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Availability of the key metabolic substrates dictates the respiratory response of cancer cells to the mitochondrial uncoupling

Alexander V. Zhdanov [a,*], Alicia H.C. Waters [a], Anna V. Golubeva [b], Ruslan I. Dmitriev [a], Dmitri B. Papkovsky [a]

[a] Biochemistry Department, University College Cork, Cavanagh Pharmacy Building, College Road, Cork, Ireland
[b] Alimentary Pharmabiotic Centre, University College Cork, Bioscience Institute, Western Road, Cork, Ireland

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ABSTRACT

Active glycolysis and glutaminolysis provide bioenergetic stability of cancer cells in physiological conditions. Under hypoxia, metabolic and mitochondrial disorders, or pharmacological treatment, a deficit of key metabolic substrates may become life-threatening to cancer cells. We analysed the effects of mitochondrial uncoupling by FCCP on the respiration of cells fed by different combinations of Glc, Gal, Gln and Pyr. In cancer PC12 and HCT116 cells, a large increase in O_2 consumption rate (OCR) upon uncoupling was only seen when Gln was combined with either Glc or Pyr. Inhibition of glutaminolysis with BPTES abolished this effect. Despite the key role of Gln, addition of FCCP inhibited respiration and induced apoptosis in cells supplied with Gln alone or Gal/Gln. For all substrate combinations, amplitude of respiratory responses to FCCP did not correlate with Akt, Erk and AMPK phosphorylation, cellular ATP, and resting OCR, mitochondrial Ca^{2+} or membrane potential. However, we propose that proton motive force could modulate respiratory response to FCCP by regulating mitochondrial transport of Gln and Pyr, which decreases upon mitochondrial depolarisation. As a result, an increase in respiration upon uncoupling is abolished in cells, deprived of Gln or Pyr (Glc). Unlike PC12 or HCT116 cells, mouse embryonic fibroblasts were capable of generating pronounced response to FCCP when deprived of Gln, thus exhibiting lower dependence on glutaminolysis. Overall, the differential regulation of the respiratory response to FCCP by metabolic environment suggests that mitochondrial uncoupling has a potential for substrate-specific inhibition of cell function, and can be explored for selective cancer treatment.

1. Introduction

A role of the mitochondria is that of a 'power plant' of the eukaryotic cell. The electron transport chain (ETC) conducts a cascade of RedOx reactions and generates proton motive force (PMF) which is utilised by F0F1 ATP synthase (complex V) to produce ATP through the oxidative phosphorylation (OxPhos). Being the most effective way of ATP production, OxPhos is tightly regulated. The efficiency of OxPhos, defined by the amount of inorganic phosphate (P*i*) utilised for ATP production per amount of O_2 consumed [1,2], may be affected by a number of factors, including the level of uncoupling between inward mitochondrial H^+ current and ATP synthesis. Indeed, a certain proportion of H^+ is always translocated to inside the matrix bypassing complex V, thus degrading the mitochondrial membrane potential ($\Delta\Psi m$). This so called 'extrinsic' uncoupling can be achieved through the activation of uncoupling proteins, a nonspecific $\Delta\Psi m$-dependent proton leak, general ion symport/antiport and chemical uncouplers [3]. The weak acid protonophore FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) [4], which provides reversible uncoupling [5] and dissipates PMF in a concentration-dependent manner, [6,7] is commonly used in the experiments with isolated mitochondria and whole cells.

Isolated mitochondria are a simple and well-established model as they are accessible to the metabolic substrates and pharmacological compounds, and independent on complex inter-compartmental transport of biomolecules, cytoplasmic metabolism and ion fluxes [8]. The substrates feeding the Krebs cycle and complexes I–V strongly affect respiration of isolated mitochondria [1,9]. Different combinations of the substrates and co-factors are used for analysis of i) the functional activity of mitochondrial enzymes and contribution of ETC complexes to the total mitochondrial respiration; ii) H^+/O, H^+/ATP and P*i*/O ratios; iii) the state III (and state IV) respiration and respiratory control ratio;

