MULTI-PARAMETRIC METABOLIC ASSESSMENT OF CELLS UNDER STRESS CONDITIONS

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DECLARATION

This thesis has not been submitted in whole or part to this or any other university for any degree, and is, unless otherwise stated, the original work of the author.

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ABSTRACT

Glycolysis, glutaminolysis, the Krebs cycle and oxidative phosphorylation are the main pathways for cell metabolism. Exposing cells to key metabolic substrates (glucose, glutamine and pyruvate 12 combinations in total); we were able to investigate the contribution of substrates to effect of stress conditions such as uncoupling with FCCP and hypoxia. A number of parameters were investigated: glycolysis, \(O_2\) consumption, \(O_2\) and ATP levels, and hypoxia inducible factor signalling in pheochromocytoma PC12 cells. Upon uncoupling with FCCP the mitochondria were depolarised similarly in all the cases, but a strong increase in respiration was only seen in the cells fed on glutamine combined with either glucose or pyruvate. Surprisingly, the response to FCCP did not correlate with ATP levels, which rapidly dropped upon uncoupling in the absence of glucose. Inhibition of glutaminolysis resulted in a reversal of the glutamine dependant effect. 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.

At reduced \(O_2\) availability (4 % and 0 % of atmospheric \(O_2\)), cell bioenergetics and local oxygenation varied drastically depending on the substrate composition. Cellular ATP and \(O_2\) levels orchestrated hypoxia inducible factor-2\(\alpha\) stabilisation. These results indicate that both supply and utilisation of key metabolic substrates can affect the pattern of hypoxia inducible factor-1/2\(\alpha\) accumulation by differentially regulating \(iO_2\) and ATP levels and Protein kinase B/mitogen-activated protein kinase/AMP-activated protein kinase pathways. Inhibition of key metabolic pathways (e.g. glutaminolysis) can modulate hypoxia inducible factor regulatory pathways, metabolic responses and survival of cancer cells in hypoxia. These data are of relevance to those studying neuronal and cancer metabolism under hypoxia.

Hypoxia leads to transcriptional activation, by hypoxia inducible factor, of pyruvate dehydrogenase kinase which phosphorylates and inhibits pyruvate dehydrogenase, a mitochondrial enzyme that converts pyruvate formed by glycolysis into acetyl-
CoA, thus feeding the Krebs cycle. The levels of pyruvate dehydrogenase (total and phosphorylated protein), pyruvate dehydrogenase kinase and hypoxia inducible factor-1α were analysed in human colon cancer cells HCT116 wild type and $\text{SCO2}^{-/-}$ (deficient in complex IV of the respiratory chain) grown for 1 week under 20.9% and 3% $\text{O}_2$. Our data on pyruvate dehydrogenase phosphorylation state indicates that regulation of the enzyme activity under normoxia and hypoxia can occur in a manner independent of the hypoxia inducible factor-1/pyruvate dehydrogenase kinase 1 axis, mitochondrial respiration and the demand of the Krebs cycle for acetyl-CoA. This is of particular relevance for those studying possible treatments with pyruvate dehydrogenase kinase 1 inhibitors for diabetes, heart disease or cancer.

Collectively these results can be applied to many (patho)physiological diseases such as reduced nutrient supply and $\text{O}_2$ during ischemia/stroke, hypoglycaemia in diabetes mellitus and liver carcinoma patients, and cancer associated changes in uncoupling protein expression levels.
LIST OF ABBREVIATIONS

AA – Antimycin A
ADP – Adenosine Diphosphate
Akt – Protein kinase B (PKB)
α-KG - α-ketoglutarate
AMP - Adenosine monophosphate
AMPK – AMP-activated protein kinase
ARNT - Aryl hydrocarbon receptor nuclear translocator
Asp - Aspartate
ATP – Adenosine Triphosphate
BPTES - bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulphide
CEB – Cell Energy Budget
COX – Cytochrome c oxidase
CRAC - Calcium release-activated calcium
CYTB – Cytochrome b
DMEM - Dulbecco's Modified Eagle's medium
DMSO – Dimethyl sulphoxide
ECA – Extracellular acidification rate
EPO – Erythropoietin
ER – Endoplasmic reticulum
Erk - mitogen-activated protein kinase (MAPK)
ETC- Electron Transport Chain
FADH2 - Flavin adenine dinucleotide
FCCP - Cyanine-p-trifluoromethoxyphenylhydrazone
FDG-PET - Positron emission tomography using 18F-fluorodeoxyglucose
FH – fumarate hydratase
Gal – D-galactose
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
Glc – D- glucose
Gln – L- glutamine
Glu - Glutamate
GLUT – Glucose transporter
GLS – Glutaminase
GTP - Guanosine-5’-triphosphate
HIF – Hypoxia inducible factor
H2O2 – Hydrogen peroxide
IDH – Isocitrate dehydrogenase
KCN – Potassium cyanide
LDH – Lactate dehydrogenase
L-ECA – Lactate-ECA
LT - Lifetime
MCU – Mitochondrial calcium uniporter
MEF – Mouse embryonic fibroblasts
MIX - Max-like protein X
MPC – Mitochondrial pyruvate carrier
mPTP – Mitochondrial permeability transition pore
mTOR - Mammalian target of rapamycin
NADH - Nicotinamide adenine dinucleotide
NADPH - Nicotinamide adenine dinucleotide phosphate
NCLX – Mitochondrial Sodium calcium lithium exchanger
NCX - Sodium/Calcium exchanger
$O_2^-$ - Superoxide
OCR – Oxygen consumption rate
OGD – Oxygen/glucose deprivation
OxPhos – Oxidative Phosphorylation
PARP - Poly(ADP-ribose) polymerase
PDH – Pyruvate Dehydrogenase
PDK – PDH kinase
PDP – PDH phosphatase
PHD – Prolyl hydorxylases
Pmf – Proton motive force
Pyr - Pyruvate
RACK - Receptor of activated protein kinase C
RCR – Respiratory control ratio
ROS – Reactive Oxygen Species
RPWI – Roswell Park Memorial Institute
RR – Ruthenium red
Ser – Serine
SCO – Cytochrome c oxidase
SDH – Succinate dehydrogenase
SOD – Superoxidase dismutase
STIM - Stromal interaction molecule
T-ECA – Total-ECA
Thr - Threonine
TMRM – Tetramethylrhodamine methyl ester
TORC1- Target of rapamycin complex 1
TPP + - Tetraohenyl phosphonium ion
TXNIP - Thioredoxin-interacting protein
Tyr - Tyrosine
UCP – Uncoupling protein
VEGF – Vascular endothelial growth factor
VHL - von Hippel-Lindau
WM – Working media
WT – Wild type
$\Delta \Psi_m$ – Mitochondrial membrane potential
2-HG – 2-hydroxyglutarate
**Thesis Objectives**

Apply metabolic cell energy budget to study:

1. Toxicity testing of molecular imprinted polymer - nanoparticles.
2. Cellular response to uncoupling.
3. Study factors involved in hypoxia inducible factor-α accumulation.
4. Investigate pyruvate dehydrogenase complex regulation.
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW
1.1 Metabolism of Mammalian Cells

Mammalian cells have several main metabolic pathways that work together in order to maintain the required supply of energy and key metabolites to the cell. These are glycolysis, the Krebs cycle, Oxidative Phosphorylation (OxPhos), glutaminolysis and the pentose phosphate pathway (Fig. 1.1). Each pathway has spare capacity which can be increased in the event of increased energy demand or one of the other pathways not working optimally. For example in hypoxic conditions where there is a reduction in oxygen, OxPhos has a reduced output which can be compensated for by an increase in glycolytic activity to maintain steady ATP levels.
Figure 1.1 Scheme of the main metabolic pathways in mammalian cells (glycolysis, glutaminolysis, pentose phosphate pathway, Krebs cycle and oxidative Phosphorylation). Glycolysis produces pyruvate (Pyr) which is transported into the mitochondria via the mitochondrial Pyr carrier (MPC), converted into acetyl-CoA and fed into the Krebs cycle. The Krebs cycle produces the reducing intermediates (NADH) required for oxidative phosphorylation (OxPhos). Complexes I, III and IV pump protons out of the mitochondrial matrix into the inner-mitochondrial space creating a gradient which is utilised by Complex V (ATP synthase) to generate ATP.
1.1.1 Glycolysis and the Krebs cycle

Glycolysis, also known as the Embden-Meyerhof pathways, is a series of reactions which convert one molecule of glucose into two molecules of pyruvate in the cytosol of the cell, generating two ATP molecules per molecule of glucose as well as NADH (Fig. 1.1). There are two phases in glycolysis, a priming phase which utilises 2 ATP molecules and an energy-yielding phase which generates 4 molecules of ATP and 2 molecules of NADH. Glycolysis is the first stage of three in the complete oxidation of glucose and is up-regulated in many different conditions such as O₂ deprivation or certain cancers (Busk et al., 2008, Dang and Semenza, 1999). Glycolysis is highly regulated by master metabolic regulators such as AMP-activated protein kinase (AMPK), Protein Kinase B (Akt) and hypoxia inducible factors (HIFs) (Hardie et al., 2012, Xu et al., 2014, Kim et al., 2006). Akt has been shown to up-regulate glycolysis in cancer cells under aerobic conditions (Elstrom et al., 2004). This is a prominent effect which is observed in many cancer cells, glycolysis is used as the main energy source even when there is sufficient O₂ present to allow energy production via OxPhos which is a more efficient way to generate ATP (Warburg et al., 1927). This is termed the Warburg effect, which is characterised by increased lactate production, glycolytically derived pyruvate is converted into lactate (Xu et al., 2014). This was thought to be due to dysfunctional respiratory chain activity in these cells; however it is now known that cancer cells have functional mitochondria, observed in glioblastoma cancer stem cells, epithelial ovarian cancer stem cells and human breast cancer cells (Obre and Rossignol, 2015, Jose et al., 2011, Janiszewska et al., 2012, Pastò et al., 2014). The increased glycolytic activity observed in certain cancer cells is thought to fulfil metabolic requirements necessary for cell growth. The Krebs cycle can be maintained through α-ketoglutarate (α-KG) derived from glutamine through glytaminolysis (Wise et al., 2008, Fan et al., 2013). Pyruvate formed from glycolysis enters the mitochondria and is converted into acetyl-CoA by pyruvate dehydrogenase (PDH) which is fed into the Krebs cycle, also known as the citric acid cycle or the tricarboxylic acid cycle. Acetyl-CoA is converted into citrate by citrate synthase, which is then transformed into cis-Aconitate and then D-isocitrate by aconitase the latter forming
NADH. \( \alpha \)-KG is then formed using isocitrate dehydrogenase forming NADH; \( \alpha \)-KG dehydrogenase then converts \( \alpha \)-KG into succinyl-CoA by succinyl-CoA synthetase. The cycle continues forming succinate, fumarate and malate by succinyl-CoA synthetase, succinic dehydrogenase and fumarase respectively. GTP is formed during the conversion of succinyl-CoA to succinate and FADH\(_2\) is formed in the conversion of succinate to fumarate. Malate dehydrogenase transforms malate in oxaloacetate forming NADH and finally this is converted into citrate by citrate synthase completing the cycle. Intermediates of the Krebs cycle are used for macromolecule synthesis, citrate is exported for lipid synthesis and, \( \alpha \)-KG and oxaloacetate are used to provide non-essential amino acids to build proteins and nucleotides, demand of which can have an impact on Krebs cycle function (Kaplan et al., 1993). Glutaminolysis is able to maintain Krebs cycle activity when demand for intermediates for anabolic processes forming \( \alpha \)-KG from glutamine to be used as a carbon source for the Krebs cycle (DeBerardinis et al., 2008, Obre and Rossignol, 2015). NADH and FADH\(_2\), generated by the Krebs cycle, are utilised as electron carriers in the electron transport chain (ETC) during OxPhos.

The Krebs cycle enzyme isocitrate dehydrogenase (IDH) which converts isocitrate into \( \alpha \)-KG is implicated in cancer. There are three isoforms; IDH1, IDH2 and IDH3. Mutations of IDH1 and IDH2 are common in various different cancers including; gliomas and acute myeloid leukaemia (Dang et al., 2010). IDH1 resides prominently in the cytoplasm and reactions catalysed by IDH1 are reversible. IDH2 and IDH3 are located in the mitochondrial matrix. Reactions catalysed by IDH2 are reversible but IDH3 catalysed reactions are irreversible. IDH3 can be activated by Ca\(^{2+}\), ADP and citrate, and it can be inhibited by ATP, NADH and NADPH (Losman and Kaelin, 2013). When mitochondrial glutamate levels are high, such as during hypoxia when glucose-derived citrate production is not optimal, glutamate is converted into \( \alpha \)-KG by glutamate dehydrogenase (GDH). This causes an increase in the \( \alpha \)-KG: isocitrate ratio, and under these circumstances reductive carboxylation of glutamine into citrate catalysed by IDH2 is favoured to support hypoxic growth. This can also be observed in pseudo-hypoxic cells, which are von-Hippal Lindau (VHL) deficient (Gameiro et al., 2013). IDH2 requires NADPH to work in reverse
mode; electrons can be transferred from NADH to NADP\(^+\) as there is a high level of NADH in hypoxic cells or those with a defective ETC. This switch in IDH2 catalytic direction has been demonstrated in the glioblastoma cell line SF188 during hypoxia, along with an increase in 2-hydroxyglutartate (2HG) (Wise et al., 2011). Another study using renal 143B cells, both WT and then a mutant deficient in the gene encoding cytochrome b (CYTB) (component of complex III of the ETC), showed cells with defective ETC preferentially use reductive carboxylation as a major pathway for production of fatty acids (Mullen et al., 2012). It was also shown in the same study using UOK262 derived from a tumour in a patient with hereditary leiomyomatosis and renal cell cancer, that reductive carboxylation occurs in FH deficient cells. Reductive glutamine metabolism can also be mimicked in cells with normal mitochondrial function, in this case mouse embryonic fibroblasts (MEFs) with the use of respiration chain inhibitors such as antimycin A or rotenone (Mullen et al., 2012).

1.1.2 Oxidative Phosphorylation

The OxPhos pathway consists of an ETC made up of five complexes (I-V), located on the inner mitochondrial membrane. Complexes I, III and IV pump protons from the mitochondrial matrix into the intermembrane space, creating an electrochemical and proton gradient, the proton motive force (pmf), whereby the mitochondrial matrix is more negative inside (150mV) than the intermembrane space, as shown in Fig 1.2. This gives a mitochondrial membrane potential (\(\Delta \Psi_m\)) which can be assessed (e.g. by staining with tetramethylrhodamine methyl ester (TMRM) to visualise the pmf, and ultimately mitochondrial function) (Leonard et al., 2015). Depolarisation of the \(\Delta \Psi_m\) plays a role in apoptosis (Brand and Nicholls, 2011a). This gradient can be exploited by ATP synthase (complex V), which allows protons to move into the mitochondrial matrix providing energy for ADP + Pi to form ATP (Ferguson, 2000, Nicholls and Ferguson, 2013).
Complex I, also known as NADH-coenzyme Q oxidoreductase, is the first enzyme in the ETC, and catalyses the oxidation of NADH generated in the Krebs cycle and glycolysis. Electrons from NADH are transferred to Coenzyme Q, thereby transferring four protons across the inner mitochondrial membrane (Chen et al., 2010). Complex II, sometimes referred to as succinate-Q oxidoreductase or succinate dehydrogenase is also a Krebs cycle enzyme that catalyses the oxidation of succinate to fumarate and reduces ubiquinone; this complex is not involved in the production of the proton gradient (Siebels and Dröse, 2013). Co-enzyme Q then donates electrons to cytochrome b in Complex III, also called ubiquinol cytochrome c oxidoreductase, which catalyses the reduction of cytochrome c by the electrons donated from cytochrome b, along with four protons being pumped into the mitochondrial inter-membrane space (Bazil et al., 2013). The terminal electron acceptor, Complex IV, also termed cytochrome c oxidase (COX), is where cytochrome c reduces cytochrome a₃, consuming four protons in the process and reducing O₂ to H₂O. Complex IV also pumps four protons across the inner mitochondrial membrane (Balaban et al., 2005). Complex V, also known as ATP synthase,
synthase, then utilises this proton gradient generated to phosphorylate ADP creating ATP (Arnold, 2012).

Studies have shown that ETC disorders may have a live birth prevalence of 1 in 5000, with predicted figures of 13.1 in 100 000 live births affected and 1 in 7634 for ETC disorders with onset at any age (Skladal et al., 2003). Diagnosis of these disorders is difficult, many symptoms overlap and some patients have multiple metabolic defects. Deficiency in complex I, the most common of the respiratory chain complexes, manifests as Leigh syndrome, cardiomyopathy, stroke, lactic acidosis or ataxia (Calvo et al., 2010). Complex II deficiency is very rare and not well defined (Jain-Ghai et al., 2013).

Complex III can be pharmacologically inhibited using antimycin A (AA) blocking the Q₁ site, disrupting the internal transfer of electrons within the complex. Mutations of the gene involved in the maturation of complex III, BCS1L, can cause Björnstad syndrome, a disorder in which there is abnormal hair and hearing problems, and GRACILE syndrome, which is a profound multisystem organ failure in which infants are not expected to live beyond a few months (Kotarsky et al., 2010, Hinson et al., 2007). In this disorder the BCS1L is broken down quickly, leading to a deficiency in complex III, especially in the liver and kidneys, which results in an energy deficit that damages the organs (Kotarsky et al., 2010, Hinson et al., 2007). Cardiac dysfunction as a result of myocardial infarction can lead to decreased protein levels of complex III. This was shown in mitochondria isolated from failing hearts in Wister rats; the degree of cardiac dysfunction dictated the level of mitochondrial impairment (Chinopoulos et al., 2010).

Complex IV can be inhibited by cyanide, azide and carbon monoxide. There are several genetic mutations that can occur in some of the assembly factors for complex IV including; Surfeit locus protein1, synthesis of cytochrome c oxidase (SCO) 1, SCO2, COX10, COX15, COX20, COA5 and leucine-rich PPR motif-containing protein (Rahman et al., 1996). Associated disorders include; Leigh syndrome, cardiomyopathy, leukodystrophy, anemia and sensorineural deafness. Leigh disease is one of the most common respiratory chain disorders, and is characterised
by a subacute necrotising encephalomyelopathy (Rahman et al., 1996). Most cases are associated with a COX deficiency, but some have been reported to have pyruvate dehydrogenase complex deficiency/mutations or complex I deficiency (Rahman et al., 1996).

ATP synthase deficiency is rare and can range in severity from mild cases causing; maternally inherited Leigh syndrome or neuropathy, ataxia and retinis pigmentosa syndrome or severe cases such as, neonatal mitochondrial encephalo(cardio)myopathy (Jonckheere et al., 2012). ATP synthase is also able to work in reverse mode in times of low pmf, working to restore the $\Delta \Psi_m$ which is achieved at the expense of ATP hydrolysis (Martin et al., 2014). This process is regulated by the inhibitor protein IF$_1$, which binds to the Fi domain of ATP synthase, selectively inhibiting reverse mode (Bason et al., 2014). IF$_1$ is activated when the pH becomes too acidic, below pH 6.5 and becomes inactive above pH 8.0 (Bason et al., 2014, Pullman and Monroy, 1963). ATP synthase can be pharmacologically inhibited by oligomycin and is also implicated in intrinsic apoptosis. The oligomycin-sensitivity conferring protein, a subunit of ATP synthase, is where cyclophilin D interacts and triggers the mitochondrial permeability transition pore (mPTP) to open, one of the stages in the mitochondrial apoptosis pathway (Antoniell et al., 2014). It works by adjusting the Ca$^{2+}$ level required for mPTP opening to occur (Bernardi, 2013).

Both complex I and complex III have certain amount of proton leak which, combined with available O$_2$, forms superoxide anion (O$_2^-$) (Balaban et al., 2005, Bandyopadhyay et al., 1999). This is usually metabolised into hydrogen peroxide (H$_2$O$_2$) by superoxide dismutase (SOD). If H$_2$O$_2$ comes into contact with a transient metal, a hydroxyl radical (OH·) is formed. It has been shown, that in mitochondrial isolated from either the brain or heart of a SOD2 null mouse decreased complex II activity is present, which interestingly caused decreased reactive oxygen species (ROS) levels indicating that complex II is a major site for ROS generation (Chinopoulos, 2011). Complex I is the most sparse of the ETC complexes with the quantity of each complex increasing from complex I to complex IV with ratios of complexes I, II, III, IV and ATP synthase shown to be 1.1 : 1.3 : 3.0 : 6.7 : 3.5 in
bovine heart mitochondria (Schägger and Pfeiffer, 2001). ROS induces damage of the F₀ complex, and is thought to be implicated in neurodegenerative diseases such as Parkinson’s disease, ischemia/reperfusion injury and atherosclerosis (Musatov and Robinson, 2012). Increases in ROS and O₂⁻ can lead to the release of cytochrome c from the mitochondria, initiating apoptosis without the involvement of the mPTP (PETROSILLO et al., 2003).

