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Circumventing the Crabtree Effect: A method to induce lactate consumption and increase oxidative phosphorylation in cell culture

Alexandra I. Mot[a], Jeffrey R. Liddell[a], Anthony R. White[a,b], Peter J. Crouch[a,b,*]

[a] Department of Pathology, University of Melbourne, Australia
[b] Florey Institute of Neuroscience and Mental Health, University of Melbourne, Australia

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ABSTRACT

Most cells grown in glucose-containing medium generate almost all their ATP via glycolysis despite abundant oxygen supply and functional mitochondria, a phenomenon known as the Crabtree effect. By contrast, most cells within the body rely on mitochondrial oxidative phosphorylation (OXPHOS) to generate the bulk of their energy supply. Thus, when utilising the accessibility of cell culture to elucidate fundamental elements of mitochondria in health and disease, it is advantageous to adopt culture conditions under which the cells have greater reliance upon OXPHOS for the supply of their energy needs. Substituting galactose for glucose in the culture medium can provide these conditions, but additional benefit can be gained from alternate *in vitro* models. Herein we describe culture conditions in which complete autonomous depletion of medium glucose induces a lactate-consuming phase marked by increased Mito-Tracker Deep Red staining intensity, increased expression of Kreb's cycle proteins, increased expression of electron transport chain subunits, and increased sensitivity to the OXPHOS inhibitor rotenone. We propose these culture conditions represent an alternate accessible model for the *in vitro* study of cellular processes and diseases involving the mitochondrion without limitations incurred via the Crabtree effect.

1. Introduction

Glycolysis is an inefficient mechanism for generating ATP relative to mitochondrial oxidative phosphorylation (OXPHOS) (Lunt and Vander Heiden, 2011), and most cells within the body therefore rely on OXPHOS to meet the bulk of their energy requirements. However, the presence of glucose in commonly used cell culture conditions favours glycolysis over OXPHOS due to allosteric modulation of glycolytic enzymes by glucose (Rodriguez-Enriquez et al., 2001) and the binding of hexokinase to mitochondrial porin which accelerates glycolysis (Golshani-Hebroni and Bessman, 1997). As a consequence, most cells grown in glucose-containing medium generate almost all their ATP via glycolysis despite abundant oxygen supply and the presence of functional mitochondria, a phenomenon known as the Crabtree effect (Crabtree, 1929). This phenomenon is not restricted to cells cultured in high glucose-containing medium (~20 mM) but also occurs in cells cultured in low glucose-containing (~5 mM) medium (Rodriguez-Enriquez et al., 2001).

To enhance extrapolation from cell culture experiments to *in vivo* conditions pertaining to energetically demanding cells and/or conditions arising from decreased mitochondrial function, it is therefore useful to adapt the cell culture environment such that cells are less glycolytic and more dependent on OXPHOS. One way of achieving this is by substituting glucose in the culture medium with galactose, usually at concentrations of 5–10 mM (Aguer et al., 2011; Allen et al., 2014; Bird et al., 2014; Dott et al., 2014; Marroquin et al., 2007; Robinson et al., 1992; Warburg et al., 1967). The slow oxidation of galactose to pyruvate via glycolysis forces cells to rely on mitochondrial OXPHOS to generate sufficient ATP for survival (Dott et al., 2014). Other carbohydrates including fructose and maltose have been used to replace medium glucose for the purpose of stabilising medium pH (Imamura et al., 1982) but these models have not been widely utilised.

To date the galactose model is the most widely used way to circumvent the Crabtree effect. However, limitations of this model are

Abbreviations: AMPK, α5′-adenosine monophosphate-activated protein kinase alpha; ATP5A, ATP synthase subunit alpha; BCA, bicinchoninic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; COX5B, cytochrome c oxidase subunit 5B; 2-DG, 2-deoxyglucose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OXPHOS, oxidative phosphorylation; PCNA, proliferating cell nuclear antigen; pMEFs, primary mouse embryonic fibroblasts; SDHB, succinate dehydrogenase subunit B; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; TOM20, mitochondrial import receptor subunit TOM20 homolog.

* Corresponding author at: Department of Pathology, University of Melbourne, 3010, Melbourne, Australia.
E-mail address: pjcrouch@unimelb.edu.au (P.J. Crouch).

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associated with increased cellular metabolic stress and an accelerated ageing phenotype (Aguer et al., 2011; Barker et al., 1999; Choi et al., 2013; Coban et al., 2014; Li et al., 2014) and the fact that some cell types are unable to utilise galactose (Elkalaf et al., 2013). Additional benefit can therefore be gained from alternate approaches to overcome limitations associated with the Crabtree effect. Herein we describe culture conditions in which complete autonomous depletion of medium glucose forces cells to utilise lactate via mitochondrial OXPHOS to supply their energy needs. These conditions represent an alternate accessible model for the *in vitro* study of cellular processes and diseases involving the mitochondrion without limitations incurred via the Crabtree effect.

2. Materials and methods

2.1. Materials

Cell culture reagents were from Life Technologies and chemicals were from Sigma-Aldrich unless otherwise specified.

2.2. Cell culture conditions

Primary mouse embryonic fibroblasts (pMEFs) were obtained from E14 C57BL/6 mice as previously described (Garfield, 2010). At passage 1 cells were frozen and stored in liquid nitrogen. For each experiment cells were thawed then allowed to reach confluency, then passaged and seeded into 24-well plates (Nunc) at 1×10^4 cells/cm^2 in DMEM containing 3.5 mM glucose and supplemented with 10% (v/v) foetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 mM L-glutamine, and 9.7 mM mannitol (Ajax Finechem). Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2.