Abbreviations: Akt, protein kinase B (PKB); α-KG, α-ketoglutarate; AMPK, AMP-activated protein kinase; BPTES, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide; $\Delta\Psi m$, mitochondrial membrane potential; $\Delta\Psi p$, plasma membrane potential; ΔpH, mitochondrial proton gradient; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulphoxide; ECA, extracellular acidification; Erk, mitogen-activated protein kinase (MAPK); ETC, electron transport chain; FBS, fetal bovine serum; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Gal, D-galactose; Glc, D-glucose; Gln, L-glutamine; GLS1, kidney-type glutaminase; Glu, glutamate; GLUT, glucose transporter; GSH, glutathione; HS, horse serum; iO_2, intracellular oxygen; MEFs, mouse embryonic fibroblasts; NGF, nerve growth factor; OCR, oxygen consumption rate; OxPhos, oxidative phosphorylation; PMF, proton motive force; PMPI, plasma membrane potential indicator; Pyr, pyruvate; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; TMRM, tetramethyl rhodamine methyl ester; P*i*, inorganic phosphate; WM, working media

* Corresponding author at: Cavanagh Pharmacy Building, College Road, Cork, Ireland. Tel.: +353 21 4901699; fax: +353 21 4901698.
E-mail address: a.zhdanov@ucc.ie (A.V. Zhdanov).

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and iv) mito-toxicity of the new pharmacological compounds. The O_2 consumption rate (OCR) in isolated mitochondria may be set to different levels by simple addition of the appropriate substrates and drugs affecting respiration. Thus, maximal mitochondrial respiration can be achieved by addition of FCCP to the mitochondria in state III [8].

However, the processes observed in isolated mitochondria may be significantly different from that taking place in intact cells, which have undisturbed cellular networks and environment and represent a more physiologically relevant model for experiments on bioenergetics and metabolism [8]. Mitochondrial respiration in cells is regulated by many factors and the results of uncoupling under physiological conditions are not easy to interpret. Changes in the transport of metabolites and ions across the plasma membrane, a decrease in mitochondrial and increase in cytosolic Ca^{2+}, activation of glycolysis, cytosolic and extracellular acidification, can all strongly affect respiratory responses to FCCP, and even careful optimisation of FCCP concentration for each cell type [8] may not eliminate indirect effects of this drug on cellular respiration and function in general. Thus, dissipation of the mitochondrial ATP flux upon uncoupling can rapidly activate 'non-mitochondrial' metabolic pathways involved in production and preservation of energy (e.g. AMPK) [10]. Therefore, shortages in basic metabolic substrates can contribute to cellular responses to mitochondrial uncoupling and escalate energy stress. Thus, uncoupling becomes life-threatening when glucose (Glc) is replaced with galactose (Gal), as glycolysis can no longer maintain steady ATP levels.

In cancer cells Glc supply becomes essential, since glycolysis produces large amounts of ATP regardless of high availability of O_2 (Warburg effect) [11]. In turn, most of the pyruvate (Pyr), instead of conversion into Acetyl-CoA and utilisation in the Krebs cycle [12], is converted to lactate and extruded from the cell. Some intermediates of glycolysis (e.g. phosphoenolpyruvate) are also re-directed to anabolic reactions producing materials for actively proliferating cancer cells [13–15]. To further accelerate anaplerotic reactions and ATP production, cancer cells addictively utilise glutamine (Gln), and more than half of ATP can be produced through Gln-driven OxPhos [16–19]. As a result, Gln-driven mitochondrial respiration in many cancer cells is active even at high Glc levels [20], and increases further upon replacement of Glc with Gal.

Considering the complexity of the bioenergetic network, one can anticipate that respiration of resting cells supplied with different substrates may not inform correctly on their ability to respond to mitochondrial uncoupling in a classical way, i.e. by prominent and sustained increase in respiration. Here, using rat pheochromocytoma PC12 cell and other cell lines, we studied how the availability and utilisation of major metabolic substrates modulate the respiratory response of cancer cells to mitochondrial uncoupling.