Low levels of ROS are important in multiple signalling pathways (Thannickal and Fanburg, 2000). However, when levels become too high there are certain cellular defences against ROS which include a number of antioxidant enzymes, including SOD, catalase, Haeme peroxidases and glutathione peroxidase, which act as a first line of defence (Hayes and McLellan, 1999). The second line of defence is provided by glutathione S-transferase, ascorbate, aldo-keto reductase and aldehyde dehydrogenase, which aim to protect cells from DNA, protein and lipid damage (Jo et al., 2001). Products from ROS reacting with these enzymes are ejected from the cell via the glutathione S-conjugate transporter (Hayes and McLellan, 1999, Bandyopadhyay et al., 1999). NADPH, which is required for the regeneration of glutathione from its oxidised to reduced form, is another crucial element in ROS scavenging (Jo et al., 2001). Levels of ROS are often increased in cancer cells, however so are levels of ROS scavengers. Many studies using antioxidants as cancer preventatives have shown little benefit or even detrimental effects (1994, Chandel and Tuveson, 2014).

1.1.3 Cell Bioenergetics

The area of cell bioenergetics has been studied for many years, and it is still of great importance today. Cell metabolism can be altered in many ways, some of which are still unknown. There are many drugs that can affect the mitochondria, and that would have repercussions on bioenergetics, including non-steroidal anti-inflammatory drugs (NSAIDs) and anti-cancer treatments such as Tamoxifen (Mahmud et al., 1996, Mandlekar and Kong, 2001). A bioenergetic approach can be
utilised in many treatment areas including anti-cancer therapeutics, stroke, and ischemia-reperfusion.

Increased glucose utilisation is an accepted marker of certain cancers. Increased glucose uptake has been exploited for diagnostic purposes in a clinical setting. The technique is called positron emission tomography using 18F-fluorodeoxyglucose (FDG-PET) (Gatenby and Gillies, 2004, Semenza, 2010). It has been shown to be useful in a number of different cancers, such as breast cancers, certain lymphomas, colorectal cancers and lung cancer (Alberini et al., 2009, Jerusalem et al., 2001, Fernandez et al., 2004, Machtay et al., 2013). However as with all techniques FDG-PET has its limitations. It shows areas with increased glucose uptake, including hypoxic regions where there is restricted O2 for OxPHOS and compensatory increases in glycolysis occur. However, hypoxic centres are often observed in solid tumours. The method is unable to distinguish between readings from hypoxia fuelled increases in glucose uptake, and that driven by cancer. There are also certain types of cancer which still gain much of their ATP from OxPhos, which has been shown using cervical carcinoma SiHa cells (Busk et al., 2008).

Other biochemical intermediates are used clinically as diagnostic tools in oncology. Since the increase in glycolysis can be correlated with patient prognosis, the ratio between the mitochondrial enzyme ATP synthase and the glycolytic enzyme glyceraldehyde-6-phosphate dehydrogenase gives a bioenergetic index that can be used to assess tumour aggressiveness (Cuezva et al., 2002, Chacko et al., 2013).

In each metabolic pathway there is spare capacity, which represents a potential for an increased rate of ATP generation. For example in some cancerous tumours, the centre of the tumour becomes hypoxic (i.e. deprived of oxygen without a blood supply). Under these conditions the concentration of O2 is inadequate for OxPhos to function at a normal rate, and the ATP deficit is rectified by up-regulation of glycolysis. In hypoxic conditions the ∆Ψm can decrease, which may cause a degree of mitochondrial uncoupling, whereby the electrochemical gradient created by the ETC is altered (Aley et al., 2005). As a result of this uncoupling, protons are able to cross through the inner mitochondrial membrane and complexes I-V are inhibited.
(Solaini et al., 2010). This results in the cessation of OxPhos and causes ATP synthase/complex V to function in reverse mode to back pump protons out of the mitochondrial matrix, utilising ATP in an attempt to maintain the electron chemical gradient at the expense of ATP (Nicholls and Budd, 2000a, Bason et al., 2014). This can occur naturally via activation of uncoupling proteins (UCPs) within cells, located on the mitochondrial inner membrane, which are able to dissipate the proton gradient, uncoupling the cell (Brand and Esteves, 2005). These proteins, prevalent in brown fatty adipose tissue, can be activated in cold conditions to generate heat or by superoxide and activation can lead to decreased ROS levels, protecting the cell (Echtay et al., 2002, Brand and Esteves, 2005).

Mitochondrial uncoupling causes the pmf to decrease, resulting in maximal respiration and a much lower rate of OxPhos. The reduction in the ΔΨm causes a number of changes including; reduced metabolite transport across the mitochondrial membrane, a decrease in mitochondrial and an increase in cytosolic Ca$^{2+}$, activation of glycolysis, cytosolic acidification and dissipation of the mitochondrial ATP flux (Brand and Esteves, 2005).

Pharmacological reagents such as cyanine-p-trifluoromethoxyphenylhydrazone (FCCP) or 2,4-dinitrophenol can induce mitochondrial uncoupling (Hynes et al., 2006). These reagents can cause maximal respiratory rates in the cell to be achieved (Hynes et al., 2006). Medicines including the diabetic drug, troglitazone and the statin, cerivastatin, have been withdrawn from the market as a result of organ toxicity associated with decreases in the ΔΨm, which illustrates the importance of the investigation of mitochondrial uncoupling (Bova et al., 2005, Kaufmann et al., 2006). Mitochondrial uncoupling in certain metabolic conditions / diseases can cause mitochondrial malfunction and ultimately cell death (Beeson et al., 2010, Chan et al., 2005).
1.2 Regulation of Metabolism

1.2.1 Metabolic Substrates

Otto Warburg showed in the 1930’s that cancer cells have an altered metabolism compared to normal cells, exhibiting a substantially increased glycolytic activity, with an increased production of lactate from pyruvate instead of its conversion into acetyl-CoA and utilisation in the Krebs cycle producing reducing equivalents for OxPhos. Warburg’s observations of the increased utilisation of glucose for glycolysis lead him to believe that the changes in cells which become cancerous are metabolic rather than genetic. The increased glycolytic rate occurs even when there are sufficient levels of oxygen for OxPhos and is sometimes referred to as aerobic glycolysis (Warburg et al., 1927). This phenomenon is of importance, as OxPhos is a far more efficient, although slower way to generate ATP, creating approximately 36 ATP molecules per molecule of glucose rather than a net of 2 ATP molecules created per molecule of glucose during glycolysis. When cells predominately use glycolysis for metabolism, the pyruvate formed is converted into lactate in order to maintain sufficient NAD⁺ levels for glycolytic requirements (Lunt and Vander Heiden, 2011). There are several factors which contribute to the Warburg effect, which include the transcription factor, HIF-1, oncogenic transcription factor MYC, oncogenic GTP-binding protein RAS, oncogenic non-receptor tyrosine kinase v-SRC, tumour suppressor transcription factor p53 and von Hippel-Lindau tumour-suppressor protein (pVHL) (Dang and Semenza, 1999). The increase in glycolytic activity in cancer cells has been shown to be strongly associated with hypoxia (Cairns et al., 2011). However there are conflicting data, in a study analysing 32 different cancer cell lines measuring lactate and O₂ showing glycolytic and OxPhos contributions to ATP generation respectively, average glycolytic activity in cancer cells was shown to be very similar to that observed in normal cells. However, the authors admitted the cells were not directly comparable and cells of the same tissue should be directly compared (Zu and Guppy, 2004). There are cancer cells with functional mitochondria in which high levels of OxPhos are still present, observed in glioblastoma cancer stem cells, epithelial ovarian cancer stem cells and human breast cancer cells (Jose et al., 2011, Janiszewska et al., 2012, Pastò et al.,
It is thought that the cancer cells environment is an important factor in regulation of metabolism, it has been shown using HTB-126 human breast cancer cells that deprivation of glucose in cancer cells can increase OxPhos 5-fold, while O₂ deprivation can decrease OxPhos activity, this effect was not observed in non-cancerous human breast (HTB-125) control cells (Smolkova et al., 2010).

There are many oncogenic factors which when mutated can cause metabolic changes (Table. 1.1). MYC is a constitutively expressed regulator gene that gains function in cancer, causing lactate dehydrogenase (LDH) expression to rise, and increasing glycolytic activity and lactate production (Dang and Semenza, 1999). RAS is activated by mutations, gaining function in cancer. It works to increase glycolysis and vascular endothelial growth factor (VEGF) expression (Dang and Semenza, 1999). V-SCR is an oncogenic retrovirally transduced gene that increases glycolysis and induces VEGF mRNA through HIF-1. P53 is mutated in cancer causing HIF-1α to be stabilised, hexokinase II to be activated and induction of apoptosis under hypoxic and acidic conditions (Ke and Costa, 2006). HIFs, pVHL and p53 will be discussed later.

**Table 1.1 Oncogenic factors which when mutated regulate different aspects of metabolism.**

<table>
<thead>
<tr>
<th>Oncogenic Factor</th>
<th>Mutational effect on metabolism</th>
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<tbody>
<tr>
<td>MYC</td>
<td>Increases glycolytic activity and lactate production through LDH up-regulation.</td>
</tr>
<tr>
<td>RAS</td>
<td>Increase glycolysis and vascular endothelial growth factor (VEGF) expression.</td>
</tr>
<tr>
<td>V-SCR</td>
<td>Increases glycolysis and induces VEGF transcription through HIF-1.</td>
</tr>
<tr>
<td>P53</td>
<td>HIF-1α stabilisation, hexokinase II activation and induction of apoptosis.</td>
</tr>
<tr>
<td>VHL</td>
<td>HIF-1α stabilisation under normoxic conditions.</td>
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AMPK is a master metabolic regulator which plays a crucial role in cellular energy homeostasis, responding to changes in the AMP/ATP ratio (Yuan et al., 2013, Mihaylova and Shaw, 2011). Activation of AMPK is achieved by AMP or ADP binding to the γ subunit causing a conformational change which allows phosphorylation to occur (Hardie, 2011). Once active, AMPK switches on major ATP generating pathways whilst limiting energy consuming anabolic pathways, as shown in Fig 1.3. It stimulates glycolysis by up-regulating the expression of glucose transporters (GLUT1 and GLUT4) and phosphorylates and activates 6-phosphofructo-2-kinase which synthesises the glycolysis activator fructose-2,6-bisphosphate (Marsin et al., 2000). AMPK also promotes fatty acid uptake and fatty acid oxidation and increasing mitochondrial biogenesis (Merrill et al., 1997, O’Neill et al., 2013). Decreases in ATP utilisation occur from phosphorylation of acetyl CoA carboxylase (ACC1), inhibiting fatty acid synthesis, phosphorylation of glycogen synthase and reducing glycogen synthesis. It also results in inhibition of sterol regulatory element-binding protein preventing lipid synthesis and inhibition of gluconeogenesis (Hardie, 2011, Bultot et al., 2012). AMPK is also involved in autophagy (Mihaylova and Shaw, 2011).
**Figure 1.3 Schematic of metabolic pathway regulation.** AMPK is activated when there is an increase in AMP or ADP. Activation causes the inactivation of Acetyl CoA carboxylase (ACC), leading to a reduction in lipid metabolism. AMPK activates tuberous sclerosis 1 (TSC1) which inhibits Rhed GTPase preventing mTORC1 activation. However activation of Akt by mTORC2, phosphoinositide-3-kinase (PI3K) or hypoxia can inhibit TSC1, allowing activation of mTORC1 which can activate the transcription factor hypoxia inducible factor 1α (HIF-1α). Both hypoxia and mitogen-activated protein kinase (ERK) can also activate HIF-1α which has a number of downstream targets.

Mammalian target of rapamycin (mTOR) is crucial element of cell growth and survival. mTOR exists as two complexes mTORC1 and mTORC2 each with a different role (Xu et al., 2014). mTORC1 plays a role in anabolic processes (e.g. protein synthesis) and is regulated by nutrients and growth factors. mTORC2 stimulates phosphoinositide-dependent kinase 1, which activates the...
serine/threonine kinase Akt, and in addition directly activates Akt by phosphorylation on Ser473 (Sarbassov et al., 2005, Porstmann et al., 2008, Yuan et al., 2013). Akt is another cell metabolism regulator in the PI3K/Akt/mTOR pathway. Phosphoinositide 3-kinase (PI3K) activates Akt which activates mTORC1 (Karar and Maity, 2011). Akt is normally found in an inactive state, with expression induced by oncogenic factors. Akt controls metabolism by stimulating glucose uptake by influencing the translocation of GLUT4 to the plasma membrane, increasing GLUT1 expression with a resultant increase in the expression of the enzymes involved in glycolysis and translocation of HKI/II to the mitochondrial outer membrane to increase glycolytic activity (Barnes et al., 2005). This is summarised in Fig 1.3.

There is also a phenomenon termed the Crabtree effect, whereby normal cells have depressed mitochondrial respiration as a result of increased glycolytic activity (Marroquin et al., 2007). The effect can be reversed by switching the cell medium from one containing glucose to another that contains galactose, which causes no net gain of ATP to be derived from glycolysis and forces the cells back into generating ATP via OxPhos (Marroquin et al., 2007, Rossignol et al., 2004).

In cancer cells, up-regulation of glycolytic activity occurs generally as a result of hypoxia. Hypoxia up-regulates many genes that are involved in glycolysis, including those coding for glucose transporters, LDH, pyruvate dehydrogenase kinases (PDKs) and hexokinase II (Lu et al., 2008, Waters et al., 2014, Yasuda et al., 2004, Szablewski, 2013). VEGF is also induced to promote angiogenesis (Yasuda et al., 2004). GLUT1 and GLUT5 are overexpressed in colon cancers (Pauwels et al., 2000, Szablewski, 2013).

### 1.2.2 Starvation and Hypoxia

Tumours often have a hypoxic centre which can lead to metabolic changes. HIFs are stabilised under reduced O2 and become active transcription factors (mechanism discussed later). This causes many survival and adaptive genes to be
induced or activated (Demidenko and Blagosklonny, 2011, Karar and Maity, 2011, Kim et al., 2006, Yee Koh et al., 2008). HIF-1 expression, increased in hypoxia, causes increases in GLUT 1 and GLUT 3 as well as many of the enzymes crucial to glycolysis, such as phosphofructokinase L, aldolase A, (Glyceraldehyde 3-phosphate dehydrogenase) GAPDH, phosphoglycerate kinase 1, enolase 1 and LDH A (Wenger, 2002, Szablewski, 2013).

In the inherited cancer, von Hippel-Lindau (VHL) disease, the pVHL tumour suppressor protein is mutated (Koh et al., 2008). pVHL is involved in the degradation of HIF-α, so mutations causing a reduction in this protein’s activity can lead to HIF-α stabilisation under normoxic conditions, as well as activation of genes for cell survival such as those encoding angiogenic factors such as VEGF (Cockman et al., 2000, Gameiro et al., 2013, Kamura et al., 2000).

1.2.3 Metabolic Enzymes

Pyruvate is the product of glycolysis and is pivotal in terms of metabolic pathways. There are two main routes pyruvate can take; it can either converted into acetyl-coA by pyruvate dehydrogenase (PDH) resulting in oxidation to CO₂, or it can be converted into lactate by LDH (Patel and Roche, 1990, Rardin et al., 2009). Another possible pathway is the conversion into oxaloacetate by pyruvate decarboxylase in an anaplerotic reaction aimed to replenish Krebs cycle intermediates such as alanine. It has been shown that high HKII along with low pPDH expression in colorectal tumours was associated with increased aggressiveness and survival (Hamabe et al., 2014).

The PDH complex is comprised of a catalytic PDH subunit (E1), a dihydrolipooyl acetyltransferase (E2), a dihydrolipooyl dehydrogenase (E3) and a E3-binding protein (E3BP) (Patel and Roche, 1990). The PDH complex requires certain substrates are co-factors. The PDH E1 subunit decarboxylates pyruvate uses thiamine pyrophosphate (TPP) as a cofactor; the remaining hydroxyethyl fragment is then transferred from TPP to lipoamide cofactor which is attached to E2 forming
acetyllipoamide. All of these reactions are catalysed by the PDH E1 subunit. Next E2 and E3 catalyse the transfer of the acetyl residue to coenzyme A regenerating lipoamide using FAD and NAD$^+$ as a cofactor (Martin et al., 2005).

The PDH complex is tightly regulated by phosphorylation/dephosphorylation reactions that occur on one of three serine residues on the Elα subunit, Ser-232, Ser-293 and Ser-300 and result in a change of metabolic fate for pyruvate (Rardin et al., 2009). Instead of being utilised in the Krebs cycle providing intermediates for OXPHOS it can undergo gluconeogenesis to produce glucose in times of energy stress (Foretz et al., 2010). Alternatively, in times of ischemic assault, it may be converted into lactate for metabolic fuel for oxidative cancer cells (Draoui and Feron, 2011).

Activation of the PDH complex occurs via pyruvate dehydrogenase phophastase (PDP), which works to catalyse the de-phosphorylation of PDH, resulting in activation of the enzyme (Korotchkina and Patel, 2001, Pratt and Roche, 1979, Rardin et al., 2009). There are two isoforms, PDP1 and PDP2, both of which are Mg$^{2+}$ dependent (Lawson et al., 1993). PDP is found loosely associated to the PDH complex, and only fully binds to the complex when activated. Only PDP1 is activated by Ca$^{2+}$, with maximal rate of PDP1 activity achieved at a mitochondrial Ca$^{2+}$ concentration of approximately 3 µM (Turkan et al., 2004). NADH inhibits PDPs activity (Huang et al., 1998). PDP1 is highly expressed in the testes and skeletal muscle, while PDP2 is not, whereas in the kidneys and liver the reverse is true, with only PDP2 expressed in these organs (Huang et al., 1998). Both PDP1 and PDP2 are expressed in the brain and heart. Both PDP isoforms can be inhibited by NADH (Huang et al., 1998).

PDKs are responsible for the phosphorylation/inactivation of the PDH complex, which means that they have a critical regulatory role in the bioenergetic fate of the cell. PDKs exist as a homo- or hetero-dimer of various isoforms in their active state, depending on expression levels of the different isoforms (Abbot et al., 2005, Bao et al., 2004, Lu et al., 2008). In order to inactivate PDH, PDK must bind to the inner lipoyl domain 2 or the outer lipoyl domain 1 of E2. There are four isoforms of PDK
(1-4), which have differential expression levels in different tissues. PDK2 and PDK4 are the prevailing isoforms in the heart and skeletal tissue, and these are also the only tissues in which PDK3 is expressed (Bowker-Kinley et al., 1998). High levels of PDK2 are found in the heart, skeletal muscle and liver. PDK4 can be found in high levels in the brain, kidneys and liver. PDK1 is highly expressed in the heart (Bowker-Kinley et al., 1998). Both PDK1 and PDK3 are activated by HIFs during hypoxia, whereas, PDK2 and PDK4 are activated in times of metabolic and hormone deficiencies (Lu et al., 2008). PDKs are active in their phosphorylated state. All but PDK3 can be inhibited by high levels of pyruvate (Huang et al., 1998). PDK3 has the highest activity of all PDKs and is found in very low levels in most tissues (Bowker-Kinley et al., 1998). All of the isoforms are controlled by metabolic by-products of PDH activity and can be inhibited by high levels of ADP, NAD+ and activated by high ATP, NADH, and acetyl-coA. Increases in PDK2 and PDK4 can be observed upon glucose deprivation (Abbot et al., 2005). PDK activity is increased in many different cancer cells (Board et al., 1990). Therefore, activation of the PDK can be achieved pharmacologically using the pyruvate analogue dichloroacetate, which inhibits PDK (Pratt and Roche, 1979). This is also a phenomenon that is prevalent in Alzheimers’s disease, diabetes and heart disease (Yao et al., 2009, Naik et al., 2012, Dugan et al., 2013, Michelakis et al., 2008, Piao et al., 2010). In these diseases, increased PDK levels cause decreased PDH activity and a decrease in mitochondrial biogenesis is observed. Dichloroacetate has shown to increase PDH activity in PDH-deficient cells, however it should be noted that this was only in cells in which the mutation affected E1α stability (Fouque et al., 2003, Morten et al., 1999).

There is a complimentary pairing whereby during starvation decreased PDP2 expression levels occur together with an activation of PDK2 and PDK4 ensuring survival by hyper-phosphorylation of the PDH complex, to allow pyruvate to be conserved for use in gluconeogenesis if required. This has been observed in diabetic patients (Huang et al., 2003). Activation of PDK2 and PDK4 occurs as a result of a build-up of acetyl-CoA and NADH from fatty acid oxidation, which promotes the binding PDK2 and PDK4 to PDH (Maj et al., 2006).
Pyruvate transport into the mitochondria is provided by the recently identified mitochondrial pyruvate carrier (MPC), which is located on the inner mitochondrial membrane (Bricker et al., 2012, Herzig et al., 2012). Coupled to the electrochemical gradient, transport of pyruvate is associated with symport of one proton or exchange of one hydroxide ion. The MPC complex is made up of one MPC1 core with several MPC2 subunits attached (Bricker et al., 2012, Herzig et al., 2012).

