic flux is essential to understanding cellular metabolism, and the measurement of oxygen consumption and extracellular acidification by adherent cells is now routine using instruments such as the Seahorse XF Analyzer [1–4]. The rate of mitochondrial oxygen consumption is reliably calculated as total cellular oxygen consumption rate minus any oxygen consumption that is insensitive to specific inhibitors of mitochondrial electron transport [5–7]. In small wells in polystyrene plates, the oxygen diffusion between the wells, the polystyrene, and the atmosphere is considerable and must be accounted for [4]. With these constraints, oxygen consumption rate is a direct and quantitative measure of mitochondrial electron transport rate.

Extracellular acidification is an equally accessible measurement of metabolic activity. A major component of extracellular acidification is the glycolytic production of lactate [8]. At physiological pH around 7, glucose is uncharged, whereas lactate (pK_a 3.86) exists primarily as the carboxylate anion. Net conversion of glucose to lactate$^-$ at neutral pH necessarily releases protons and acidifies the medium, so extracellular acidification rate is commonly used as a direct and quantitative measure of glycolytic rate [1,2,8–11]. However, the relationship between extracellular acidification and glycolytic rate is confounded by other acidification mechanisms. Specifically, CO_2 generated in the tricarboxylic acid cycle can be spontaneously or enzymatically hydrated to carbonic acid, H_2CO_3, which dissociates to HCO_3^- + H^+ in aqueous media at physiological pH. Conversion of one glucose molecule to lactate yields 2 lactate$^-$ + 2 H^+, whereas complete oxidation of one glucose to CO_2 yields 6 HCO_3^- + 6 H^+, so the extracellular acidification when a glucose molecule is oxidized to CO_2 is three times greater than the extracellular acidification when it is converted to lactate.

Abbreviations: RQ, respiratory quotient (CO_2 produced/O_2 consumed); OCR, oxygen consumption rate; ECAR, extracellular acidification rate; PPR, proton production rate; FCCP, carbonylcyanide 4-(trifluoromethoxy)-phenylhydrazone; KRPH, Krebs–Ringer phosphate HEPES medium

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Here, we show that CO_2 produced by respiration in the wells of a polystyrene multiwell plate is completely retained in the wells, causing almost stoichiometric acidification. The acidification can be calculated from the rate of respiration, the respiratory quotient, the net H^+ production per CO_2 generated, and the pH buffering power of the medium. Analysis of extracellular acidification by C2C12 myoblasts reveals that CO_2 production accounts for most or all of the extracellular acidification by cells given no substrate or with added pyruvate or palmitate, and 34% of the total extracellular acidification by cells oxidizing added glucose. In other cell lines, CO_2 also contributes substantially to extracellular acidification, even in many of those reported to be highly glycolytic.

2. Materials and methods

2.1. Reagents

Chemicals were from Sigma. Cell culture reagents and consumables were from Corning. Seahorse XF consumables were from Seahorse Bioscience.

2.2. Mitochondria

Rat skeletal muscle mitochondria were isolated according to [12]. Rats were used in accordance with IACUC standards under a protocol approved by the Buck Institute Animal Care and Use Committee. Rates of oxygen consumption and acidification were measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience) [13] in MAS-1 medium (2 mM HEPES, 10 mM KH_2PO_4, 1 mM EGTA, 70 mM sucrose, 220 mM mannitol, 5 mM $MgCl_2$, 0.2% fatty-acid-free bovine serum albumin (Sigma A3803)) containing 4 μM carbonylcyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP), 2.5 μg/mL oligomycin, 5 mM dichloroacetate, 5 mM aminooxyacetate, 5 mM fluoroacetate, and 2 μM atpenin A5. Oxygen consumption was corrected for rotenone-insensitive rates. Extramitochondrial acidification rate was corrected for background by subtraction of rates in sample-free wells.

2.3. Cells

Mouse C2C12 myoblasts [14] were cultured in Dulbecco's modified Eagle medium (DMEM) with 11.1 mM glucose, 2 mM glutamine, 10% v/v fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Twenty-four hours prior to assay, cells were plated in 100 μL culture medium at 20,000 cells/well in a 24-well polystyrene Seahorse V7-PS Flux plate with no additional coating. 25 min prior to assay, cells were washed three times with and then incubated in a final volume of 500 μL Krebs–Ringer phosphate HEPES (KRPH) medium (2 mM HEPES, 136 mM NaCl, 2 mM NaH_2PO_4, 3.7 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.1% w/v fatty-acid-free bovine serum albumin, pH 7.4 at 37 °C). At assay start, medium was replaced with fresh KRPH containing 500 U/mL carbonic anhydrase (Sigma C2624) and either pyruvate (10 mM), glucose (10 mM), carnitine and palmitate (0.5 mM each), or no substrate. Cell respiratory control was assayed by stepwise addition of A: 2 μg/mL oligomycin; B: 0.5 μM FCCP; C: 1 μM rotenone, 1 μM myxothiazol, 5 mM 2-deoxyglucose; D: HCl. Two measurement cycles of 2 min mix, 1 min wait, and 5 min measure were carried out after each addition, with three cycles following the final addition. Following the assay, samples were removed for lactate analysis, and wells were washed three times with 250 μL BSA-free KRPH. Twenty-five microliters of RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EGTA, 1 mM EDTA, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 0.1% v/v SDS, pH 7.4 at 22 °C) was added. Cells were incubated on ice for 30 min and agitated on a plate shaker at 1200 rpm for 5 min. Protein concentration was measured by BCA assay. Protein content was ~4 μg/well.