2. Experimental procedures

2.1. Materials

O_2-sensitive probes MitoXpress®-Xtra [21], MitoXpress®-Intra NanO2 [22] and pH-sensitive probe pH-Xtra [23] were from Luxcel Biosciences (Cork, Ireland). Glutaminase inhibitor, BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide) [24] was kindly provided by Dr. Takashi Tsukamoto (John Hopkins University, MD). Mitochondrial membrane potential indicator Tetramethyl rhodamine methyl ester (TMRM), Lipofectamine 2000 and Opti-MEM I were from Invitrogen Life Technologies (Carlsbad, CA). Plasma membrane potential indicator (PMPI) [25] was from Molecular Devices (Sunnyvale, CA). ECL Prime Western blotting reagent was from GE Healthcare Life Sciences (Waukesha, WI), pre-made acrylamide gels, running and transfer buffers were from GeneScript (Piscataway, NJ), BCA™ Protein Assay kit was from Thermo Fisher Scientific (Rockford, Ill). The mitochondria-targeted Ca^{2+} biosensor, $_{mito}$Case12 [26] was from Evrogen JSC (Moscow, Russia). CellTiter-Glo® ATP Assay was from Promega (Madison, WI). Mineral oil (type 37) was from Cargille Laboratories (Cedar Grove, NJ). Dulbecco's Modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) media, nerve growth factor (NGF), collagen IV, FCCP, D-glucose, D-galactose, L-glutamine, sodium pyruvate and other reagents were from Sigma-Aldrich.

2.2. Composition of the media and experimental conditions

Rat pheochromocytoma PC12 cells, human colon cancer HCT116 cells and mouse embryonic fibroblasts (MEFs) were from American Tissue Culture Collections (ATCC, Manassas, VA). PC12 cells were maintained in suspension in RPMI 1640 medium supplemented with 10 mM HEPES (pH 7.2), 2 mM L-Gln, 10% horse serum (HS), 5% fetal bovine serum (FBS), 100 U/ml penicillin/100 μg/ml streptomycin (P/S) in humidified atmosphere of 5% CO_2 and 95% air at 37 °C. HCT116 and MEFs were maintained in the same conditions in DMEM medium supplemented with HEPES, L-Gln, 10% FBS and P/S.

PC12 cells were differentiated as described previously [26]. Briefly, for experiments with OCR, ECA and iO_2, cells were seeded at 5×10^4 cells/well on 96-well plates (Greiner Bio One, Frickenhausen, Germany) coated with 0.01% collagen IV, and differentiated for 3–5 days in RPMI supplemented with $NaHCO_3$, L-Gln, 1% horse serum, P/S, and 100 ng/ml NGF. For live cell confocal imaging cells were seeded at 2.5×10^4 cells per ~1 cm^2 dish differentiated on glass bottom mini-dishes (MatTek, Ashland, MA) coated with a mixture of collagen IV (0.007%) and poly-D-lysine (0.003%). For protein analysis cells were seeded at 5×10^5 cells per well and differentiated for 5 days on 12-well plates (Corning Life Sciences, NY) coated with collagen IV.

HCT116 and MEFs were seeded in a growing medium at 2.5×10^4 cells/well on 96-well plates (Greiner) coated with 0.01% collagen IV, grown for 2 days prior to analysis.

Working media (WM) were prepared as follows. Powder DMEM (Sigma, cat. No 5030) was reconstituted in deionised water and filter-sterilised. From this plain DMEM, 12 different WM were composed by addition of 100 nM NGF, 10 mM Glc, 10 mM Gal, 2 mM Gln and 1 mM Pyr as shown in Table 1. No serum was added. All WM contained 20 mM HEPES, pH 7.2, except for ECA measurements.

Prior to the experiments, growth or differentiation media were replaced with one of the WM, and the cells were incubated in 5% CO_2 at 37 °C for 2 h. To inhibit glutaminolysis, BPTES (10 μM) was applied to the samples 1 h prior to and kept during the experiments. To uncouple respiration, cells were treated with 1 μM FCCP, optimal for all cell lines, as determined in separate experiment (Supplemental Fig. S1).

2.3. O_2 consumption rate (OCR) assay

Measurement of OCR and iO_2 (see Section 2.5) was performed using a well-established phosphorescence quenching technique [27,28]. Developed for the assessment of O_2 consumption by biological specimens on a conventional fluorescence spectrometer or plate reader [21], a water-soluble phosphorescent O_2-sensitive probe MitoXpress®-Xtra was validated [23,29,30] and used in a number of studies [31–36]. These works demonstrate that the phosphorescence quenching is a

Table 1
Composition of the WM.

Components	WM											
	1	2	3	4	5	6	7	8	9	10	11	12
D-glucose	+	+	+	+								
D-galactose					+	+	+	+				
L-glutamine	+	+			+	+			+	+		
Pyruvate		+	+			+	+			+	+	
NGF[a]	+	+	+	+	+	+	+	+	+	+	+	+

[a] Only for PC12 cells.