In some cancers there are mutations in metabolic enzymes involved in the Krebs cycle or the ETC. There are certain metabolic enzymes which act as tumour suppressors. Two of these are the Krebs cycle enzymes fumarate hydratase (FH) and succinate dehydrogenase (SDH) (Selak et al., 2005). Mutations in both of these enzymes can cause pseudo-hypoxia, activating HIF-α under normoxic conditions (MacKenzie et al., 2007). SDH is also a part of complex II of the ETC, and mutations in this key enzyme are associated with hereditary paraganglioma with phaeochromocytome, which arises from inhibition of prolyl hydroxylases (PHDs) causing HIF-α stabilisation as a result of decreased hydroxylation (Gottlieb and Tomlinson, 2005, Baysal et al., 2000). There are two proposed methods of PHD inhibition, firstly by ROS created as a result of ETC malfunction without complex II. The second mechanism proposes a build-up of succinate in the mitochondria as a result of SDH not functioning properly and the conversion into fumarate not occurring, with the excess succinate being able to act as a secondary messenger, inhibiting PHD activity (Gottlieb and Tomlinson, 2005). The latter is more likely to be the correct hypothesis as the same PHD inactivation occurs in hereditary leiomyomatosis and renal cell cancer caused by FH mutations, and FH is unlikely to be involved in ROS production (Gottlieb and Tomlinson, 2005, Tomlinson et al., 2002). In this case fumarate builds up as a result of FH mutations, causing the inhibition of SDH, thereby increasing succinate levels. It has been shown that the inhibition of PHDs by both fumarate and succinate is α-ketoglutarate (α-KG) dependant. Fumarate and succinate interfere with the binding of α-KG, which is a required co-substrate, to PHD (Selak et al., 2005). Using cell-permeating α-KG derivatives, normal PHD activity can be regained and normal HIF-1α hydroxylation can occur (MacKenzie et al., 2007).
Another metabolic enzyme implicated in cancer is isocitrate dehydrogenase (IDH), which converts isocitrate into α-KG within the Krebs cycle. There are three isoforms; IDH1, IDH2 and IDH3. Mutations of IDH1 and IDH2 are common in various different cancers including; gliomas and acute myeloid leukaemia (Dang et al., 2010). IDH1 resides prominently in the cytoplasm and reactions catalysed by IDH1 are reversible. IDH2 and IDH3 are located in the mitochondrial matrix. Reactions catalysed by IDH2 are reversible but IDH3 catalysed reactions are irreversible. IDH3 can be activated by Ca\(^{2+}\), ADP and citrate, and it can be inhibited by ATP, NADH and NADPH (Losman and Kaelin, 2013). When mitochondrial glutamate levels are high, such as during hypoxia when glucose-derived citrate production is not optimal, glutamate is converted into α-KG by glutamate dehydrogenase (GDH). This causes an increase in the α-KG:isocitrate ratio, and under these circumstances reductive carboxylation of glutamine into citrate catalysed by IDH2 is favoured to support hypoxic growth. This can also be observed in pseudo-hypoxic cells, which are VHL deficient (Gameiro et al., 2013). IDH2 requires NADPH to work in reverse mode; electrons can be transferred from NADH to NADP\(^+\) as there is a high level of NADH in hypoxic cells or those with a defective ETC. This switch in IDH2 catalytic direction has been demonstrated in the glioblastoma cell line SF188 during hypoxia, along with an increase in 2-hydroxyglutarate (2HG) (Wise et al., 2011). Another study using renal 143B cells, both WT and then a mutant deficient in the gene encoding cytochrome b (CYTB) (component of complex III of the ETC), showed cells with defective ETC preferentially use reductive carboxylation as a major pathway for production of fatty acids (Mullen et al., 2012). It was also shown in the same study using UOK262 derived from a tumour in a patient with hereditary leiomyomatosis and renal cell cancer, that reductive carboxylation occurs in FH deficient cells.

Reductive glutamine metabolism can also be mimicked in cells with normal mitochondrial function, in this case mouse embryonic fibroblasts (MEFs) with the use of respiration chain inhibitors such as AA or rotenone (Mullen et al., 2012).

Glutamine is transported into the cell via the glutamine transporter SN1 or amino acid transporters ASCT1/ASCT2, and these all involve the co-transportation of Na\(^{2+}\). Glutamine, is then transported into the mitochondria by the mitochondrial...
glutamine carrier (Albrecht et al., 2000), or converted into glutamate in the cytosol and transported into the mitochondria in a glutamate carrier or via a glutamate-aspartate anti-porter, which is part of the malate-aspartate shuttle (Albrecht et al., 2000). Once in the mitochondria glutamine is converted into glutamate by glutaminase and then further transformed into α-KG which can be fed into the Krebs cycle.

Glutamine plays a role in glucose regulation; α-KG produced via glutaminolysis and the Krebs cycle can activate glucose transport into the cell. Alpha-KG causes the transcriptional repression of thioredoxin-interacting protein (TXNIP) via the activation of the transcriptional factor MondoA protein (Han and Ayer, 2013). TXNIP is a glucose sensitive MondoA target involved in a negative feedback loop restricting glucose uptake. MondoA:MIX (Max-like protein X) complexes accumulate in response to high levels of intracellular glucose and repress TXNIP, which reduces glucose uptake (Stoltzman et al., 2011). Ultimately glucose stimulates TXNIP activation and while glutamine represses this glucose fuelled activation, thereby increasing glucose transport into the cell (Stoltzman et al., 2011, Han and Ayer, 2013).

1.2.4 Calcium and Calcium Transporters

Calcium is important for many different cellular functions including; buffering system, secondary messenger, apoptosis and metabolism (Denton, 2009, Griffiths and Rutter, 2009, Nicholls, 2005, Orrenius et al., 2003, Tarasov et al., 2012). Due to this Ca$^{2+}$ needs to be stored and then mobilised when necessary. Intracellular Ca$^{2+}$ is stored predominantly within the endoplasmic reticulum (ER) and the mitochondria (Deng et al., 2009). Mitochondrial Ca$^{2+}$ is stored as a Ca$^{2+}$-phosphate complex within the matrix (Nicholls, 2005). This complex is easily dissociable and the Ca$^{2+}$ is liberated from the phosphate when there is a reduction in the ΔΨm and exits the mitochondria via efflux transporters and the phosphates leaves via the phosphate transporter (Nicholls, 2005). Ca$^{2+}$ signalling and transporters are shown in Fig 1.4.
**Figure 1.4 Schematic Ca\(^{2+}\) signalling and transporters.** Voltage-gated Ca\(^{2+}\) channels (vGCC), N-methyl-D-aspartate (NMDA) receptors, nicotinic acetylcholine (n-Ach) receptors, and Orai are involved in Ca\(^{2+}\) entry into the cell. The Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) and the plasma membrane Ca\(^{2+}\) ATPase (PMCA) are responsible for Ca\(^{2+}\) efflux. Ca\(^{2+}\) is stored in the endoplasmic reticulum (ER) and can enter via stromal interation molecule (Stim) interacting with Orai forming a Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel or sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). Ca\(^{2+}\) is released from the ER via the inositol triphosphate (IP\(_3\)) or ryanodine (Ry) receptors. The mitochondrial Ca\(^{2+}\) uniporter (MCU) is responsible for mitochondrial Ca\(^{2+}\) influx and the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger for mitochondrial Ca\(^{2+}\) efflux. Ca\(^{2+}\) is bound to Ca\(^{2+}\) binding proteins in the cytosol.
Rapid Ca$^{2+}$ transmission is of great importance, mitochondria-associated membranes allow rapid transfer of Ca$^{2+}$ from the endoplasmic reticulum (ER) to the mitochondria. Activation of inositol triphosphate (IP$_3$) or ryanodine receptors on the ER results in Ca$^{2+}$ release into the mitochondria, which is taken up by Ca$^{2+}$ uniporters (Hayashi et al., 2009). Mitochondria-associated membranes are important for stimulation of oxidative metabolism, activation of manganese superoxide dismutase, ER stress and autophagy (Hopper et al., 2006, Hamasaki et al., 2013).

Ca$^{2+}$ plays a crucial role in excitable cells. In neurons Ca$^{2+}$ release triggers neurotransmitter release and synaptic plasticity, in myocytes within skeletal and cardiac tissue Ca$^{2+}$ is necessary for muscle contraction (Holbro et al., 2009). If the mitochondria become overloaded with free Ca$^{2+}$ this can be the start of the intrinsic apoptotic pathway initiating programmed cell death (Fulda and Debatin, 0000).

Increased mitochondrial Ca$^{2+}$ levels activate the opening of the mPTP, which allows cytochrome c to be released binding with caspases forming the apoptosome initiating the caspase cascade resulting in apoptosis (Brunelle and Letai, 2009). Mitochondrial apoptosis can be driven by B-cell CLL/lymphoma 2 (BCL-2)proteins (Brunelle and Letai, 2009).

Ca$^{2+}$ can regulate metabolism, increases in intracellular Ca$^{2+}$ can activate some metabolic dehydrogenases, PDH as well as the Krebs cycle enzymes isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Denton, 2009, Griffiths and Rutter, 2009). Activation of these enzymes increases Krebs cycle activity, increasing NADH levels, providing more reducing intermediates for OXPHOS. Ca$^{2+}$ has also been shown to regulate key cell survival pathways. Hypoxia inducible factor 1α (HIF-1α), can be regulated in an O$_2$ independent manner by the receptor of activated protein kinase C (RACK1) pathway; in which dimerised RACK1 binds to HIF-1α in the presence of spermidine/spermine-N$^1$-acyetyltransferase 1, heat-shock protein 90 and the E3 ligase complex to promote HIF-1α degradation (Hawley et al., 2005). However calcineurin, a Ca$^{2+}$ and calmodulin dependent phosphatase activated by Ca$^{2+}$ can dephosphorylate RACK blocking the dimerisation required for RACK mediated HIF-1α degradation (Koh et al., 2008, Yee Koh et al., 2008).
similar manner AMPK can be activated by Ca
\(^{2+}\)/calmodulin-dependant protein kinase (Hawley et al., 2005).

When ER Ca
\(^{2+}\) levels are decreased Ca
\(^{2+}\) can enter the ER via the sarcoplastic/endoplasmic reticulum Ca
\(^{2+}\)-ATPase (SERCA), or stored operated channels which function through co-ordinated operation of stromal interaction molecule (STIM) and calcium release-activated calcium (CRAC) channel protein (Orai) and are ubiquitously expressed in both excitable and non-excitable cells (Deng et al., 2009, Mancarella et al., 2011). Redistribution of the stromal interaction molecule (STIM), an ER Ca
\(^{2+}\) sensor, occurs when ER Ca
\(^{2+}\) levels are low, causing aggregation and translocation of these proteins to ER junctions where they couple with Orai which opens the newly formed CRAC channels allowing Ca
\(^{2+}\) entry into the ER (Deng et al., 2009). In times of oxidant stress or hypoxia this mechanism is inactivated and the STIM/Orai complexes uncoupled, caused by hypoxia induced decreased cytosolic pH, possibly protecting cells from Ca
\(^{2+}\) overload (Mancarella et al., 2011). As a result of which, upon hypoxia there is an increase intracellular Ca
\(^{2+}\) concentration, which can increase Ca
\(^{2+}\)/calmodulin-dependant protein kinase activation of AMPK (Mungai et al., 2011). It has been shown that Ca
\(^{2+}\) is also able to regulate HIF signalling in a hypoxia independent manner by the receptor of activated protein kinase C (RACK1) pathway; in which dimerised RACK1 binds to HIF-1\(\alpha\) in the presence of spermidine/spermine-N\(^1\)-acetyltransferase 1, heat-shock protein 90 and the E3 ligase complex to promote HIF-1\(\alpha\) degradation (Liu et al., 2007). However calcineurin, a Ca
\(^{2+}\) and calmodulin dependent phosphatase activated by Ca
\(^{2+}\) can dephosphorylate RACK blocking the dimerisation required for RACK mediated HIF-1\(\alpha\) degradation (Koh et al., 2008, Yee Koh et al., 2008).

1.2.4.1 Mitochondrial Transporters Controlling Calcium Fluxes

There are many cellular Ca
\(^{2+}\) transporters transporting Ca
\(^{2+}\) into the cell; voltage-gated Ca
\(^{2+}\) channels, N-methyl-D-aspartate receptor, nicotinic acetylcholine receptor and transient receptor potential canonical, and out of the cell; Na
\(^{+}\)/Ca
\(^{2+}\) exchanger and plasma membrane Ca
\(^{2+}\) ATP-ase (Grienberger and Konnerth). Calcium can then be either transported into the ER via ryanodine or inositol triphophate receptors
or into the mitochondrial via the mitochondrial calcium uniporter (MCU) (Grienberger and Konnerth, Mallilankaraman et al., 2012).

Another Ca\(^{2+}\) transporter is the leucine-zipper-EF hand-containing transmembrane region Ca\(^{2+}/H^+\) antiporter involved in Ca\(^{2+}\) influx. The Ca\(^{2+}/H^+\) antiporter transports Ca\(^{2+}\) in a 1:1 ratio with H\(^+\) efflux, it is electrogenic and so the H\(^+\) released from the mitochondria during respiration can drive mitochondrial Ca\(^{2+}\) entry (Santo-Domingo and Demaurex, 2010). The Ca\(^{2+}/H^+\) antiporter can be inhibited by ruthenium red (Santo-Domingo and Demaurex, 2010).

The uniporter is located on the inner mitochondrial membrane and has a low affinity for Ca\(^{2+}\) (Mallilankaraman et al., 2012). Uptake of Ca\(^{2+}\) is driven by \(\Delta \Psi_m\) and uptake is able in part to control the rate of energy production by regulating certain enzymes (Denton, 2009, Griffiths and Rutter, 2009). The MCU depends on a large electrochemical gradient to function (Mallilankaraman et al., 2012). It was shown by Kirichok et al that the probability of the Ca\(^{2+}\) channel being open in cells with a \(\Delta \Psi_m\) of -200 mV was 99% whereas a reduced \(\Delta \Psi_m\) of -80 mV had only 11% probability of the MCU allowing Ca\(^{2+}\) entry into the mitochondria (Kirichok et al., 2004, Hoffman et al., 2014). The MCU is regulated by MICU1 and MICU2 (Patron et al., 2014). MICU1 is involved in MCU opening in response to increases in extramitochondrial Ca\(^{2+}\) levels causing rapid increases in mitochondrial Ca\(^{2+}\) (Csordas et al., 2013). Whereas, MICU2 has an inhibitory effect ensuring MCU has a low affinity for Ca\(^{2+}\), preventing Ca\(^{2+}\) cycling and possible overload (Mallilankaraman et al., 2012, Patron et al., 2014). The MCU can be pharmacologically inhibited by ruthenium red (Mallilankaraman et al., 2012).

The mitochondrial sodium/ calcium exchanger (NCLX) is responsible for mitochondrial Ca\(^{2+}\) efflux (Boyman et al., 2013, Palty et al., 2010). It is localised to the inner mitochondrial membrane and has low affinity and high capacity for Ca\(^{2+}\) (Palty et al., 2010). NCLX expression has been linked to enhanced mitochondrial Ca\(^{2+}\) efflux (Palty et al., 2010). NCLX can be inhibited using CGP-37157 (Palty et al., 2010). The NCLX is electrogenic and is able to switch to reverse mode, pumping Ca\(^{2+}\) into the mitochondria and Na\(^+\) out, depending on the \(\Delta \Psi_m\) and cytosolic and...
mitochondrial matrix levels of Na\(^+\) (Kim and Matsuoka, 2008, Smets et al., 2004). Therefore under conditions where the \(\Delta\Psi_m\) is depolarised the NCLX does not aid in mitochondrial Ca\(^{2+}\) efflux (Smets et al., 2004).

1.3. **CELL/TISSUE OXYGENATION AND HYPOXIA**

Oxygen levels can vary throughout tissue and vessels. Physiologically normoxic O\(_2\) concentrations across a range of tissues have a partial pressures in the range of 10-45mmHg (Aragones et al., 2009). Individual cells generally have a range of O\(_2\) partial pressures between 10-20 mmHg (1.3 - 2.5 % O\(_2\)) with mitochondria having O\(_2\) partial pressures under 10 mmHg (under 1.3 % O\(_2\)) (Carreau et al., 2011). Oxygen gradients exist in tumours, the further the tumour is away from any blood supply the lower the O\(_2\) concentration will be, there are often regions with very low O\(_2\) concentrations almost reaching anoxia (Dmitriev et al., 2012).

There can be variation within a tissue or cell monolayer, with oxygen gradients between the extracellular and intracellular regions of the cell (Dmitriev et al., 2012). For this reason, it is important to know where you are measuring. There are a number of different approaches to quantify oxygen levels within tissues or cells. We can use oxygen sensitive probes to measure both intracellular and extracellular O\(_2\) levels (Fercher et al., 2011, O'Riordan et al., 2007, Zhdanov et al., 2010a, Dmitriev et al., 2012). In many tumours there is poor vascularisation, leading to an inadequate supply of O\(_2\), which results in hypoxic regions. Within these regions activation of a number of genes to aid in cell survival ensues, for example VEGF responsible for angiogenesis (Yasuda et al., 2004).

In addition to being a critical area of research in the cancer field, hypoxia is of great importance to those studying ischemia/reperfusion which can occur as the result of a stroke, cardiac arrest, neonatal hypoxic–ischemic encephalopathy in infants from complications during labour (Sanderson et al., 2013). The changes which occur as a result of ischemia cause damage, however the major injury is as a result of ROS created during reperfusion and opening of the mPTP following myocardial
ischemia/reperfusion (Schriewer et al., 2013). During ischemia there is a cessation of OxPhos which causes a reduction in the $\Delta \Psi_m$, depletion of ATP as a result, prevents ATP synthase working in reverse mode to restore the $\Delta \Psi_m$ (Sanderson et al., 2013). Therapies to reduce ischemia/reperfusion induced damage centre around reducing ROS production, or increasing antioxidant levels (Cheng et al., 2002, Valerio et al., 2011). The levels of cytosolic and mitochondrial Ca\textsuperscript{2+} are of great importance during ischemia/reperfusion, when cytosolic Ca\textsuperscript{2+} increases the mitochondria uptake the excess Ca\textsuperscript{2+} which can drive the opening of the mPTP and initiate apoptosis (Davidson et al., 2012).

P53, a tumour suppressor gene, has been implicated in many different cancers with a number of mutations in this gene are found (Muller and Vousden, 2013). P53 is involved in many processes including: cell cycle arrest, senescence, apoptosis and DNA repair (Kelly et al., 2003, Muller and Vousden, 2013, Speidel, 2010). P53 has been implicated to act both directly or indirectly with HIF in times of oxygen deprivation (Naito et al., 2010, Sermeus and Michiels, 2011). Depending on the duration and level of hypoxia P53 levels have been shown to change, either increasing as a result of interactions with HIF-1$\alpha$ or murine double minute 2 or decreasing through carboxyl terminus of Hsp70-interating protein mediated degradation (Naito et al., 2010, Sermeus and Michiels, 2011).

P53 accumulates as a result of DNA damage, hypoxia or oxidative damage and translocates to the mitochondria where it either inhibits or activates Bcl-2 family members (Speidel, 2010). Bcl-2 is pro-apoptotic and if activated directly causes mitochondrial outer membrane permeablisation, triggering caspase release and initiating the caspase cascade, resulting in apoptosis (Speidel, 2010). P53 facilitates apoptosis as a result of decreases in Guanosine-5'-triphosphate (GTP) after renal ischemia reperfusion in males Sprague-Dawley rats (Kelly et al., 2003).
1.3.1 HIFs

In hypoxia many transcription factors are activated, the major ones being hypoxia inducible factors (HIFs). HIFs exist as heterodimer complexes of α and β subunits, HIF-α levels are generally low under normoxic conditions as they are constantly degraded in the presence of O₂ by prolyl hydroxylases (PHD1, 2 & 3), which hydroxylate two prolyl residues in the oxygen-dependant degradation domain (ODD), this process occurs rapidly (Jewell et al., 2001). There are three isoforms of HIF-α, HIF-1α, HIF-2α and HIF-3α. Hydroxilation under normoxia occurs predominately by PHD2 (Berra et al., 2003a), silencing of PHD2 is adequate to allow stabilisation of HIF-α under normoxic conditions, whereas knockdown of PHD1 or PHD2 is not (Berra et al., 2003b). PHDs can be induced by HIF-1 creating a feedback loop. This process is shown in Fig 1.5.