ZR75 mammary ductal carcinoma [15] and SJSA-1 osteosarcoma [16] cells were cultured in RPMI 1640 with 10% v/v FBS; MCF7 mammary adenocarcinoma cells [17] were cultured in low-glucose (5.5 mM) DMEM with 10% v/v FBS. All lines were assayed in a minimal TES buffer containing 3.5 mM KCl, 120 mM NaCl, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 1.2 mM Na_2SO_4, 2 mM $MgCl_2$, 15 mM glucose, 20 mM TES, and 0.3% w/v BSA at pH 7.4 at 37 °C. I6 embryonic stem cells and neural stem cells were cultured and assayed as described [18]. Primary rat cortical neurons prepared as in [19] were assayed in minimal TES as above modified with 1 mM $MgCl_2$ and 0.4% w/v BSA. Dispersed rat islets prepared as in [20] were assayed in RPMI medium devoid of phenol red, riboflavin and folic acid, containing 3 mM glucose, 2 mM glutamine, 5 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM TES, and 1% v/v FBS, pH 7.4 at 37 °C. INS1E pancreatic insulinoma cells [21] were cultured in RPMI 1640 with 11 mM glucose, 10% v/v FBS, 10 mM HEPES, 2 mM glutamine, 50 μM β-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C, 5% CO_2, and assayed in minimal TES as above modified with 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, 5 mM $NaHCO_3$, 10 mM Na-TES, 2 mM glucose, and 2 mM glutamine. Mouse cortical synaptosomes were isolated and assayed as in [22].

2.4. Lactate measurement

End point lactate concentration was determined in a 96-well plate by measuring the initial velocity (2 min) of $NAD^+ \rightarrow NADH$ by lactate dehydrogenase. Immediately following the extracellular flux assay, 100 μL of assay medium was diluted 1:1 with 2× hydrazine buffer (1× composition 500 mM Tris, 10 mM EDTA, 200 mM hydrazine, 2 mM NAD^+, 20 U/mL lactate dehydrogenase, pH 9.8 at 22 °C). NADH fluorescence at 340 nm excitation/460 nm emission was monitored in a BMG Pherastar FS microplate reader and calibrated against known lactate concentrations from 0 to 50 μM. Lactate in glucose-containing wells was ~40 μM.

2.5. Calculations

The respiratory quotient (RQ; CO_2 produced/O_2 consumed) for complete or partial oxidation of a substrate is reaction pathway-independent and can be calculated from the relevant balanced equation. Similarly, the maximum H^+ released per O_2 consumed by respiration can be calculated (if all of the CO_2 generates HCO_3^- in the well, see Results):

For conversion of pyruvate$^-$ plus malate^{-2} entirely to citrate^{-3} (Fig. 1A):

$$C_3H_3O_3^- + C_4H_4O_5^{-2} + O_2 \rightarrow C_6H_5O_7^{-3} + CO_2 + H_2O \quad RQ = 1/1 = 1.00$$
$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \quad \max H^+/O_2 = 1/1 = 1.00$$

For complete oxidation of glucose

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad RQ = 6/6 = 1.00$$
$$6CO_2 + 6H_2O \rightarrow 6HCO_3^- + 6H^+ \quad \max H^+/O_2 = 6/6 = 1.00$$

For complete oxidation of glycogen

$$[C_{24}H_{42}O_{21}]n + 24nO_2 \rightarrow 24nCO_2 + 21nH_2O \quad RQ = 24n/24n = 1.00$$
$$24nCO_2 + 24nH_2O \rightarrow 24nHCO_3^- + 24nH^+ \quad \max H^+/O_2 = 24n/24n = 1.00$$

For complete oxidation of pyruvate$^-$

$$C_3H_3O_3^- + H^+ + 2.5O_2 \rightarrow 3CO_2 + 2H_2O \quad RQ = 3/2.5 = 1.20$$
$$3CO_2 + 3H_2O \rightarrow 3HCO_3^- + 3H^+ \quad \max H^+/O_2 = (3–1)/2.5 = 0.80$$

For complete oxidation of palmitate$^-$

$$C_{16}H_{31}O_2^- + H^+ + 23O_2 \rightarrow 16CO_2 + 16H_2O \quad RQ = 16/23 = 0.70$$
$$16CO_2 + 16H_2O \rightarrow 16HCO_3^- + 16H^+ \quad \max H^+/O_2 = (16–1)/23 = 0.65$$