Primary adult human fibroblasts were obtained from superficial skin biopsies of the right inner forearm of a healthy person as previously reported (Solski et al., 2012). All culture conditions were as described above for the pMEFs.

Primary mouse brain astrocytes were obtained from new born C57BL/6 pups as previously described (Hare et al., 2013). Cells were seeded into 48-well plates at 15×10^4 cells/cm^2 and allowed to reach maturity until day 15. Experiments were commenced on day 15 (thereafter referred to as experimental day 0) when the medium was replaced with DMEM containing 5.0 mM glucose and supplemented with 10% (v/v) foetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cultures were maintained at 37 °C in a humidified incubator with 10% CO_2.

These three cell types were chosen based on their versatility to manipulations in culture conditions. For all cell types the volume of media used was equivalent to 20 mL per T75 flask (266 μL/cm^2). All mouse work was approved by the University of Melbourne Biosciences Animal Ethics Committee. All human cell work was approved by the University of Melbourne Biosciences Human Ethics Committee.

2.3. Lactate and glucose content of cell culture medium

Lactate and glucose levels in the cell culture medium were determined using methods based on those previously described (Liddell et al., 2009). Aliquots of medium were collected from the cell cultures at the start of the culture period (day 0) then further aliquots collected once daily for 7 days (pMEFs) or 13 days (human fibroblasts). For astrocyte cultures medium aliquots were collected on days 0, 0.25, 1, and 5. Modifications made to the protocols described by Liddell et al. (2009) involved the use of glucose and lactate standard curves instead of extinction coefficients.

2.4. BCA assay for quantification of protein content

Total protein levels within each well of the culture plates were measured at each medium collection time point (once daily, on days 0–7) for the pMEFs. For all cell types protein levels were measured at the completion of all 2-deoxyglucose (2-DG) or rotenone treatments. Results are expressed relative to vehicle control treated cells to account for differences in cell confluency between glucose-consuming and lactate-consuming phases. Following medium collection, cells were washed twice with chilled PBS. Protein was solubilised using 300 μL per well of 100 mM NaOH and incubated on an orbital shaker at room temperature for 2 h. Protein concentration was measured using a Bicinchoninic Acid (BCA) Assay Kit (Thermo Scientific) by comparison to a bovine serum albumin standard curve, as per manufacturer instructions. While protein content is influenced by a multitude of factors, robust changes in total cellular protein are a good indicator of both cell proliferation and cell viability (Engelhard et al., 1991; Vichai and Kirtikara, 2006).

2.5. Phase contrast microscopy

Phase contrast microscopy was used to monitor changes in pMEF confluency and morphology daily during the experiment (days 0–7). Representative images of the cells were taken using a light microscope and a Canon Powershot A2300 digital camera on day 1 (glucose-consuming phase) and day 5 (lactate-consuming phase).

2.6. Western blotting for protein analyses

pMEFs grown in T75 flasks (Nunc) were pelleted (720 RCF, 3 min) and lysed in PhosphoSafe Extraction Buffer (Merck) supplemented with 1 mM phenylmethyl sulphonyl fluoride (PMSF) and 1.6 mM deoxyribonuclease I from bovine pancreas (DNase I, Roche). Following centrifugation (15,000 RCF, 3 min), lysates at equal protein concentration were mixed with 4 x gel loading buffer [250 mM Tris, 20% (v/v) glycerol, 8% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue] then heated at 95 °C for 5 min. Denatured and reduced proteins were separated on 4–12% Bis Tris gels (NuPage) at 200 V for 42 min. Resolved proteins were transferred onto PVDF membranes at 20 V for 7 min using the iBlot transfer system (Invitrogen). PVDF membranes were blocked with PBST [PBS supplemented with 0.05% (v/v) Tween-20] containing 4% (w/v) skim milk powder. Membranes were incubated with primary antibody overnight at 4 °C then secondary antibody for 2 h at room temperature before visualising chemiluminescence of protein bands using ECL Advance (GE Healthcare) and a MicroChemi imager (DNR Bio-Imaging Systems). Primary antibodies to proliferating cell nuclear antigen (Cell Signaling #2586, 1:1500), citrate synthase (Abcam #ab96600, 1:800), cytochrome c oxidase subunit 5 B (Abcam #ab180136, 1:600), ATP synthase subunit alpha (Abcam #ab176569, 1:600), succinate dehydrogenase subunit B (Abcam #ab175225, 1:600), mitochondrial import receptor subunit TOM20 homolog (Protein Tech #11802-1-AP, 1:600), superoxide dismutase 2 (Abcam #ab13533, 1:5000), superoxide dismutase 1 (Abnova #PAB0725, 1:2000), phospho(Thr172)-5′-adenosine monophosphate-activated protein kinase alpha (Cell Signaling #2535, 1:400), 5′-adenosine monophosphate-activated protein kinase alpha (Cell Signaling #2532, 1:600), and glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling #2118, 1:5000) were used. HRP-linked anti-rabbit IgG (Cell Signaling #7074, 1:5000) and HRP-linked anti-mouse IgG (Cell Signaling #7076, 1:5000) secondary antibodies were used. Relative abundance of proteins was determined using ImageJ 1.38 × software.