Hydroxylated HIF-α undergoes proteasome-mediated degradation facilitated by VHL ubiquitin E3 ligase which tags HIF-1α with ubiquitin marking it for degradation (Pioli and Rigby, 2001). PHD hydroxylation is O₂ dependant, meaning HIF-α stabilisation occurs in hypoxic conditions. HIF-β is constitutively active and is present in two forms, HIF-1β also known as the aryl hydrocarbon receptor nuclear translocator (ARNT1) and HIF-2β or ARNT2. Once HIF-α is stabilised, it is translocated into the nucleus where is dimerises with HIF-β to form an active transcription factor which can go on to induce many cell survival and adaptation genes needed in order for the cells to survive the hypoxic environment. In hypoxic conditions oxygen levels decrease along with the activity of PHDs, allowing HIF-1α to accumulate (Demidenko and Blagosklonny, 2011), shown in Fig 1.5.
Under normoxia HIF constantly undergoes proteasomal-mediated degradation. In the presence of O₂ prolyl hydroxylases (PHDs) hydroxylate two prolyl residues in the oxygen-dependant degradation domain of HIF. Once hydroxylated, von Hippel-Lindau (VHL) ubiquitin E3 ligase tags HIF-1α with ubiquitin marking it for degradation proteasome-mediated degradation. Under hypoxic conditions the O₂ dependant PHDs no long function, meaning there is no VHL binding and HIF-α is stabilised. Once stabilised HIF-1α will translocate to the nucleus where it will form a dimer with HIF-1β. HIF-1α which has not undergone hydroxylation allows CREB-Bind protein/p-300 (CBP/p-300) recruitment, resulting in gene transcription.

Once activated HIFs are involved in the activation of a number of metabolic proteins including; GLUT1 and GLUT3, phosphofructokinase L, aldolase A, (Glyceraldehyde 3-phosphate dehydrogenase) GAPDH, phosphoglycerate kinase 1,
enolase 1 and LDH A (Wenger, 2002). HIF activation also stimulates numerous angiogenic factors such as VEGF providing blood supply and nutrients to a tumour. Both VEGF and erythropoietin (EPO) are expressed in neurons and are both neurotrophic and neuroprotective (Brines et al., 2000, Góra-Kupilas and Josko, 2005, Morishita et al., 1997, Storkebaum et al., 2004). However their roles are not only in the central nervous system, increases in mRNA expression of both EPO and VEGF have been implicated in persistent pulmonary hypertension of the newborn (PPHN) and cyanotic congenital heart disease (CCHD) (Lemus-Varela et al., 2010).

The VEGF family has a number of members, VEGF A-F and placenta growth factor (PIGF) (Keifer Jr et al., 2014). These are involved in angiogenesis, neuronal development, lymphangiogenesis and vascular permeability (Keifer Jr et al., 2014). VEGF plays an important role in response to ischemia in conditions such as heart disease or stroke (Liu et al., Lemus-Varela et al., 2010). VEGF gene therapy is currently under investigation for a number of conditions including: myocardial ischemia, amyotrophic lateral sclerosis (a progressive neurodegenerative disease), cerebral ischemia and peripheral arterial disease (Won et al., 2013, Keifer Jr et al., 2014, Isner et al., 1996).

EPO initially discovered as a hematopoietic growth factor is also regulated by HIF. EPO is of great importance to cardiac adaptation under hypoxia, both acute and chronic, through HIF-1α transcription (El Hasnaoui-Saadani et al., 2013). In normal physiology the kidney has renal EPO producing cells which are increased in number in response to decreases in O₂, in an oxygen dependent manner (Haase, 2013). During embryogenesis the liver is the primary site for EPO production, a function which is maintained in adults and EPO is produced in response to hypoxia or pharmacological induced HIF induction (Fried, 1972, Haase, 2013).

Translation of HIFs can be upregulated by Akt and Erk pathways (Koh et al., 2008). HIF-2α has been shown to be dependent on Akt2 and HIF-1α dependent on Akt3 (Toschi et al., 2008). AMPK is stimulated under hypoxic conditions inhibiting the activity of mTOR, a crucial element of cell growth and survival, which affects protein synthesis and transcription, thereby decreasing HIF production (Liu et al., 2006).
The activity of both HIF-1 and HIF-2 is dependent on mTOR, which is the catalytic subunit of mTORC1 and mTORC2, while HIF-1α requires both of these complexes for activation; mTORC2 is the only essential component for HIF2α activation (Toschi et al., 2008). mTORC1 is involved in anabolic processes, such as protein synthesis and is regulated by nutrients and growth factors. mTORC2 activates phosphoinositide-dependent kinase 1 which can activate the serine/threonine kinase Akt (Sarbassov et al., 2005, Porstmann et al., 2008, Yuan et al., 2013). Akt is an important metabolic regulator which can increase glucose uptake by inducing GLUT4 translocation and GLUT1 expression, it can also activate mTORC1, influencing HIF-1α activity (Karar and Maity, 2011).

HIF has been implicated in cancer and is correlated with a poor prognosis (Dong et al., 2013, Wan et al., 2010, Span et al., 2011). Tumours often have a hypoxic centre which can lead to metabolic changes. Metabolic substrates play important roles in cancer cells during hypoxia, high levels of glycolysis and glutaminolysis are important for cell survival. Glutamine and glucose are important in HIF-signalling during hypoxia, deprivation of one or both of these substrates can lead to inhibition of HIF-1α accumulation under hypoxic conditions (Kwon and Lee, 2005). Under glucose and oxygen deprivation, down-regulation of HIF signalling occurs as a result of depleted ATP levels, causing disruption of translational processes (Zhdanov et al., 2013). Increased expression of HIF-2α is observed in PC12 cells, and is strongly associated with development of pheochromocytoma (Span et al., 2011).

1.4. METHODS OF ASSESSMENT OF CELL METABOLISM AND BIOENERGETICS

Metabolic assessment can be used to perform toxicity testing, which is of vital importance for any potential therapeutic agent and in order to comply with regulations. Scientists need to be able to reduce the number of animals used, refine experiments so no unnecessary pain is caused to animals, using other means of testing materials. There is also a need to test non-lethal effects that compounds may have on cells, assessing the metabolic changes that may occur within the cell. There are many drugs that are known to cause mitochondrial toxicity, including
some chemotherapeutic agents, antibiotics and antiretroviral drugs. There are many different ways in which drug-induced mitochondrial toxicity can occur; inhibition of ETC complexes can occur by drugs such as antipsychotics or local anaesthetics. Mitochondrial toxicity can also be as a result of: ROS generation from doxorubicin, disruption to mitochondrial protein synthesis and biogenesis by antivirals or uncoupling reagents such as NSAIDs (Chan et al., 2005).

Mitochondrial toxicity can go undetected using tradition methods such as ATP, MTT and LDH assays. These assays only work to test cell viability; a compound may in fact be toxic but not lead to apoptosis or necrosis. Mitochondrial toxicity is one of the causes of late stage drug attrition in the pharmaceutical industry, so methods to assess this characteristic at an early stage of drug development are important (Zhang et al., 2009).

The cell energy budget (CEB) platform has many applications; it can be used to perform toxicity testing, to differentiate the utilisation of different metabolic pathways (bioenergetics), and to perform in-depth metabolic studies. The CEB system can determine the contributions of the main metabolic pathways of OxPhos, Krebs cycle and glycolysis to ATP production. The platform uses O$_2$ and pH sensitive probes to assess the different metabolic pathways (Hynes et al., 2009a, O’Riordan et al., 2007, Zhdanov et al., 2011b).

Both within our lab and outside, it has been shown that the CEB platform can be used to assess mitochondrial toxicity in both eukaryotic cells and bacteria (Hynes et al., 2006, Jasionek et al., 2010, Bansal et al., 2013, Beeson et al., 2010).

1.4.1 Traditional methods

Earlier research consisted of measurement on both intact cells and isolated mitochondria in vitro. Measurements include: trypan blue exclusion, NAD(P)H, ATP, ADP/ATP ratio, pmf, ΔΨ$_m$, Ca$^{2+}$ and ROS fluorescent indicators (Kadish et al., 1968). These measurements do not give information on the contribution of different metabolic pathways and may miss changes due to compensatory up-regulation of
other metabolic pathways, for example in the case of mitochondrial toxicity up-regulation of glycolysis occurs to compensate for decrease OxPhos.

Previous oxygen consumption methods used O₂ electrodes to measure reductions in dissolved O₂. The Clark type O₂ electrodes are comprised of a platinum cathode and anode connected via an electrolyte solution (Brand and Nicholls, 2011b). Reduction of O₂ results in an electrical current which can then be converted into a concentration of dissolved O₂ (Brand and Nicholls, 2011b). For this technique isolated mitochondria are used and manipulations are performed. Addition of ADP is used to achieve state 3 respiration which can be used as a measure of OxPhos capacity. State 4, a resting rate of respiration can be accomplished by adding oligomycin to eliminate ATP turnover (Rosenthal et al., 1987, Berman and Hastings, 1999). A final State 3, in which a rate of uncoupled respiration can be measured is provided by FCCP treatment (Rosenthal et al., 1987, Berman and Hastings, 1999). With these measurements the respiratory control rate (RCR) can be calculated which is the rate of state 3 respiration divided by the rate of stage 4 respiration (Rosenthal et al., 1987). The main disadvantage is that this technique is predominately performed on isolated mitochondria and a constant stirring of the sample is required.

The use of intact cells provides a more informative and complex picture of events with greater physiological relevance. Fluorescence live cell imaging can allow monitoring of ΔΨᵢ, ΔΨₘ and Ca²⁺ or ROS indicators (Zhdanov et al., 2011a, Zhdanov et al., 2010b).

Colourmetric techniques are available for glucose measurement using an enzymatic procedure studying glucose oxidation by glucose oxidase (Kadish et al., 1968). Glycolytic activity can be estimated measuring ATP levels with the ATP synthase inhibitor, oligomycin. The level of respiration decreases and the rate of glycolysis is up-regulated, in this case the majority of ATP produced will be from glycolysis (Brand and Nicholls, 2011b). Oligomycin blocks the transport of protons through ATP synthase, thereby inhibiting ATP production via OxPhos.
Glycolytic rates can be assessed in perfused heart tissue by monitoring $^3$H$_2$O production from [5-$^3$H/U-14C]-glucose addition. Metabolism causes $^3$H to be transformed into water and labelled glucose to be converted into glucose-6-phosphate (Neely et al., 1972). $^{14}$CO$_2$ and $^3$H$_2$O production can be measured determining glucose oxidation and glycolysis respectively (Lopaschuk et al., 1993). Another measure of glycolytic activity is to measure glucose uptake using the radio-labelled glucose analog 2-deoxyglucose which can be metabolised into glucose-6-phosphate (Zhao et al., 2008). This technique can be performed on both adherent and non-adherent cells (Zhao et al., 2008). Measurement of glucose transporters can provide information about glucose uptake, GLUT1 protein levels can be accessed via Western blot. Increased GLUT1 protein levels can indicate increased levels of glycolysis, it is also a commonly used hypoxia marker, increased in O$_2$ deprivation conditions (Meijer et al., 2012).

Assessment of the pmf can also give indications as to mitochondrial activity. It is split into two components, $\Delta \Psi_m$ and pH gradient, the former is more dominant contributor (Brown and Brand, 1986). Experiments using the ionophore nigercin allow measurement of pmf solely from $\Delta \Psi_m$. Indirect methods are required to measure $\Delta \Psi_m$ which involve adding lipophilic membrane-permeant cations, such as TPP$^+$ (tetraohenylphophonium ion) monitoring cation uptake via fluorescence or absorbance (Brown and Brand, 1986).

1.4.2 New approaches

Each metabolic pathway has a considerable spare capacity which can be utilised when another pathway is not functioning optimally or in times of increased energy demand. A schematic of the interconnecting metabolic pathways is shown below is Fig. 1.6, which illustrates how each metabolic pathway does not work to full capacity. Therefore one pathway can increase ATP production to compensate for decreased ATP synthesis by another to maintain constant ATP levels. As shown in Fig. 1.6, lactate levels give an indication to glycolytic activity; CO$_2$ levels can show Krebs cycle activity and measuring O$_2$ levels and the oxygen consumption rate.
(OCR), the contribution of OXPHOS to ATP production can be elucidated. The relative contribution of glycolysis, Krebs cycle and OxPhos varies between cell types and conditions. Metabolic malformations or disorders can result in altered metabolic pathway contributions.

![Diagram of the main metabolism pathways in mammalian cells.](image)

**Figure 1.6. Diagram of the main metabolism pathways in mammalian cells.**

Metabolic pathways work together to maintain a constant supply of ATP for the cell. Each pathway has a spare capacity which can be utilised in times of energy stress in other metabolic pathways. For example in hypoxia where OxPhos is down regulated glycolysis is up-regulated to maintain constant ATP levels within the cell.

New approaches for measuring metabolic pathways within cells give a more comprehensive overview of cellular metabolism. One more modern approach is the XF system in which mitochondrial respiration and glycolysis in live cells can be measured using solid-state sensors, enabling the measurement of both OCR and ECA simultaneously (Laderoute et al., 2014). One disadvantage of this system is that it is costly, a special machine (XF analyser) with XF microplates has to be purchased to use this system (Invernizzi et al., 2012, Wu et al., 2007).

There are several systems available for measuring respiratory activity in cells by optochemical sensing approaches (Domenis et al., 2011, Gero et al., 2014, Comelli
et al., 2011). One such system allows O$_2$ measurement in differing O$_2$ ranges from physiological down to hypoxic levels (Trumbeckaite et al., 2013). This system can also be combined with fluorometry allowing measurement of ROS, Ca$^{2+}$, ATP, pH or ΔΨ$_m$. This system is also expensive, requiring the purchase of a special Oxygraph-2k instrument (Trumbeckaite et al., 2013, Comelli et al., 2011). Within our lab, the CEB platform has been developed which is a useful series of methods for investigation of perturbed metabolism and mitochondrial and glycolytic disorders. With this system investigation of changes in the contribution of different metabolic pathways to ATP generation by studying various fluxes of each system is possible (Zhdanov et al., 2011a, Zhdanov et al., 2011b).

In our system the level of cellular respiration can be assessed by measuring oxygen levels using either extracellular or intracellular Pt-porphyrin based phosphorescent probes (Dmitriev et al., 2012, Fercher et al., 2011). Optical signals of these probes can be read conveniently in standard 96 well microtitter plates on a conventional time–resolved fluorescence plate reader. Phosphorescence intensity values measured at two delay times can be converted into lifetime (LT) values (Eq. 1.1) and then into O$_2$ concentration or OCR values with an equation obtained from probe calibration (Eq. 1.2 and 1.3) as shown in Fig 1.9. The probes are reversibly quenched by O$_2$, so as the O$_2$ levels in the sample are depleted by cellular respiration probe signal increases.

$$\tau = (t_2 - t_1) / \ln(F_1/F_2)$$  \hspace{1cm} (Eq. 1.1)

For the intracellular O$_2$ probe MitoXpress-Intra®, loading occurs as shown in Fig. 1.7. O$_2$ is calculated using eq. 1.2 which was obtained from calibrations performed in the laboratory. O$_2$ is calculated as µM per well in a 96 well plate, 4-8 wells are used per condition and at least 3 repeat experiments are conducted and the averages calculated.

$$O_2 = 18576 \exp(-LT/6.8794) \quad (r^2 = 0.9863) \quad (Dmitriev et al., 2012)$$ \hspace{1cm} (Eq. 1.2)
Figure 1.7. Diagram of well in a 96 well plate loaded with MitoXpress-Intra.
The MitoXpress-Intra probe is a cell penetration nanoparticle probe.

MitoXpress-Xtra® extracellular O$_2$ probe (OCR measurement), loading occurs as shown in Fig.1.8 and O$_2$ is calculated using:

\[ O_2 = 4455.46 \exp(-LT/7.48284) \quad (r^2 = 0.99325) \quad (\text{Dmitriev et al., 2012}) \]

(Eq. 1.3)

Figure 1.8. Diagram of well in a 96 well plate loaded with MitoXpress-Extra.
The MitoXpress-Extra probe is located in the media and the well is sealed with mineral oil to prevent O$_2$ diffusion.
Figure 1.9. **Processing OCR data A.** Raw phosphorescence lifetime (LT) values for MEF cells treated with water (control), AA (antimycin A) (negative control) and test sample (PEG1100 NP). AA treatment inhibits respiration giving the lowest LT and highest O₂ value. LT Values are then converted into **B.** Extracellular O₂ values using equation 1.3 obtained from calibration. AA values are steadily maintained at 200 µM showing no oxygen consumption, PEG1100 treated cells have a steeper rate of O₂ consumption compared with the control. **C.** The slopes are then calculated and OCR determined, these show the greater OCR in the PEG1100 treated cells compared with the control indication up-regulation of OxPhos under these conditions.

The level of cell oxygenation is inversely related to the respiratory activity. The measurement of extracellular O₂ allows assessment of the oxygen consumption rate (OCR) whereas measuring intracellular O₂ provides accurate quantitative real-time intracellular O₂ values, whilst allowing transient changes to be observed.
Using the CEB platform, extracellular acidification (ECA) is measured using a long-decay emitting pH-sensitive lanthanide probe. There are two assay formats used, lactate-related (L-ECA) and total-ECA (T-ECA). In the T-ECA a layer of heavy oil covers the wells of on the 96 well plate and combined extracellular acidification generated by CO₂ from the Krebs cycle and as a result of lactate production from glycolysis is measured. It should be noted that small amounts of CO₂ are also produced from the pentose phosphate pathway (Wamelink et al., 2008). L-ECA performed without oil allows CO₂ to escape in the atmosphere, measuring only the contribution of lactate to extracellular acidification, as shown in Fig.1.10 (Hynes et al., 2009b, Zhdanov et al., 2011b). With dual measurement of L-ECA and T-ECA the contribution of glycolytic and non-glycolytic ECA can be elucidated. The phosphorescence LT of the pH-Xtra probe increases with [H⁺] elevation and decreased pH. The resulting LT signals can be converted into pH values using Eq. 1.4, obtained from probe calibrations.

\[ \text{pH} = \frac{1893.4 - \text{LT}}{227.54} \]  

(Eq. 1.4)

**Figure 1.10. Diagram of wells in a 96 well plate loaded with pH-Extra.**

Lactate-ECA (L-ECA) had an open system and the probe is in the media, however total-ECA (T-ECA) a layer of mineral oil seals the well preventing CO₂ diffusion allowing measurement of lactate and CO₂.

pH is then converted into H⁺ and the rate calculated.

The CEB measurements are usually combined with ATP assessment using a luminescent kit (Promega CellTiter-Glo®Luminescent Viability Assay). Together with
ATP measurement, the CEB platform allows real time measurement of metabolic changes. Stimulations with pharmacological reagents which allow maximal (FCCP) and minimal (AA) respiration to be observed and which also shifts glycolytic pathway function. Oligomycin can be used to inhibit OxPhos by blocking ATP synthase function; however this causes an increase in pmf. Dual treatment of FCCP/oligomycin allows respiratory activity with uncoupled mitochondria to be assessed without compensatory hydrolysis of ATP by ATP synthase to maintain $\Delta \Psi_m$.

As demonstrated by the investigation of the role of enzyme fumarate hydratase (FH) in cellular ATP production using FH knockdown MEF cells, the CEB platform is an effective tool for assessing mitochondrial defects (O'Flaherty et al., 2010). A striking decrease in oxygen consumption was observed in FH$^{-/-}$ cells compared with wild type (WT) cells. Uncoupling of these cells did not increase OCR but a two fold increase was seen in WT cells (O'Flaherty et al., 2010). A compensatory increase in glycolytic flux was observed in these cells, illustrated by increase L-ECA, which increased further upon uncoupling of the mitochondria (Zhdanov et al., 2011b). Manipulation of culture medium (glucose + or -) was able to distinguish differences in ATP responses in FH$^{-/-}$ and WT cells (O'Flaherty et al., 2010). These results show practical use of the CEB platform for bioenergetics research.
CHAPTER 2. MATERIALS AND METHODS
2.1 Materials

O₂-sensitive probes MitoXpress®-Xtra, MitoXpress®-Intra NanO₂ and pH-sensitive probe pH-Xtra were from Luxcel Biosciences (Cork, Ireland). Glutaminase inhibitor, BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide) 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 (PMP1) was from Molecular Devices (Sunnyvale, CA). Amersham 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), PhosStop Phosphatase Inhibitor and complete Protease Inhibitor Cocktail Tablets were from Roche (Ireland), BCA™ Protein Assay kit and PageRuler Plus Prestained Protein Ladder were from Thermo Fisher Scientific (Rockford, Ill). The mitochondria-targeted Ca²⁺ biosensor, mitoCase12 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). SMARTpool: ON-TARGETplus PDK1 siRNA and ON-TARGETplus Non-targeting siRNA #1 from Dharmacon, Thermo Scientific. SV Total RNA Isolation System, ImProm-II™ RT System and PCR Master Mix were from Promega (Madison, WI). Dulbecco's Modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) media, McCoy's 5A modified media, nerve growth factor (NGF), collagen IV, FCCP, D-glucose, D-galactose, L-glutamine, sodium pyruvate, HEPES, fetal bovine serum, horse serum, oligomycin, antimycin A and other reagents were from Sigma-Aldrich.
### Table 2.1 Cell seeding densities used.

<table>
<thead>
<tr>
<th>Cells</th>
<th>96 well (cells/well)</th>
<th>12 well (cells/well)</th>
<th>6 well (cells/well)</th>
<th>1 cm² dish (cells/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20.9% O₂</td>
<td>3% O₂</td>
</tr>
<tr>
<td>PC12</td>
<td>5*10⁴</td>
<td>5*10⁵</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>MEF</td>
<td>4*10⁴</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>HCT116</td>
<td>4*10⁴</td>
<td>n/a</td>
<td>1.2*10⁵</td>
<td>3*10⁵</td>
</tr>
<tr>
<td>HCT116 SCO2/-</td>
<td>4*10⁴</td>
<td>n/a</td>
<td>2*10⁴</td>
<td>3*10⁵</td>
</tr>
</tbody>
</table>

### Table 2.2 Constituents of working media.  Nerve growth factor was only added to media used for PC12 cells to cause differentiation in these cells making them a neuronal cell modal. All media were serum-free and all but the media used for ECA experiments contained 20 mM HEPES (pH 7.2).

<table>
<thead>
<tr>
<th>Components</th>
<th>Working media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>10 mM D-glucose</td>
<td>+</td>
</tr>
<tr>
<td>10 mM D-galactose</td>
<td></td>
</tr>
<tr>
<td>2 mM L-glutamine</td>
<td>+</td>
</tr>
<tr>
<td>1 mM Pyruvate</td>
<td>+</td>
</tr>
<tr>
<td>100 ng/ml Nerve growth factor</td>
<td>+</td>
</tr>
</tbody>
</table>
### Table 2.3 Cellular stains for microscopy measurements.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Concentration</th>
<th>Function</th>
<th>Staining Procedure</th>
<th>Excitation, emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitocase12</td>
<td>5 ng/ml</td>
<td>Stains mitochondrial Ca(^{2+})</td>
<td>Lipofectamine transfection</td>
<td>488nm, 500-540nm</td>
</tr>
<tr>
<td>Fluo4-AM</td>
<td>2.5 µM</td>
<td>Stains cytosolic Ca(^{2+}) (\text{Ca}^{2+})</td>
<td>Add 2.5 µM for 60 min, wash, leave for 30 min and measure</td>
<td>488nm, 500-540nm</td>
</tr>
<tr>
<td>TMRM</td>
<td>20 nM</td>
<td>Stains ΔΨm</td>
<td>Add 20 nM 30 min prior to measurement</td>
<td>543nm, 555-600nm</td>
</tr>
<tr>
<td>PMP1</td>
<td>20 nM</td>
<td>Stains ΔΨp</td>
<td>Add 20 nM 30 min prior to measurement</td>
<td>543nm, 555-600nm</td>
</tr>
<tr>
<td>JC1</td>
<td>2.5 µM</td>
<td>Stains ΔΨm</td>
<td>Add 2.5 µM for 30 min, wash and measure</td>
<td>488nm, 500-540nm</td>
</tr>
<tr>
<td>mtAlpHi</td>
<td>5 ng/ml</td>
<td>Intracellular pH</td>
<td>Lipofectamine transfection</td>
<td>488nm, 500-540nm</td>
</tr>
</tbody>
</table>
### Table 2.4 Antibodies used for Western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>M.W. (kDa)</th>
<th>Secondary</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>118</td>
<td>Goat</td>
<td>R &amp; D</td>
</tr>
<tr>
<td>Hif-2α</td>
<td>120</td>
<td>Mouse</td>
<td>R &amp; D</td>
</tr>
<tr>
<td>pPDH E1-α subunit (Ser293)</td>
<td>43</td>
<td>Rabbit</td>
<td>Abcam</td>
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<tr>
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### Table 2.5 Immunostaining Antibodies

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<td>55</td>
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2.2 METHODS

2.2.1 Cell Culture

Rat pheochromocytoma PC12 cells, human colon cancer HCT116 cells and mouse embryonic fibroblasts (MEFs) were obtained from American Tissue Culture Collections (ATCC, Manassas, VA). A mutant cell line, HCT116 SCO2−/−, which is deficient in synthesis of cytochrome c oxidase SCO2, the assembly unit for complex IV of the ETC, were kindly donated by P. M. Hwang (NIH). The HCT116 SCO2−/− cells were created using targeting vectors which were delivered by recombinant adenovirus (Sung et al., 2010). Both alleles of the SCO2 gene were disrupted in the HCT116 cell line creating a homozygous knockout.

MEF cells were normally cultured in Dulbecco’s Modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 10 mM HEPES (pH7.2), 2 mM L-glutamine (L-Gln), 100 U/ml penicillin /100 µg/ml streptomycin (P/S). PC12 cells were grown in RPMI 1640 medium supplemented with 10 mM HEPES (pH7.2), 2 mM L-Gln, 10% horse serum (HS), 5% FBS and P/S. Once seeded PC12 cells were grown in RPMI medium supplemented with 1% HS, HEPES (pH7.2), 2 mM L-Gln, P/S and 100 ng/ml nerve growth factor (NGF) and left to differentiated for 5 days prior to the start of each experiment (differentiation made PC12 cells into a neuronal cell modal.). HCT116 and HCT116 SCO2−/− cells were grown in McCoys medium supplemented with HEPES, L-glutamine, 10% FBS and P/S. All cells were grown in humidified atmosphere of 5% CO2 and 95% air at 37°C unless otherwise stated.

For experiments in 96 well plates, all wells were coated with 0.01% collagen IV. MEF cells were seeded at a density of 4*10⁴ cells /well, PC12 cells were seeded at 5*10⁴ cells /well, HCT116 and HCT116 SCO2−/− cells were seeded at 2.5-4.0*10⁴ cells /well. For protein isolation of PC12 cells were seeded in a 12 well plates at a density of 5*10⁵ PC12 cells / well. HCT116 cells were seeded in 6 well plates at a density of 1.2*10⁵ cells / well at 20.9 % O₂ and a density of 3*10⁵ when grown under 3% O₂, HCT116 SCO2−/− cells were seeded at 2*10⁵ cells / well under 20.9 % O₂ and 3*10⁵ cells / well at 3% O₂ (different densities to account for differences in growth under different O₂ conditions). For siRNA transfection HCT116 and HCT116
SCO2−/− cells were grown at either atmospheric (20.9 %) or hypoxic (3 %) O₂ for 3 days and then seeded on day 4 in 6 well plates. Cells were then grown in respective O₂ environments for a further 3 days. Different seeding densities in different cell lines and plates are shown in Table 2.1.

Working media (WM) was used in metabolic substrate experiments. It was made using reconstituted DMEM powder in Milli-Q water and filter sterilised. Twelve different WM were made using different combinations of 10 mM glucose (Glc), 10 mM galactose (Gal), 2 mM glutamine (Gln) and 1mM pyruvate (Pyr), all media contained 100 nM NGF and all were serum-free. All but the media used for ECA experiments contained 20 mM HEPES (pH 7.2). WM are show in table 2.2. The medium was replaced at the start of each experiment and the cells grown for 2 hours.

2.2.1.1 Fluorescent Microscopy

For microscopy, PC12 cells were seeded at a density of 2.5*10⁴ cells/ 1cm² on glass bottom Matek mini-dishes with a collagen IV (0.0075) and poly-D-lysine mix coating and differentiated for 5 days with NGF. HCT116 cells were seeded at 1.5*10⁴ cells/dish and HCT116 SCO2−/− cells were seeded at 2.2*10⁴ cells / dish.

All probes were loaded in serum free OPTIMEM except TMRM and PMPI which were maintained in the cells normal media at 20 nM for the duration of the experiment. Transfection was performed using Lipofectamine-2000 and serum free OptiMEM medium for Mitocase12 and mtAlpHi. The probe was diluted to a concentration of 5 ng/ml in serum free OPTIMEM, in a separate tube 1 µL lipofectamine-200 was added to 40 µl of serum free OPTIMEM. The tubes were left for 5 minutes and then combined. The combined mixture was left for a further 20 minutes to allow complexes to form. In the meantime the cells were washed with serum free OPTIMEM 3 times and the media was replaced with serum free OPTIMEM (160 µl/dish). Once probe/lipofectamine complexes had formed the solution was added drop-wise onto the dish (40 µl/dish). The dishes were left for 10-14 hours and then the media was replaced with normal seeding medium. Measurements were then conducted (excitation 488 nm, emission 500-540).
To stain cells for cytosolic Ca\(^{2+}\) 2.5 µM Fluo4-AM was used, the probe was diluted in serum free OPTIMEM and left for 60 minutes, the cells were then washed with normal seeding media 3 times and the left for 30 minutes and measured (excitation 488 nm, emission 500-540 nm). The process was the same for JC1 (ΔΨm stain). To assess ΔΨm and ΔΨp, TMRM and PMPI were used (20 nM), staining started 30 minutes prior to measurement and probes were maintained at 20 nM throughout the experiment (excitation 543 nm, emission 555-600 nm). Staining was conducted in normal seeding medium.

A list of probes, function and excitation and emission wavelengths can be found in Table 1. Microscopy measurements were conducted using an Olympus FV1000 confocal laser scanning microscope under controlled conditions of CO\(_2\), humidity and temperature. In all experiments the differential interference contrast and fluorescence images were collected kinetically with a 60× oil immersion objective in two planes using 0.5 µm step and 20–30 s intervals. The resulting z-stacked images were analysed using FV1000 Viewer software (Olympus), Excel, Adobe Photoshop and Illustrator. The staining details are described in table 2.3.

Immunofluorescence analysis of PC12 cells was conducted in Ibidi chambers. Cells were washed 3 times with PBS, fixed with 4% paraformaldehyde in PBS for 10 minutes, then washed 3 times with PBS, quenched with 50 mM NH\(_4\)Cl/PBS for 15 minutes. Cells were then washed with PBS 3 times, permeabilised with 0.25% TritonX-100 for 10 minutes, blocked with 5% FBS/PBS for 30 minutes, incubated with Novus anti-HIF-1α (1 in 200 dilution) primary made up in 5% FBS/PBS for 50 minutes. Cells were washed 5 times with PBS and then incubated with Alexa-fluorescent 488 conjugated secondary antibodies (1 in 500 dilution) for 50 minutes in the dark, and washed 5 times with PBS. Cells were counterstained with 300 nM DAPI for 10 minutes, washed once with PBS, dipped in deionised water and mounted with ProLong® Gold and air-dried (Table 2.5). The intensity and localisation of HIF-1α was analysed on an Olympus FV1000 confocal laser scanning microscope. DAPI and Alexa-488 images were collected with a 60X oil immersion objective in eight planes with 0.5 µm steps in sequential mode, using standard excitation and emission wavelengths. The resulting single plane (DIC) and z-stacked
fluorescent images were analysed using FV1000 Viewer software (Olympus), Adobe Photoshop and Illustrator. Assistance was given with the microscopy from Dr Alexander Zhdanov.

2.2.1.2 siRNA transfection

Transfection of siRNA was conducted on cells grown in either 6 or 12 well plates. Cells were washed with antibiotic and serum free OPTIMEM medium and transfected using Lipofectamine-2000 as stated above in 2.2.1.1. Concentrations of 10 nM siRNA or control scrambled siRNA were used. Serum was added 4 hours after transfection and media replacement to complete media was done after approximately 16 hours. The cells were then grown for 72 hours and either protein or RNA isolated. For siRNA transfection under hypoxic conditions, cells were placed into the hypoxia workstation set to 3% O₂ on day 1, cells were then seeded on day 3, transfection took place on day 4 and isolation of either protein or RNA was conducted on day 7, 72 hours post transfection.

2.2.2 Cell Based Assays

2.2.2.1 Oxygen Consumption Rate (OCR)

OCR is measured using the phosphorescent O₂-sensitive probe, MitoXpress. Cells were grown and treated as stated; media was then replaced with 100 µl of air-equilibrated medium containing 200 nM MitoXpress probe. The wells were covered in 150 µl pre-warmed mineral oil to provide an O₂-impermeable seal. The plate was then quickly read on a TR-F reader Victor 2 (PerkinElmer Life Sciences) pre-set at 37°C and measured at 20.9% or 4% atmospheric O₂ (i.e. with TR-F reader placed in the hypoxia chamber). Each sample was measured every 3-5 min for over 60 min. Two intensity values were taken at delay times of 30 and 70 µs with a gate time of 100 µs. Intensity signals were converted into phosphorescence lifetime (τ) values as follows: \( \tau = (t_2-t_1)/\ln(F_1/F_2) \), where \( F_1 \), \( F_2 \) are the TR-F intensity signals at delay times \( t_1 \) and \( t_2 \). Initial rates of cell deoxygenation were calculated as
nmole/min per 10^6 cells. Average O_2 levels were calculated and then OCR was calculated O_2 consumed by cells in 1 min per 1 mg of total soluble protein.

2.2.2.2 Extracellular Acidification (ECA)

Cells were seeded in 96 well plates coated with 0.01 % collagen IV, PC12 cells were differentiated for 5 days and MEF, HCT116 and HCT116 SCO2^-/- cells were left for 24 hours after seeding. Firstly cells were washed with and then replaced with 150 µl un-buffered DMEM supplemented with 1 mM sodium pyruvate, 10 mM glucose and 2 mM L-glutamine (not the case for different substrate work). Cells were then incubated under CO_2 free conditions at 37ºC for 2-2.5 hours. Medium was then replaced with buffered DMEM containing 10 mM HEPES, 1 mM sodium pyruvate, 10 mM glucose and 2 mM L-glutamine and placed back into the CO_2 free incubator for 30 min – 1 hour. After a period of at least 3 hours under CO_2-free conditions the medium was changed to 100 µl buffered DMEM which contained 1 µM pH-Xtra probe. For measurement of lactate-ECA (L-ECA) the plate was then read on a TR-F reader Victor 2 (PerkinElmer Life Sciences) pre-set at 37ºC and measured at 20.9 %, 4 %, 3 % or 1 % atmospheric O_2. To measure Total-ECA (T-ECA) a layer of 150 µl of pre-warmed to 37ºC heavy mineral oil was added to seal the plate after the addition of the medium with probe, immediately prior to measurement. The plate was read for at least 1 hour using 340 ± 35 nm excitation and 615 ± 8.5 nm emission, two intensity signals were measured at delay times of 100 and 300 µs with a measurement window of 30 µs. Intensity values were converted into probe fluorescence life-time (τ) values τ=(t_2-t_1)/ln(F_1/F_2), t_1 and t_2 relate to the two delay times and F_1 and F_2 the intensity signals at those corresponding times, τ is then converted into pH and then H^+ values.

2.2.2.3 Intracellular Oxygen (iO_2)

Cells were seeded in 96 well plates coated with 0.01% collagen IV, after 24 hours for MEF and HCT116 cells and 4 days of differentiation for PC12 cells, medium which contained 10 µg/ml NanO2 probe was added to the cells, cells were then incubated for further 16-24 hours at 37ºC. After which cells were washed with 150 µl medium, then replacement with 300 µl fresh medium was done, and the plate
was read on a TR-F reader Victor 2 (PerkinElmer Life Sciences) pre-set at 37°C and measured at 20.9 %, 4 % or 3 % atmospheric O₂ (i.e. with TR-F reader placed in the hypoxia chamber). Each sample well was measured every 3-5 min over 6 hours, taking two intensity readings at delay times of 30 and 70 µs and gate time 100 µs (excitation/emission: 340/642 nm). Intensity signals were converted into lifetime using the equation above (OCR section). There is no correction for back diffusion, experiments conducted at 21% O₂ and 4% O₂ are not directly compared so corrections for differences in potential back diffusion in various O₂ conditions are not required.

2.2.2.4 ATP

ATP levels were assessed using Promega CellTiter-Glo assay. Cells were grown on 96 well plates and treated as stated, equal amounts of CellTiterGlo reagent as media in the well was added to each well to lyse the cells. The plate was then shaken for 2 min and each sample was then transferred into a white well plate and read on Victor 2 plate reader under standard luminescence settings. For all metabolic assays protein concentrations were assessed using BCA protein assay kit. Data was then corrected to protein content for each sample (BCA assay is described below in 2.2.3.1).

2.2.2.5 Nanoparticle Toxicity Testing

Two sets of nanoparticles (NPs) were assessed, firstly bare molecular imprinted polymer (MIP) NPs made up of methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TRIM), pentaerythitol-tetrakis-(3-mercaptopropionate) (PETMP) and N,N-Diethyldithiocarbamic acid benzyl ester and then with a poly(ethylene glycol) (PEG) methacrylate shell with a molecular weight or either 1100 or 4000 kDa (Poma et al., 2013, Moczko et al., 2013). Secondly a group of MIP NPs which dissolved in H₂O (100 ml) in the presence of 30 g of trypsin-derivatised glass beads and composed of 39mg N-isopropylacrylamide (NIPAm), 2 mg N,N'-Methylenebisacrylamide (BIS), 33 mg N-tert-Butylacrylamide (TBAm) and 2.2 µL Acrylic acid. These MIP NPs are referred to as Tyrpsin NPs, some fluorescent Rhod/Trypsin NPs were made by
addition of 1% in mol (compared to the total moles in solution) of Methacryloxyethyl thiocarbamoyl rhodamine to the Trypsin NPs.

2.2.3 Protein Isolation and Western Blot analysis

Cells were seeded in either 12 or 6 well plates. After treatment cells were placed on ice and washed twice with PBS and then lysed with lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1 % IGEPAL CA-630, 50 mM HEPES (pH = 7.5) phosphatase inhibitors and protease inhibitors. Plates were kept on ice on a shaker for 15 minutes and then scraped collecting the lysate. Lysate was centrifuged for 10 min at 16,000 g at 4°C. Protein concentrations were measured by BCA protein assay kit and lysates normalised. Either 8 % or 4-20 % polyacrylamide gel electrophoresis was used to separate the proteins, which were then transferred onto a 0.2 µm PVDF membrane using a wet mini-transfer system and blocked with either 5 % milk /TBST or 5 % BSA/TBST depending on the primary antibody used, for 2 hours at room temperature. Incubation with primary antibodies was conducted in either 5 % milk /TBST or 5 % BSA/TBST at 4°C overnight and then 2 hours at room temperature for horse radish peroxidise conjugated secondary antibodies. Visualisation of the blots was done using ECL prime reagents using a LAS-3000 imager (FujiFilm) with Image Reader LAS-3000 2.2 software. Data was analysed quantitatively using Image J software, normalising signals to α-tubulin. Images were processed using Picasa, Photoshop and Illustrator programs. A list of antibodies used for Western blotting can be found in Table 2.4.

2.2.4 BCA Protein Assay

Prior to preparation of lysates for Western blot, protein concentrations were quantified using the Thermo Fisher Scientific BCA Protein Assay Kit, as per manufactures instructions. Protein concentrations were tested along with a range of standards, a working reagent was added and the plate incubated at 3 °C for 30 min, absorbance was then measured at 562 nm. Protein concentrations were
calculated from the standard curve and concentrations normalised to the same level. Protein concentrations were also obtained from iO$_2$, oxygen consumption rate and extracellular acidification experiments and the data normalised according to concentrations.

2.2.5 RNA Isolation and Reverse Transcription and q-PCR

RNA was isolated using Macherey Nagel/ Fisher NucleoSpin RNA II kit. Cells were washed with PBS and then isolated according to kit guidelines.

Reverse transcription (RT) was completed using a Promega kit as per manufactures protocol. Briefly, RNA was diluted to 1.5 µg in Nuclease-free water and 0.5 µg oligo(dT)$_{15}$ primers added, tubes were then incubated at 70 °C for 5 min followed by 5 min on ice. An RT buffer containing, 2 mM MgCl$_2$, 0.5 mM dNTP, 1 unit RNase inhibitor and 160 units reverse transcriptase was added to the RNA and incubated at 42 °C for 1 hour. The reverse transcriptase was then heat inactivated by incubating the tubes at 70°C for 15 min. A negative control without RNA added was also done.

Real-Time PCR experiments were conducted using the Maxima SYBR green/qPCR Master Mix kit. Each well contained; 150 ng cDNA, 0.325 µM of forward and reverse Primer, 10 µl Maxima SYBR Green qPCR Master Mix (2x), 0.5 µM ROX and made up to 20 µl with nuclease-free water. Real-time PCR was then performed on an Applied Biosystems 7300 Real Time PCR System using the following settings; stage 1, 10 min at 95 °C, stage 2, 45 cycles of; 95 °C for 15 sec denaturation, 60 °C for 20 sec annealing and 72 °C for 28 sec extension. A total sample volume of 20 µl was used with primer concentration of 0.325 µM. A list of primers used for q-PCR can be found in Table 2.5.

2.2.6 Statistical Analysis

Statistical analysis was performed using the results of 3–6 independent experiments using the programs, Excel and Minitab. Confidence levels of 0.05, 0.01 and 0.001 were deemed as statistically significant. The differences between mean
values were assessed by 2-tailed Student t-test once equality of variance among samples were checked by the Levene’s test. The differences in iO₂, TMRM, Mitochondrial Ca²⁺ (in Δ%) were evaluated using Mann–Whitney U-test. To ensure accurate data, the majority of the experiments were performed in 3–8 replicates, and repeated at least 3 times. The ECA, OCR, iO₂ and ATP levels were normalised to the total protein content in the samples. Fluorescence intensities on the confocal images (TMRM, mitoCase12, Fluo4) were examined in kinetic mode analysing 5–20 cells in 3 independent experiments. The differences between the mean values were evaluated using two-tailed Student t-test. q-PCR data was normalised to β-actin levels and corrected to normoxic WT control, averages were made of at least 3 separate experiments. Western blots were quantified using ImageJ and normalised to α-tubulin levels. If phosphorylated proteins were measured these were normalised to total protein level of the corresponding protein.
CHAPTER 3. APPLICATION OF CELL ENERGY BUDGET PLATFORM IN TOXICOLOGICAL AND METABOLIC STUDIES WITH CELL MODELS
3.1 Abstract

The cell energy budget system can determine the contributions of the main metabolic pathways of oxidative phosphorylation, Krebs cycle and glycolysis to ATP production using O\(_2\) and pH sensitive probes. Glycolysis, glutaminolysis, the Krebs cycle and oxidative phosphorylation are the main contributors to cell metabolism. Glycolysis and glutaminolysis are strongly elevated in cancer cells, providing them with ATP and building materials for tumour expansion. As a result, cancer cells can actively proliferate at deep hypoxia and often have elevated hypoxia inducible factor 2 (HIF-2) levels. However, in energy stress conditions HIF pathways and viability of cancer cells may be suppressed by deficiencies in metabolic pathways, and this can be probed by depriving the cells of key metabolic substrates. We examined the contribution of key metabolic substrates (glucose, pyruvate or glutamine - 12 combinations in total) to stress conditions, mitochondrial uncoupling with FCCP, or hypoxia (4 % O\(_2\)). The cells maintained on at least one of the substrates did not lose viability, mitochondrial Ca\(_{2+}\), membrane potential or respiration. Upon uncoupling with FCCP the mitochondria were depolarised similarly in all the cases, but a strong increase in respiration was only seen in the cells fed on glutamine combined with either glucose or pyruvate. Surprisingly, the response to FCCP did not correlate with ATP levels, which rapidly dropped upon uncoupling in the absence of glucose. Inhibition of glutaminolysis resulted in a reversal of the glutamine dependant effect. At reduced O\(_2\) availability (4 % and 0 % of atmospheric O\(_2\)), cell bioenergetics and local oxygenation varied drastically depending on the substrate composition. Cellular ATP and O\(_2\) levels, orchestrated HIF-2\(\alpha\) stabilisation. At 4 % O\(_2\), rapid and deep deoxygenation was observed in the cells lacking glycolytic ATP and maintained on glutamine and pyruvate. In these cells HIF-2\(\alpha\) levels reached maximum in 3 H and then gradually decreased. At the same atmospheric O\(_2\), only minor HIF-2\(\alpha\) stabilisation was seen in the cells fed on glucose and/or pyruvate. These results indicate that inhibition of key metabolic pathways (e.g. glutaminolysis) can modulate HIF regulatory pathways, metabolic responses and survival of cancer cells in hypoxia or upon activation of respiration.
3.2 Introduction

3.2.1 The Cell Energy Budget (CEB) platform

The CEB system can determine the contributions of the main metabolic pathways of OxPhos, Krebs cycle and glycolysis to ATP production. The platform uses O\textsubscript{2} and pH sensitive probes to assess the different metabolic pathways, as described in Ch. 1 (1.4.2) and (Hynes et al., 2009a, O’Riordan et al., 2007, Zhdanov et al., 2011b).

Toxicity testing is of vital importance for any potential therapeutic agent, there are many drugs that are known to cause mitochondrial toxicity, including some chemotherapeutic agents, antibiotics and antiretroviral drugs. Drug-induced mitochondrial toxicity can occur by; inhibition of ETC complexes (antipsychotics or local anaesthetics), ROS generation (doxorubicin), disruption to mitochondrial protein synthesis and biogenesis (antivirals) or uncoupling reagents such as non-steroidal anti-inflammatory drugs (Chan et al., 2005).

It is important to assess mitochondrial toxicity, which has been shown to cause organ toxicity. The antiretroviral drugs, nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) used in AIDS treatment prevent normal reverse transcription, interfering with mitochondrial replication, which can lead to decreased mitochondrial function within tissues and can cause myopathy, peripheral neuropathy and lactic acidosis (Walker et al., 2002, Wang and Flint, 2013). Mitochondrial toxicity can go undetected using tradition methods such as ATP, MTT and LDH assays. These assays only work to test cell viability; a compound may in fact be toxic but not lead to apoptosis or necrosis. Mitochondrial toxicity is one of the causes of late stage drug attrition in the pharmaceutical industry, so methods to assess this characteristic at an early stage of drug development are very important (Zhang et al., 2009).

Both within our lab and outside, it has been shown that the CEB platform can be used to assess mitochondrial toxicity in both eukaryotic cells and bacteria (Hynes et al., 2006, Jasionek et al., 2010, Bansal et al., 2013, Beeson et al., 2010).
Each metabolic pathway has some spare capacity, so in times where there is decreased O$_2$ for example, OXPHOS would be reduced but glycolytic activity would increase as a compensatory measure to maintain constant ATP levels. In a cell energy budget platform, assays which are performed are more sensitive and more selective than standard viability assays. A panel of molecular imprinter polymeric (MIPs) nanoparticles (NPs) imprinted with melamine were previously tested, using MTT assay and found to have no toxic effect (Supplemental 1). Here we performed a more detailed metabolic analysis of the same panel of NP using the CEB system.

The CEB can also be used to study metabolic changes in cells supplied with different metabolic substrates under differing stress conditions. It is known that cancer cells have high levels of glycolysis and glutaminolysis consuming glucose and glutamine respectively. When there are shortages in metabolic substrates from hypoxia, pharmacological treatment or metabolic disorders, certain cell responses ensue which could be fatal for cancer cells. It is known that different metabolic substrates enter different metabolic pathways. The cell energy budget system is able to distinguish differences in metabolic substrates utilisation in different metabolic pathways. Glucose is the main metabolic fuel for glycolysis, in which one molecule can be converted into two pyruvate molecules, which then can be fed into the Krebs cycle after conversion to acetyl-CoA. When glucose is replaced with galactose there is no net gain of ATP produced from glycolysis, due to the significantly slower rate of conversion into glucose-6-phosphate compared to that from glucose, and there is a compensatory increase in OXPHOS as a result (Marroquin et al., 2007, Rossignol et al., 2004). Glucose deprivation in PC12 cells has been shown to decrease ATP levels, increase reactive O$_2$ species (ROS), decrease mitochondrial membrane potential ($\Delta\Psi_m$) and lead to both apoptosis and necrosis after 24 H (Liu et al., 2003). It has been shown that there may be an increase in glucose metabolism in response to increased ROS from decreased OXPHOS activity in cancer cells (Aykin-Burns et al., 2009).

Another important metabolic substrate is glutamine, which can be converted into glutamate and then $\alpha$-ketoglutarate ($\alpha$-KG) via glutaminolysis, and utilised into the Krebs cycle. Cancer cells are considered to be addicted to glutamine, which
provides the cells with all non-essential amino acids, nitrogen for lipid and nucleotide biosynthesis, activates the TORC1 signalling, as well as being a mitochondrial substrate utilised in the Krebs cycle (Wise and Thompson, 2010). The high level of glutaminolysis in cancer cells has been exploited as a possible research area for cancer therapeutics. Studies include; inhibition of glutamine uptake by blocking transporters, inhibiting complex I to prevent NAD\(^+\) generation required for glutamine utilisation, lowering blood glutamine levels and inhibiting glutamine dependent mTOR activation (Avramis and Panosyan, 2005, Esslinger et al., 2005, Krause et al., 2002).

Metabolic substrates play important roles in cancer cells during hypoxia, high levels of glycolysis and glutaminolysis are important for cell survival. Glutamine and glucose are important in HIF-signalling during hypoxia, deprivation of one or both of these substrates can lead to inhibition of HIF-1α accumulation under hypoxic conditions (Kwon and Lee, 2005). Under glucose and oxygen deprivation, down-regulation of HIF signalling occurs as a result of depleted ATP levels, causing disruption of translational processes (Zhdanov et al., 2013). Increased expression of HIF-2α is observed in PC12 cells, and is strongly associated with development of pheochromocytoma (Span et al., 2011).

Using rat pheochromocytoma PC12 cells and other cell lines, we studied how the availability and utilisation of major metabolic substrates (glucose, glutamine, and pyruvate) modulate the respiratory response of cancer cells to mitochondrial uncoupling. We also studied different factors involves in HIFα accumulation, including the availability of key metabolic substrates, OCR, iO\(_2\), hypoxic stress, activation of AMPK, Akt and Erk, cellular ATP levels and transcription of HIF-α and PHD.
3.2.2 Aims

Using the CEB platform to perform mitochondrial toxicity testing and to investigate the contribution of key metabolites (e.g. glucose, pyruvate, and glutamine) to:

1. Cell bioenergetics and responses to uncoupling.
2. To study the factors involved in HIF-α accumulation.
3.3 RESULTS

3.3.1 Toxicity Testing of Nanoparticle Preparations

Toxicity levels of bare and PEG-1100 and PEG-4400 coated NPs were assessed by the CEB platform in MEF cells. Our results show that there was no difference to total ATP levels observed after 24 and 48 hour NP exposure to MEF cells (Fig. 3.1C). Next, intracellular O$_2$ levels were measured, there was no difference observed between the MIP NP exposed cells and the control (Fig. 3.1A-B). However, differences in oxygen consumption rate were seen (Fig. 3.1E-F). There were no OCR changes in the cells exposed to bare MIP NPs compared to control treated with deionised water, which the NPs were made up in. But there was a decrease in OCR in the PEGylated MIP NPs treated MEFs. A reduction of almost 60% in OCR was found in the MIP NPs coated with PEG1100, and a 30% reduction in the MEFs treated with MIP NPs coated with PEG4000, these changes remained relatively constant after 48 H exposure (p<0.001 for both time points) (Fig. 3.1F). Lactate-extracellular acidification (L-ECA) assay revealed an induction in glycolysis in the PEG1100 MIP NP treated cells, shown by a 40% increase in L-ECA after 24 hour exposure. No change was observed in the Bare or the PEG 4000 MIP NPs (Fig. 3.1D).
Figure 3.1. Toxicological analysis of Bare and PEG-1100/-4000 NP preparations. MEF cells were exposed to either Bare or PEG-1100/-4000 NPs for up to 48 hours, NPs were suspended in sterile water, which was used as a control. Results were normalised to control. CEB analysis; iO$_2$ profiles after (A) 24 hours and (B) 48 hours, (C) ATP values after 24 H, (D) L-ECA after 24 hours show increased glycolytic rate in PEG1100 NP treated cells and (E) OCR after 24 hours and (F) after 48 hours show decreased oxygen consumption in PEG1100 NP exposed cells. ATP, OCR and ECA were normalised to control (sterile water), all data were n=3 with error bars showing standard deviation.

Toxicity studies were also performed on another set of MIP NPs, Trypsin and Rhod/trypsin in MEF cells (Fig. 3.2). ATP and iO$_2$ were unaffected in MEF cells which had undergone up to 48 H incubation with either Trypsin or Rhod/trypsin NPs (Fig. 3.2A-C). However there was a significant reduction in OCR of 55% and 56%
from control in cells treated with Trypsin and Rhod/trypsin NPs for 24 h respectively (Fig. 3.2E). This reduced OCR was maintained for at least 48 H exposure (Fig. 3.1F). A substantial increase in ECA was observed in cells exposed to trypsin NPs (44%), while a 17% increase was seen in Rhod/trypsin treated cells (Fig. 3.2D). This is indicative of mitochondrial toxicity; the mitochondria appear to not be fully functioning and so OXPHOS capacity has decreased and glycolysis increased to allow steady ATP levels to be maintained.
Figure 3.2. Toxicological analysis of Trypsin or RHOD/Trypsin preparations. MEF cells were exposed to either Trypsin or RHOD/Trypsin NPs for up to 48 hours, NPs were suspended in sterile water, which was used as a control. Results were normalised to control. CEB analysis; iO2 profiles after (A) 24 hours and (B) 48 H, (C) ATP values, (D) L-ECA show increased glycolysis in Trypsin NPs and (E) OCR after 24 H and (F) after 48 hours decreased O2 consumption in both treatments. ATP, OCR and ECA were normalised to control (sterile water), all data were n=3 with error bars showing standard deviation.
The dramatic decrease in OCR in PEG-1100 treated cells and both Trypsin and Rhod/trypsin treated cells shows a reduction in the level of respiration in these cells (Fig. 3.1E-F and Fig. 3.2E-F). Together with the increase in L-ECA (Fig. 3.1D and Fig. 3.2D) in cells exposed to PEG-1100, Trypsin or Rhod/trypsin, showing increased levels of glycolysis in these cells to compensate for reduced OXPHOS activity, this suggests a significant level of mitochondrial toxicity as a result of exposure to these NPs, altering cell metabolism but not inducing cell death.

These results demonstrate that metabolic OCR and ECA assays offer a more sensitive in-depth metabolic analysis for toxicity assessment and can provide mechanistic information about mode of action of NP preparations and their targets within the cell. In contrast, conventional (non-specific) tests such as total ATP, LDH release and MTT assay, often miss pronounced toxic effects, especially if these are masked by compensatory pathways in the cell. This was the case with our samples.
3.3.2 Metabolic Substrates in Cell Bioenergetics

Oxygen sensitive probes were used to assess respiration and OXPHOS rates. The pH sensitive probe pH-Xtra probe was used to measure extracellular acidification, and ATP levels were quantified using a standard luminescent cell viability kit. The cell oxygenation measured using an extracellular O$_2$ probe, MitoXpress-Xtra, is inversely related to the respiratory activity in cells. The degree of the response can be seen by measuring intracellular O$_2$ levels using an intracellular phosphorescent O$_2$ probe, MitoXpress-Intra (Fercher et al., 2011, Dmitriev et al., 2012). Extracellular acidification can be assessed with the use of a long decay pH-sensitive lanthanide probe, pH-Xtra (Hynes et al., 2009a, Zhdanov et al., 2011b). Experiments were conducted as stated in Chapter 2 methods.

Working media (WM) was used in metabolic substrate experiments. It was made using reconstituted DMEM powder in Milli-Q water and filter sterilised. Twelve different WM were made using different combinations of 10 mM glucose (Glc), 10 mM galactose (Gal), 2 mM glutamine (Gln) and 1 mM pyruvate (Pyr). All media contained 100 nM NGF, and all were serum-free. All but the media used for ECA experiments contained 20 mM HEPES (pH 7.2). WM are show in table 2.2. The medium was replaced at the start of each experiment and the cells grown for 2 hours.

Using rat pheochromocytoma PC12 cells, mouse embryonic fibroblasts (MEF) and human colon cancer HCT116 cells, how the availability and utilisation of major metabolic substrates (glucose, glutamine, and pyruvate) modulate the respiratory response of cancer cells to mitochondrial uncoupling was studied. This is of importance as it has been shown that various cancer cells exhibit altered metabolism and mitochondrial function. Certain pharmacological agents are known to cause mitochondrial uncoupling, so the effect of substrate deprivation under these conditions is of particular relevance to those studying anti-cancer therapeutics. Cells were incubated with one of the 12 different WM for 2 hours, allowing time for metabolic adaptation and then treated with 1 µM FCCP, a concentration which had previously been optimised in complete media (Zhdanov et
al., 2010b). The concentration of FCCP was not optimised for each condition and extreme treatments such as without any metabolic substrates of just Gal may cause PC12 cells to be stressed prior to uncoupling. However there is a broad concentration range which PC12 cells can withstand.

Firstly, the respiratory responses of PC12 cells to mitochondrial uncoupling were studied using the intracellular O\(_2\) probe, MitoXpress-Intra, and extracellular O\(_2\) probe MitoXpress-Xtra (Fercher et al., 2011, Dmitriev et al., 2012). It is known that cell oxygenation is inversely related to respiratory activity. Basal OCR data show cells incubated with WM containing Pyr/Gln with or without Gln had the highest O\(_2\) consumption rates, with iO\(_2\) values of 93 and 96 \(\mu\)M O\(_2\) respectively (Fig. 3.3A). Cells supplied with WM containing, Glc, Gal or no substrates had the lowest OCR, with values of 127, 136 and 144 \(\mu\)M O\(_2\) cells respectively. Intracellular O\(_2\) levels upon uncoupling were then investigated, as these indicate the magnitude of respiratory response. Intracellular O\(_2\) (iO\(_2\)) results showed four distinct types of profiles (Fig. 3.3B).

The first type of response was in cells supplied with Gln with either Glc or Pyr, which showed a rapid decrease in iO\(_2\) followed by continuous, for over an hour, cell deoxygenation in response to uncoupling, suggesting an increase in mitochondrial respiration. There was a substantial \(\Delta\)iO\(_2\) in cells which had shown both high (Gal/Pyr/Gln and Pyr/Gln) and moderate (Glc/Gln, Glc/Pyr/Gln) basal OCR. The second type of response refers to cells supplied with Pyr or Gln with or without Gal and Gal/Gln. In these cells there was an inhibition of respiration and cell reoxygenation. It was unexpected to see decreased respiration in Gal/Gln supplied cells as they had a high level of basal OCR. The third type of response showed a rapid transient decrease in iO\(_2\) followed by steady minor cell deoxygenation, observed in cells supplied with Glc/Pyr. iO\(_2\) were partially restored but remained at this level for up to an hour (Fig. 3.3B). The fourth type of response displayed no change, or a slow progressive reoxygenation, seen in cells supplied with Gal or no substrates.
The levels of iO$_2$ were plotted at the time points 10 and 60 min after FCCP addition (Fig. 3.3C). A dramatic response was seen in the cells in WM containing Gln in combination with either Pyr or Glc. These cells had a sustained increased respiratory rate for at least 60 min, while other cells grown in Glc/Pyr or Glc, for instance, showed a slight increase in respiration after 10 min which was absent at the 60 min time point (Fig. 3.3C).

It was observed that after 4 hours of FCCP treatment partial detachment of cells from the collagen coated surface of the wells occurred. To investigate whether the decrease in respiration observed in cells supplied with Gal/Gln or Gln was as a result of apoptosis, poly (ADP-ribose) polymerase (PARP) levels were studied. Cleaved PARP levels are known to increase in cells undergoing apoptosis in order to try and repair damaged DNA (Li et al., 2002). PARP is cleaved after 4 hours FCCP treatment in these cells along with a decrease in the 1A/1B-light chain 3 (LC3) I/II ratio indicating autophagy occurred (Fig. 3.3D).
Figure 3.3. Differential metabolic response according to substrate composition. PC12 cells were incubated in different WM for 2 hours prior to experiment start. A. Basal OCR levels (numbers above bars oxygenation values). B. Intracellular O₂ profiles were measured using the lifetime values obtained using the MitoXpress-Intra probe, uncoupling was achieved by addition of 1 µM FCCP after baseline had been established. C. The iO₂ levels obtained (representative plots shown in (B)) were used at the time points 10 and 60 min after FCCP addition were plotted. Deep sustained deoxygenation was observed in cells supplied with Gln in combination with Pyr or Glc. D. Markers or apoptosis (poly (ADP-ribose) polymerase (PARP)) and autophagy (1A/1B-light chain 3 (LC3) I/II ratio (numbers above bars)) were studied by western blotting analysis. Results demonstrate moderate increase in the levels of autophagy (PARP is cleaved) in cells supplied with Gal/Gln after 4 hours of FCCP treatment * (p < 0.001). α-tubulin protein bands were used for normalisation. Error bars show standard deviation n=6 (A-C) and 3(D).
There are differential O$_2$ consumption rates dependent on substrate composition. In cells supplied with Glc, the basal OCR and the responses to FCCP were similar in Glc alone and Glc/Pyr (Fig. 3.4A). There were different profiles observed in cells supplied with Glc/Gln, Glc/Gln/Pyr or Gal/Gln/Pyr with steeper deoxygenation in coupled cells which showed a rapid decrease in O$_2$ levels upon uncoupling. Cells in WM containing no substrates or Gal alone demonstrated similar patterns in deoxygenation, in cells with coupled mitochondrial O$_2$ levels decreased slightly and then gradually increased, FCCP caused a small decrease (Fig. 3.4B-C). In Gal/Gln, Gal/Pyr and Pyr cells O$_2$ levels were similar to those in Glc/Pyr; however when the cells were treated with 1 µM FCCP O$_2$ levels began to rise after approximately 25-30 min. In cells supplied with just Gln upon uncoupling there was no change in O$_2$ levels (Fig. 3.4C).
Figure 3.4. Differential $O_2$ consumption according to substrate composition in resting and uncoupled PC12 cells. PC12 cells were pre-incubated with different metabolic substrates for 2 hours and then OCR was measured with or without 1 µM FCCP addition. **A.** Showing basal and uncoupled OCR for cells grown in WM containing Glc, deep sustained deoxygenation is observed in cells supplied with Glc/Gln and Glc/Gln/Pyr upon uncoupling. **B.** Cells with WM containing Gal, an inhibition of respiration was observed in uncoupled Gal/Gln cells. **C.** Cells with WM containing no sugar, no change or progressive reoxygenation can be seen in (-) uncoupled cells.
In order to elucidate whether this difference in cellular deoxygenation was in part due to differences in energy supply, ATP levels were assessed (Fig. 3.5A). Basal ATP levels were constant in all but Gal and no substrate WM treated cells. However, upon FCCP stimulation there was a reduction in ATP in all cells grown in WM which did not contain Glc, indicating the importance of the up-regulation of glycolysis to sustain constant ATP levels upon uncoupling (Fig. 3.5B). To distinguish possible differences between WM containing Glc, ECA was monitored (Fig. 3.5C). Very limited ECA was observed in cells without Glc. Resting ECA levels were lower in cells supplied with Glc/Pyr/Gln which also had one of the highest basal OCR and the highest ECA was seen in Glc/Gln which had the lowest resting OCR; showing that glycolytic activity was inversely related to OCR (Fig. 3.3A and Fig. 3.5C). Increases in ECA upon FCCP stimulation occurred in cells given Glc/Gln with or without Pyr, which produced a strong sustained respiratory response to FCCP (Fig. 3.3C).
Figure 3.5. Substrate composition dictates ATP levels after uncoupling by FCCP in PC12 cells. A. Basel ATP levels were measured after 3 hours incubation with WM. A reduction is observed in ATP level in Gal or (-) treated cells. B. ATP ratio of uncoupled/resting cells. Bars correspond to 10 min time point and numbers 60 min after FCCP addition, all cells without Glc had a dramatic decrease in ATP upon uncoupling. C. Lactate-extracellular acidification (L-ECA) in resting and uncoupled cells. L-ECA is increased upon uncoupling in cells which showed the greatest respiratory response (Glc/Gln with and without Pyr). Error bars represent standard deviation, n=4, * - (p < 0.001).

The effect of substrate composition to \( \Delta \Psi_p \) upon uncoupling was assessed using PMPI staining. No effect was seen with FCCP treatment, as a positive control cells were treated with 100 mM KCl (Fig. 3.6A-B).
Figure 3.6. The effect of substrate composition on $\Delta \Psi_p$ in PC12 cells. PC12 cells incubated with different metabolic substrates were stained with 20 nM PMPI ($\Delta \Psi_p$ stain) for 30 minutes and then measured; PMPI was maintained at 20 nM throughout the experiment. A. Representative images of $\Delta \Psi_p$ shown of PC12 cells stained with PMPI with addition of 1 µM FCCP (no effect observed) or 100 mM KCl (positive control). B. Staining occurred in a similar manner in all but Gal or no substrates, no changes in $\Delta \Psi_p$ were shown in PC12 cells with PMPI staining upon uncoupling with 1 µM FCCP, positive control of 100 mM KCl.

Mitochondrial Ca$^{2+}$ levels and $\Delta \Psi_m$ were investigated, in an attempt to elucidate the reason behind the large, sustained increase in respiration in cells in WM containing
Gln in combination with either Pyr or Glc. Increases in mitochondrial Ca$^{2+}$ are known to activate metabolism by activating key metabolic dehydrogenases (Denton, 2009). There was no change in basal $\Delta \Psi_m$ or Ca$^{2+}$ levels, except cells in WM consisting of just Gal or no substrates (Fig. 3.7A-C). All cells responded to FCCP treatment in a similar manner, with a decreased $\Delta \Psi_m$ (Fig. 3.7A-B) and decrease in mitochondrial Ca$^{2+}$ levels (Fig. 3.7B-C). The plasma membrane was not affected by FCCP; however cells with no metabolic substrates had a substantially depolarised $\Delta \Psi_p$ at rest.

In times of energy depletion or when there are changes in intracellular O$_2$, metabolic pathways can be activated; hence key metabolic markers involved in hypoxia and energy maintenance were investigated after 4 hours of FCCP treatment (Fig. 3.7D). Phosphorylation at Ser2448 of mTOR, a crucial element of cell growth and survival, decreased in all cells supplied with Glc upon FCCP addition and remained the same in cells supplied with Gal/Gln/Pyr compared with basal levels. Phosphorylation of AMPK (TH172), a master metabolic regulator responding to changes in the AMP/ATP ratio was decreased slightly in all cells after FCCP treatment (Fig. 3.7D). FCCP strongly affected the Erk pathway decreasing levels of p 44 Erk (TH 202) / p 42 Erk (Tyr 204) ratios in all cells except those fed with Gal/Gln/Pyr. The same pattern was observed with p-Akt (Ser471) levels. Phosphorylation of Erk1/2 (p-44/p-42) and Akt increased in cells supplied with Gal/Pyr/Gln. Cells given Gal/Gln did not show any phosphorylation of p-AMPK, Erk1/2 or p-Akt (Fig. 3.7D).
**Figure 3.7. The effect of substrate composition on $\Delta \Psi_m$ and mitochondrial Ca$^{2+}$ in PC12 cells.**

A. Basel $\Delta \Psi_m$ shown by TMRM staining; only cells supplied with no substrates or Gal alone had a decreased $\Delta \Psi_m$.  

B. Representative microscopy images of cells stained with 20 nM TMRM ($\Delta \Psi_m$) or 5 ng/ml mitocase12 (mitochondrial Ca$^{2+}$) which were dissipated after 1 µM FCCP addition, in a similar manner in all WM except no substrates or Gal. Bar represents 50 µm.  

C. TMRM intensity upon 1 µM FCCP decreased to a similar level in all WM within 6 min, except no substrates and Gal.  

D. Western blotting analysis of phosphorylation of mTOR(Ser2448), AMPKα(Thr172), p44 Erk(Thr202), p42 Erk(Tyr204) and Akt(Ser473) upon 1 µM FCCP treatment for 4 hours. Data is quantified in bar chart, protein phosphorylation levels were normalised to α-tubulin. Error bars show standard deviation of the probe intensities within a cell population (60-80 cells in total), n = 4, * p < 0.05 A. compared to Glc/Gln/Pyr, D. FCCP treated compared to non-treated.
We observed a prominent role of Gln in FCCP mediated respiratory responses; it is known that PC12 cells are highly glutaminolytic (Fig. 3.3B, table 3.1). In order to investigate whether there was a role of glutaminolysis in the respiratory response kidney type glutaminase 1 (GLS1) was inhibited using the 10 µM Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) with 1 hour treatment prior to, as well as being maintained in the WM during experiments. No major effect on ATP levels was observed in BPTES treated cells (Table 3.1). Treatment of cells with 10 µM BPTES lead to minimal differences in resting OCR and iO₂ levels. Addition of BPTES in uncoupled cells dissipated the sustained respiratory ‘first type’ response previously observed in cells grown in WM containing Gln in combination with either Pyr or Glc (Fig. 3.8A-C). No effect of BPTES was seen in cells in WM which did not contain Gln, showing the specificity of BPTES (Fig. 3.8C). The iO₂ profile observed in cells supplied with Gal/Pyr/Gln or Pyr/Gln changed from a type 1 to a type 2 respiratory response, with reoxygenation such as that seen in cells fed with Gal/Pyr or Pyr (Fig. 3.8A). GLS1 inhibition increased glycolytic activity in cells supplied with Glc/Pyr/Gln and Glc/Gln (2.6 fold and 1.5 fold respectively); which suggests a substantial induction in glycolysis (Fig. 3.8D). BPTES treatment did not change L-ECA after FCCP stimulation (Fig. 3.8D).

**Table 3.1 Effect of GLS inhibition on ATP levels in PC12 cells at rest and uncoupled**

<table>
<thead>
<tr>
<th>WM and treatment</th>
<th>Glc/Pyr/Gln</th>
<th>Glc/Pyr</th>
<th>Glc/Gln</th>
<th>Glc</th>
<th>Gal/Pyr/Gln</th>
<th>Gal/Gln</th>
<th>Pyr/Gln</th>
<th>Gln</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>0.99</td>
<td>1.04</td>
<td>1.04</td>
<td>1.02</td>
<td>1.06</td>
<td>0.89</td>
<td>0.95</td>
<td>0.88</td>
</tr>
<tr>
<td>Uncoupled</td>
<td>1.07</td>
<td>1.11</td>
<td>1.04</td>
<td>1.14</td>
<td>0.78</td>
<td>0.56</td>
<td>0.37</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Averaged BPTES (+) ATP and averaged BPTES (-) ATP were calculated, BPTES (+)/BPTES (-) ratio then determined, data shown in arbitrary units [a.u.].
Figure 3.8. Inhibition of GLS1 changes respiratory response in cells supplied with Gln containing WM. PC12 Cells were incubated with different metabolic substrates for 2 hours prior to the start of each experiment. A. iO$_2$ profiles with GLS1 inhibitor 10 µM BPTES or mock control DMSO. B. Changes in iO$_2$ at 10 and 60 minute time points, when glutaminolysis is inhibited the sustained deep deoxygenation response is reversed, and iO$_2$ increases. C. OCR plots for cells supplied with Gal/Pyr/Gln or Glc/Pyr with and without 1 µM FCCP and 10 µM BPTES, the change in respiratory response to FCCP is only observed in cells supplied with Gln in the WM demonstrating BPTES selectivity for GLS1, the response is also only seen in uncoupled cells. D. L-ECA BPTES/DMSO treatment. Error bars show standard deviation (n=4). * - (p < 0.01).

To see if this effect was specific for PC12 cells or cancer cells, a further two cell lines were tested, human colon cancer cells (HCT116) and the non-cancerous mouse embryonic fibroblasts (MEFs). In HCT116 the pattern of deoxygenation upon uncoupling with FCCP was similar to that in the PC12 cells, with a less prominent effect (Fig. 3.9A). The largest decrease upon FCCP treatment occurred in cells supplied with Gln with either Pyr or Glc with or without Gal (Fig. 3.9C). As
observed in PC12 cells, HCT116 cells were unable to withstand FCCP treatment when supplied with Gln or Gal/Gln (Fig. 3.9A). These cells had low iO₂ levels at rest with an increase in iO₂ levels with FCCP treatment. BPTES treatment in HCT116 cells resulted in reduction in iO₂ in all cells in WM containing Gln, supressing the previously FCCP induced sustained increase in respiration, however the responses were not as strong as those observed in PC12 cells, suggesting HCT116 cells are less sensitive to BPTES treatment (Fig. 3.9A).

In MEFs the response to FCCP was generally more rapid and transient and even in the absence of Gln, MEF cells were still able to generate a continuous and positive response to FCCP (Fig. 3.9B). The type of responses seen in MEF cells differed to that seen in the other two cell lines with iO₂ profiles being either:

The first response was characterised by a rapid increase in respiration followed by partial or complete restoration of resting iO₂ (Gln with Glc or Pyr as well as Glc/Pyr) (Fig. 3.9B). The second response showed a decrease in respiration and cell reoxygenation (no substrates, Gln and Pyr alone or with Gal). Lastly, the third response displayed no significant changes in respiration and iO₂ (Glc or Gal alone). The greatest response to uncoupling in MEF cells was from those supplied with Glc/Pyr/Gln; in these cells GLS1 inhibition was only able to blunt the response to uncoupling. In the absence of Gln, MEFs were able to give a sustained positive response to FCCP, as seen in cells supplied with Glc/Pyr (Fig. 3.9B-C).
Figure 3.9. Inhibition of GLS1 changes respiratory response in cells supplied with Gln containing upon uncoupling in HCT116 and MEF cells. A. \(\text{iO}_2\) profiles for \(\text{iO}_2\) profiles upon uncoupling in HCT116 cells, a similar pattern of respiratory response to uncoupling is observed in these cells as was seen in PC12 cells, with similar effects from GLS1 inhibition by 10 µM BPTES in cells supplied with Gln containing WM. B. \(\text{iO}_2\) profiles in uncoupled MEF cells which show a different respiratory response compared with the HCT116 and PC12 cells. C. MEF cells are able to produce a respiratory response in the absence of Gln, making them less sensitive to glutaminolysis inhibition.
3.3.3 Differential contribution of key metabolic substrates and O2 in HIF signalling

Metabolic substrates play important roles in cancer cells during hypoxia. High levels of glycolysis and glutaminolysis are important for cell survival. Gln and Glc are important in HIF-signalling during hypoxia, which has been shown by deprivation of one or both of these substrates leading to inhibition of HIF-1α accumulation under hypoxic conditions (Kwon and Lee, 2005). It has also been demonstrated that under O2 and Glc deprivation down-regulation of HIF signalling can occur via disruption of translational processes as a result of cellular ATP depletion (Zhdanov et al., 2013). Using differentiated PC12 cells the different factors which are involved in HIFα accumulation including; the availability of key metabolic substrates, OCR, iO2, hypoxic stress, activation of AMPK, Akt and Erk, cellular ATP levels and transcription of HIF-α and PHD were studied.

Self-deoxygenation profiles for PC12 cells exposed to different WM for 2 hours under normoxic conditions at 37°C were done, the WM was replaced and intracellular O2 was then monitored at 4% O2. Cells supplemented with Glc/Gln/Pyr showed the deepest and most rapid deoxygenation monitored over 5 hours, reaching 10 µM within 1 hour (Fig. 3.10A). Gal/Gln and Gln treated cells took 75-90 min, Glc/Gln/Pyr took 80-110 min and Glc/Gln cells took 130-160 min (Fig. 3.10A-C). Cells supplied with just Glc showed a much shallower curve and with no substrates showed very minor deoxygenation, similar to that seen in cells treated with antimycin A (AA), inhibiting respiration. There is good correlation between OCR and initial cell deoxygenation (r=0.92, p<10^-4). OCR is highest in cells supplied with Gln with either Gal/Pyr or Pyr and lowest in cells with just Gal or no substrates (Fig. 3.10C).
Figure 3.10. Substrate composition dictates deoxygenation profiles upon exposure to 4% O\(_2\) in PC12 cells. PC12 cells were seeded and differentiated for 5 days (MitoXpress-Intra loading occurred 16 hours prior to the start of the pre-incubation) and then pre-incubated with different metabolic substrates for 2 hours. Plates were then transferred to 4% O\(_2\) and measured. A-B. \(iO_2\) levels in cells supplied with Glc (A) or Gal (B), data recorded at 4% O\(_2\), cells were transferred from 20.9% O\(_2\) and monitored for changes in cell deoxygenation. When mitochondrial respiration is inhibited with antimycin A (AA) \(iO_2\) levels remain close to ambient. C. Initial cell deoxygenation rates calculated from slopes from (A) and (B) during the first 40 min.

From these results as one might expect HIF-2\(\alpha\) was rapidly stabilised in cells supplied with Gln with Gal/Pyr or Glc/Pyr. Cells with Glc/Gln or Gal/Gln showed an increase in HIF-2\(\alpha\) accumulation with maximum levels reached after 3 hours (Fig. 3.11A). Cells supplied with Gal were unable to stabilise HIF-2\(\alpha\) under anoxic conditions without both Gln/Pyr (Fig. 3.11A-B). Low respiring cells without Gln
showed very little HIF-2α stabilisation when exposed to 4% O₂ for up to 4 hours. A strong inverse correlation was observed between HIF-2α and iO₂ over 2-4 hours (r=-0.57 - -0.68, p<10⁻³). A large decrease in ATP was seen in all cells deprived of Glc (Fig. 3.11B).

Under anoxic conditions for 2 hours the pattern of HIF-2α stabilisation was very different (Fig. 3.11A-B). Cells supplemented with Glc showed high protein levels of HIF-2α, correlating nicely with ATP levels (r=0.89, p<10⁻⁵) cells with Gal/Pyr/Gln and Pyr/Gln showed moderate levels, which were much lower than the levels seen at 4% and small amounts of HIF-2α were observed in Gal/Gln cells. Cells supplied with Gal/Pyr showed hardly any HIF-2α in any condition (Fig. 3.11A). There was a dramatic increase in HIF-2α stabilisation in cells supplied with Glc/Pyr under anoxic conditions compared to the minimal stabilisation seen under hypoxia.
Figure 3.11. Substrate composition dictates HIF-2α stabilisation in PC12 cells exposed to different O₂ conditions. PC12 cells were incubated with different metabolic substrates for 2 hours and then maintained at 21% O₂ or moved to 4% for 2-4 hours or 0% O₂ for 2 hours, proteins were then isolated. A. Western blot of HIF-2α in cells exposed to different WM at 4% O₂ for 2-4 hours or 0% for 2 hours. B. Results of quantitative analysis of HIF-2α levels plotted, along with corresponding ATP levels which were measured in PC12 after 2 hours pre-incubation with different metabolic substrates and 0-4 hours exposure to 4% O₂ using the CellTiter-Glo assay, n = 4.

The effect of GLS1 inhibition with BPTES on respiration was studied under hypoxia, an increase in iO₂ was observed in cells supplied with Gln at 4% O₂ showing a reduction in the respiration rate (Fig. 3.12A). Significant reductions in ATP levels in cells with Gln at 4% O₂ treated with BPTES were witnessed compared to non-
treated (Fig. 3.12B). The reductions were only seen in cells supplemented with Gln, demonstrating the specificity of BPTES.

ATP levels under normoxia (19-21% O$_2$) were similar in different WM. However, after 4 hours exposure to 4% O$_2$ cells supplied in Gal/Gln/Pyr had significantly lower ATP levels compared to Glc control (p<0.01), which was lower still when Pyr was excluded in cells fed on Gal/Gln (Fig. 3.12B). The effect was greater still when cells were placed under anoxic conditions for 2 hours (Fig.3.12B).
Figure 3.12. Inhibition of GLS1 changes respiratory response in cells supplied with Gln containing WM at 4% O₂. PC12 cells were seeded and differentiated for 5 days (MitoXpress-Intra loading occurred 16 hours prior to the start of the pre-incubation) and then pre-incubated with different metabolic substrates for 2 hours. Inhibition of GLS1 with 10 µM BPTES occurred 1 hour prior to the start of the measurement. Plates were then transferred to 4% O₂ and measured. **A.** iO₂ profiles for (Glc/Pyr/Gln and Gal/Pyr), (Gln Glc/Pyr and Gal/Gln) and (Glc/Gln and Glc) with and without 10 µM BPTES treatment at 4% O₂. Reduction in respiration with increases in iO₂ was observed in cells inhibited with GLS1 and supplied with Gln. **B.** ATP levels in cells supplied with Glc, Gal/Gln/Pyr and Gal/Gln at 19-21%, 4% and 0% treated with either 10 µM BPTES or mock DMSO. Data are shown as mean with standard deviation. * show statistical significance between control cells (supplied with Glc and not treated with BPTES) (p<0.01), ** shows significance in ATP between BPTES (+) and BPTES (-) cells (p<0.01) n=3, all other results were not statistically significant.
Phosphorylated Akt data reveal an increase in all samples under hypoxic conditions compared to normoxic samples. p-AMPK (Thr172) and p-mTOR (Ser2448) showed similar levels in all O\textsubscript{2} conditions in cells in Glc/Gln/Pyr WM. Proteins involved in HIF-activation, p-Akt (Thr308 and Ser473) and p44/42 Erk1/2 (Thr202/Tyr204) showed a substantial increase in protein levels after 2 H at 4% O\textsubscript{2}, which decreased after 4 hours at 4% O\textsubscript{2} and was only very slightly increased under anoxic conditions for 2 hours. When grown with Glc/Gln/Pyr HIF-1/2α levels rose with increasing exposure to 4% O\textsubscript{2}, however decreased levels were witnessed under anoxic conditions. In cells supplied with Gal/Gln/Pyr increased HIF-1/2α protein levels were observed after 2 hours, which decreased significantly upon treatment with BPTES. In cells supplied with Glc/Pyr a substantial decrease in HIF-1/2α protein levels was seen at 4% O\textsubscript{2}, with HIF-1/2α protein levels only found under anoxic conditions.

AMPK phosphorylation (Thr308) remained relatively steady in cells supplied to Glc/Gln/Pyr in various O\textsubscript{2} conditions. However when the medium was switched to Gal/Gln/Pyr and the cells exposed to O% O\textsubscript{2} a considerable increase in phosphorylation was observed in control and BPTES treated cells. Differences in Akt phosphorylation (Thr308 and Ser 473), were also observed in Gal/Gln/Pyr WM with phosphorylation levels sustained at 4% O\textsubscript{2} for at least 4 hours whereas at Glc/Gln/Pyr levels decreased under these conditions. With GLS inhibition these Akt phosphorylation levels increased in cells supplied with Gal/Gln/Pyr, an effect not seen in Glc/Gln/Pyr supplied cells. A similar phenomenon was seen for p44 and p42 Erk phosphorylation which increased under hypoxia along with BPTES treatment (Fig. 3.13).
Figure 3.13. Effects of metabolic substrates on the levels of HIF-α and the phosphorylation of HIF-regulating proteins. A. Western blotting analysis of HIF-1/2α and protein phosphorylation levels in WM containing Glc/Gln/Pyr (A.) and WMs without Glc (B.) or Gln (C.) GLS1 activity was inhibited with 10 µM BPTES (DMSO was used as mock control). D. Analysis of the effects of Glc (green) and Gln (blue) depletion on HIF-2α levels; bands are highlighted in corresponding colours in B and C. Data are normalised to the corresponding values in Glc/Gln/Pyr medium at 19% O₂ (1 a.u.). E. Analysis of the effects of GLS1 inhibition on HIF-2α levels in WM containing Gal/Gln/Pyr (red in B). Data are normalised to the corresponding values in cells treated with mock at 19% O₂ (1 a.u.). Qualitative data corrected to total protein levels shown in supplemental 2A-C. * - show significant difference (p < 0.05) from corresponding values: in Glc/Gln/Pyr medium (D); without BPTES (E).
Immunofluorescence shows the effects of Glc and Gln on nuclear HIF-1α accumulation at different O₂ levels. Analysis revealed similar levels of HIF-1α in cells supplied with Glc/Gln/Pyr and Gal/Gln/Pyr and grown under 4% O₂, however in cells with Glc/Pyr WM HIF-1α levels were only observed under anoxic conditions. Nuclear HIF-1α levels were higher in cells supplied with Glc/Pyr compared with Glc/Gln/Pyr cells under 0% O₂. Treatment with BPTES caused a decrease in HIF-1α levels in Glc/Gln/Pyr cells, though the drop was much more dramatic in Gal/Gln/Pyr treated cells (Fig. 3.14a-B).

**Figure 3.14. Effects of metabolic substrates on the levels of HIF-α and the phosphorylation of HIF-regulating proteins.** A. Immunofluorescence images and of the most prominent effects of Glc and Gln on nuclear HIF-1α accumulation (Novus HIF-1α primary antibody (50 minutes) and Alexa-488 fluorescent secondary antibody (50 minutes)) at different O₂ availability. Cells were incubated for 4 hours at 4% O₂ or 2 hours at 0%. DAPI was used for nuclear counterstaining. B. Quantitative analysis of HIF-1α accumulation from analysing 5–20 cells in 3 independent experiments. Error bars represent SD. * - show significant differences under normoxia in Glc/Gln/Pyr medium. Bar in A is 20 µM.

The levels of AMPKα and Akt phosphorylation were assessed under conditions of oxygen/glucose deprivation (OGD). Protein levels of HIF-2α were significantly higher in Glc/Gln compared with Gal/Gln supplied cells under hypoxic conditions. We found that AMPK phosphorylation was significantly higher in Gal/Gln supplied cells exposed to anoxia compared with Glc/Gln cells (Fig. 3.15 C, D).
phosphorylation was substantially higher in Glc/Gln WM compared to Gal/Gln after 2 hours at 4 % O₂, decreasing to similar levels after 4 hours at 4 % O₂. P-Erk 1/2 protein levels were significantly higher in Gal/Gln WM compared to Glc/Gln. Under anoxic conditions a large increase in p-AMPKα levels was observed in all WM without Glc, conversely, p-Akt levels decrease (Fig. 3.15 C, D).
**Figure 3.15.** AMPKα and Akt phosphorylation and effect of AMPK inhibition on cell viability upon OGD. PC12 cells were incubated with different metabolic substrates for 2 hours and then maintained at 19% O₂ or moved to 4% or 0% O₂ for differing time points, proteins were then isolated. 

A. Western blotting analysis of HIF-2α and phosphorylation of HIF-regulating proteins in Glc/Gln or Gal/Gln media at different atmospheric O₂.

B. Quantitative analysis of A normalised to corresponding values in normoxic cells supplied with Glc/Gln. In WM containing Gal/Gln at 4% O₂, Akt/Erk phosphorylation is increased; however HIF-2α levels are significantly lower, strong increase in p-AMPKα and decrease in p-Akt are highlighted by quadrangles.

C. Western blotting analysis shows a large increase in p-AMPKα levels under anoxia in all WM without Glc.

D. Conversely, p-Akt levels decrease. In A, B and D, n = 3; in C, n = 4. Phosphorylated proteins are corrected by total proteins shown in supplemental 2A-D. Error bars represent SD. * (p < 0.01)
3.4 Discussion

Glycolysis and OxPhos are the two main ATP generating pathways in mammalian cells. When Glc is absent, increases in OxPhos generated ATP enables the cell to survive (Marroquin et al., 2007). We found in the presence of Gln and Pyr cells are able to survive without Glc, increasing OXPHOS flux and mitochondrial respiration (Fig. 3.3C and 3.4B-C). These cells had the highest OCR, which was decreased with the addition of Glc as glycolysis was still able to produce ATP (Fig. 3.3A, Fig. 3.5A).

During uncoupling, upon FCCP addition the ΔΨm is dissipated, mitochondrial Ca2+ levels decrease, cytosolic pH increases and OxPhos is no longer able to produce ATP. In fact ATP synthase works in reverse mode, utilising ATP in order to attempt to restore the ΔΨm, observed in many different physiological conditions such as cancer and ischemia (Ivanes et al., 2014, Pasto et al., 2014). A deep and sustained deoxygenation was observed in cells supplied with Gln with Glc or Pyr when uncoupled with FCCP, demonstrating cancer cells glutaminolytic nature (Fig. 3.3C). Surprisingly, even without Glc uncoupled cells supplied with Gln/Pyr were able to produce this response.

The reasoning behind the deep and sustained deoxygenation in cells supplied with Gln in combination with Glc or Pyr was investigated studying multiple factors including ATP, ΔΨp, ΔΨm, mitochondrial Ca2+ levels, extracellular acidification, key metabolic proteins, metabolic substrate entry into the cell and mitochondria and glutaminolysis. ATP was not the limiting factor to the deoxygenation response to FCCP observed, ATP levels were only sustained in those cells supplied with Glc upon uncoupling (Fig. 3.5B). The respiratory response to FCCP did not correlate with ATP levels, which dropped rapidly upon uncoupling in the absence of Glc, indicating that mitochondrial respiration does not depend on ATP levels (Fig. 3.5A-B). Even cells supplied with Gal/Gln/Pyr or Gln/Pyr were able to produce a substantial respiratory response to FCCP which lasted for at least 1 hour (Fig. 3.3C).
Although the mitochondrial membrane potential was similar in all cases, a strong increase in respiration was seen only in the cells fed on Gln combined with either Glc or Pyr (Fig. 3.3C, Fig. 3.6A-B). Gln transport into the cell and mitochondria was investigated to see why Gln needed to be combined with either Pyr or Glc for this response to uncoupling. Gln is transported into the cell via the Gln transporter SN1 and the amino acid transporters ASCT1/ASCT2 (Fig. 3.16) (Bode, 2001). These all involve Na\(^{2+}\) co-transport, which could be affected by ΔΨ\(_p\) depolarisation. However depolarisation of the ΔΨ\(_p\) was not observed making it likely plasma membrane Gln transport would be unaffected. Gln is transported into the mitochondria via the mitochondrial Gln carrier which is dependent on ΔpH, causing FCCP to suppress this transport (Albrecht et al., 2000). However cytosolic Gln can be converted into glutamate (Glu) which can be transported into the mitochondria via the Glu transporter or the Glu-Aspartate (Asp) antiporter in the malate-asp shuttle (Fig. 3.17). Transport via the Glu-Asp antiporter requires NADH; meaning cells without Glc or Pyr would not be able to transport Gln into the mitochondria this way. However this antiporter is electrogenic, requiring an electrochemical potential across the mitochondrial membrane, which is dissipated upon uncoupling (LaNoue and Tischler, 1974). After FCCP addition transport of Gln is no longer achieved by the Glu-Asp antiporter, and either Glc or Pyr are necessary for the mitochondrial Gln transporter to be functional and Gln to enter the mitochondria (Fig. 3.16).

Glc enters the via through Glc transporters GLUT1 and GLUT4, Glc is then converted into Pyr, which is able to enter through the H\(^+\)-linked monocarboxylate transporter (MCT). Pyr is co-transported with H\(^+\) into the mitochondria through the mitochondrial pyruvate carrier (MPC) where it is converted into acetyl-CoA and then eventually α-ketoglutarate (α-KG) (Bricker et al., 2012). As Pyr transport is H\(^+\)-coupled, the rate will decrease upon uncoupling (Herzig et al., 2012). This explains the lack of respiratory increase in FCCP treated cells supplied with Glc, Pyr or Glc/Pyr (Fig. 3.3B-C).
Figure 3.16. Transport of metabolic substrates across the plasma membrane. Gln is transported into the cell via the glutamine transporter SN1, or the amino acid transporter ASCT1 or ASCT2, all of which involved Na\(^+\) co-transportation. Pyr enters the cell via a proton linked monocarboxylate transporter (MCT). Glc enters the cells via the Glc transporters GLUT-1 and GLUT-4 and is utilised in the glycolytic pathway to produce Pyr.

It is of importance that in the presence of Gln, α-KG transport can activate Glc transport into the cells. α-KG acts by causing transcriptional repression of the glucose sensitive thioredoxin-interacting protein (TXNIP), which is normally induced in response to high levels of Glc, providing a negative feedback loop (Fig. 3.17). α-KG acts via the activation of the transcriptional factor MondoA protein (Stoltzman et al., 2011, Han and Ayer, 2013). In the absence of Gln, α-KG levels are decreased, decreasing Glc uptake via the formation of MondoA:MIX (Max-like protein X) complex which causes MondaA to activate TXNIP expression (Fig. 3.17). Therefore in the presence of Gln, Glc fuelled TXNIP induction is repressed and Glc transport increased (Fig. 3.13) (Stoltzman et al., 2011, Han and Ayer, 2013).
Figure 3.17. Transport of metabolic substrates across the mitochondrial membrane. Gln is transported into the mitochondria via a Gln/H⁺ antiporter, or is converted into glutamate (Gln) and enters by the malate-aspartate (Asp) shuttle and is transformed into α-ketoglutarate (α-KG). Pyr is transported by the mitochondrial pyr carrier (MPC). 1 µM FCCP depolarises ΔΨm and dissipates ΔpH, decreasing Gln and Gln mitochondrial entry. Thioredoxin (TXN) plays an important role in protecting the cell against oxidative stress. TXN-interacting protein (TXNIP) down regulates the expression and activity of TXN which can be transcriptionally activated by Mondo A, inhibiting Glc uptake. Glutaminolytically derived α-KG causes transcriptional repression of TXNIP via activation of MondoA:Mix (MondoA:Max-like protein X) which inhibits TXNIP and therefore prevents TXNIP GLUT inhibition.

It should also be noted that FCCP causes a decrease in mitochondrial Ca²⁺ levels (Fig. 3.7 B), which are known to regulate the mitochondrial enzymes pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH) and α-KG dehydrogenase (α-KGDH) (Denton, 2009). Decreased activity of PDH and IDH as a result of FCCP treatment can lead to a reduction of α-KG formed. It would also mean less
reducing equivalents are formed which would have an impact on ATP and ROS production. Increased levels of ROS would be of particular concern to cells in WM without Gln, one of the precursors for the antioxidant glutathione (Roth et al., 2002). It has been shown that glutathione and superoxide dismutase are reduced after FCCP treatment, along with an increase in ROS after 48 hours (Han and Park, 2011).

As expected, glycolytic activity was inversely related to OCR, demonstrated by Glc/Pyr and Glc which had the lowest basal OCR of those cells supplied with Glc, also had the highest basal L-ECA values (Fig. 3.3A, Fig. 3.5C). The most significant increase in L-ECA upon uncoupling was observed in cells supplied with Glc/Gln, possibly as a result of the repression of TXNIP by α-KG increasing Glc uptake (Fig. 3.5C, Fig. 3.15) (Stoltzman et al., 2011, Han and Ayer, 2013).

It is known that cancer cells are highly glutaminolytic, inhibition of glutaminolysis with BPTES decreased the respiratory response to FCCP in both PC12 and HCT116 cells (Fig. 3.8A-C and 3.9A-C). Glycolytic activity was increased in response to GLS1 inhibition in cells supplied with Glc/Gln with or without Pyr (Fig. 3.8D). However, in MEFs a different pattern of substrate dependent responses to uncoupling was observed (Fig. 3.9B-C). Glc and Pyr had a larger role to play in the respiratory response in these cells compared to PC12 and HCT116 cells, indicating a reduced role of glutaminolysis which was highlighted by the smaller decrease in response to uncoupling as a result of GLS1 inhibition (Fig. 3.9B).

A reduction in the phosphorylation of Erk p44/p42 (Thr202/Tyr204), Akt (Ser473), AMPKα (Thr173) and mTOR (Ser2448) after FCCP addition in cells supplied with Glc with Gln or Pyr was observed (Fig. 3.7D). The reduction in Erk and Akt phosphorylation was more marked in cells supplied with Glc/Gln/Pyr or Glc/Gln, the cells which demonstrated the strong respiratory response to FCCP. This effect may be as a result of an increased NAD/NADH ratio together with Ca^{2+} affecting the phosphorylation of these proteins. The increase in AMPKα phosphorylation in cells supplied with Glc was surprising as ATP levels were not significantly altered in these cells with FCCP addition (Fig. 3.7D). AMPK can be activated by increased ROS levels.
as well as an increase AMP/ATP ratio, it is possible that a reduction in ROS levels is sufficient to decrease AMPK phosphorylation (Dugan et al., 2013, Hardie et al., 2012). In the absence of Glc, in cells fed with Gal/Gln a dramatic decrease in p-AMPK levels was observed, which can be explained by induction of apoptosis shown by a very substantial decrease in PARP levels (Fig. 3.3D).

Next, the factors involved in HIF-α accumulation were studied including the availability of key metabolic substrates, OCR, IO₂, hypoxic stress, activation of AMPK, Akt and Erk, cellular ATP levels and transcription of HIF-α and PHD. Cellular HIF-1/2α levels can increase dependent on level and duration of hypoxia or metabolic deprivation. HIF pathways are important in metabolic regulation, giving them a pivotal role under hypoxia or metabolic substrate deprivation. Levels of IO₂ and metabolic substrates are also able to modulate these pathways. Gln was the one of the main contributors to HIF-2α stabilisation at 4% O₂, as it dictates the levels of cell deoxygenation, which was demonstrated in cells without Gln which were unable to stabilise HIF when transferred from 19 % to 4 % O₂ (Fig. 3.11A-B).

For HIF to be stabilised O₂ levels need to decrease below 40 mmHg (50 µM) which did not occur without Gln, however in the presence of Gln O₂ levels went under 10 µM (Fig. 3.10A-B) (Semenza, 2012). Cells supplied with Gal/Gln/Pyr were deoxygenated to below 10 µM and remained at that level for the first 2 hours of hypoxic exposure, allowing HIF accumulation to occur (Fig. 3.10B and 3.11A). Whereas cells supplied with Glc/Pyr showed lower cell deoxygenation and lower levels of HIF accumulation. However, cells supplied with Glc/Gln showed high levels of cell deoxygenation but lower levels if HIF-2α which suggests that substrate dependent effects other than cellular O₂ play a role in HIF accumulation under hypoxia (Fig. 3.10A, Fig. 3.11A).

Glutaminolysis was shown to be important for respiratory responses at 4 % O₂ as well as during FCCP treatment (Fig. 3.12A). Without Gln or with BPTES treatment inhibiting GLS1 causing cellular α-KG levels to decrease which may lead to a decrease in PHD activity, increased HIF stabilisation occurs under these conditions (Seltzer et al., 2010). However, this phenomenon was not observed in BPTES
treated cells supplied with Glc/Gln/Pyr or Glc/Pyr (Fig. 3.13A, B). In these cells intracellular O₂ levels are sufficient for PHD hydroxylation (Fig. 3.12A). It has been shown that Pyr can activate HIF via interactions with the α-KG binding site on PHDs (Lu et al., 2005); this may clarify why we saw higher HIF-α levels in Glc/Gln (Fig. 3.11A), which generate Pyr via glycolysis, supplied cells compared with Gal/Gln.

Substrate availability can determine the rate of HIF-α synthesis (Fig. 3.11A-B). This is likely to be from differences in ATP levels and HIF translation pathways (Fig. 3.11B and 3.13A-D). In hypoxia a large variability in cell bioenergetics and local oxygenation was observed, which was dependent on substrate composition. It is known that glucose is required for increased glycolytic flux necessary for cell survival under hypoxic conditions (Callahan et al., 1990). In the cells deprived of Glc, ATP continuously decreased at 4% O₂ and dropped dramatically at 0% O₂ (Fig. 3.11B). Under anoxic conditions the main driver for HIF accumulation was ATP availability (Fig. 3.11B). Investigation of the effect of OGD on AMPKα and Akt phosphorylation revealed that an increase in p-AMPK levels in PC12 cells is associated with reduced production of HIF-α upon OGD, whereas Akt/Erk phosphorylation and O₂-dependent accumulation of HIF-α are not tightly linked.

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. These results also show that both supply and utilisation of key metabolic substrates (Glc, Gln and Pyr) can affect the pattern of HIF-1/2α accumulation by differentially regulating iO₂ and ATP levels and Akt/Erk/AMPK pathways. Inhibition of key metabolic pathways (e.g. glutaminolysis) can modulate HIF regulatory pathways, metabolic responses and survival of cancer cells in hypoxia. This data is of relevance to those studying neuronal and cancer metabolism under hypoxia. These studies can relate to a number of different physiological conditions such as, deprivation of nutrients and O₂ as a result of ischemia/stroke, diabetes mellitus, liver carcinoma and cancer associated changes in uncoupling protein expression levels (Kalogeris et al., 2014, Heller and Chow, 2014, El-Serag et al., 2008).
3.5 Conclusions

To conclude these results demonstrate that the CEB platform is able to distinguish between the contributions of the main metabolic pathways; OXPHOS, Krebs cycle and glycolysis to ATP production and can be used to investigate toxicities from exogenous compounds, offering a more in-depth and more sensitive analysis compared to existing toxicity testing methods. The CEB platform can also be used to distinguish differences in bioenergetics between both cells and treatments, making it a valuable research technique. It can also be used to perform in-depth metabolic studies.

Please note that Chapter 4 (pp.109-134) is unavailable due to a restriction requested by the author.
OVERALL CONCLUSIONS

In this project it has been demonstrated that the CEB platform is a very useful tool for bioenergetics research and mechanistic studies of cell metabolism can be used to delineate the contributions of the main metabolic energy generating pathways. This system allows comprehensive assessment of mitochondrial toxicity, offering a more selective and sensitive analysis compared to conventional toxicity assays such as MTT, ATP, cell viability. But it has shown it can also be used to differentiate metabolic variances in ATP production between cell lines and/or treatments.

Using the CEB platform an in-depth study was performed, which showed that metabolic substrates are able to dictate that respiratory response to energy stress (FCCP uncoupling), and that mitochondrial uncoupling has a potential for substrate-specific inhibition of cell function. This approach and analytical methodology is applicable to a number of different cell models and research areas such as deprivation of nutrients and O₂ as a result of ischemia/stroke, diabetes mellitus and cancer associated changes in uncoupling protein expression levels. 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.

The results indicate that both supply and utilisation of key metabolic substrates (Glc, Gln and Pyr) can affect the pattern of HIF-1/2α accumulation by differentially regulations iO₂ and ATP levels and Akt/Erk/AMPK pathways. Inhibition of key metabolic pathways (e.g. glutaminolysis) can modulate HIF regulatory pathways, metabolic responses and survival of cancer cells in hypoxia. This data is of relevance to those studying neuronal and cancer metabolism under hypoxia. However it can be applied to many (patho)physiological diseases such as reduced nutrient supply and O₂ during ischemia/stroke, hypoglycaemia in diabetes mellitus patients and liver carcinoma (Kalogeris et al., 2014, Heller and Chow, 2014, El-Serag et al., 2008).
The last study revealed a new regulatory pathway for PDH E1-α phosphorylation. It has been shown that phosphorylation of PDH E1-α can occur in a manner independent of the HIF-1/PDK1 axis, mitochondrial respiration and the demand for acetyl-CoA in the Krebs cycle. These findings are of particular importance to those who are investigating the use of PDK1 inhibitors as possible treatments for patients with diabetes, heart disease or cancer.
**Future Work**

To complete this work further experiments are required. Firstly PDH activity needs to be measured to check that there is not a increased in PDH E1α phosphorylation to compensate for increase PDH activity in these cells under hypoxic conditions, however this is unlikely. PDH activity can be tested using kit such as the one sold by Abcam. A definitive reason for the increased phosphorylation of PDH in SCO2<sup>-/-</sup> under normoxia needs to be investigated. PDH levels should be studied in a larger range of O<sub>2</sub> concentrations to find when PDH becomes phosphorylated or if it is a gradual effect. A possible reasoning for the change in PDH phosphorylation status in the SCO2<sup>-/-</sup> cells is connected with the hypothesis that SCO2<sup>-/-</sup> cells undergo reductive carboxylation under hypoxic conditions. If this were the case glycolytically derived acetyl-CoA would not be needed as it would be generated through reverse Krebs cycle from α-KG formed through glutaminolysis. This would explain why these cells are unable to survive when glutaminolysis is inhibited as the reductive carboxylation is unable to form necessary lipids required for growth. This can be tested using carbon labelling techniques combined with mass spectrometry.
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11b. Comparative bioenergetic assessment of transformed cells


**APPENDIX**

**Supplemental 1.** MTT test performed on both HaCaT and HT1080 cells, showing good levels of biocompatibility after 24 hours exposure to various MIP NP conditions. Data obtained from Francesco Canforotta in Leicester University, UK.
Supplemental 2. Western Blotting analysis of total mTOR, Akt, Erk1/2 and AMPK protein levels. PC12 cells were incubated in different metabolic substrates for 2 hours and then retained at 21% O₂ or transferred to 4% or 0% O₂ for the shown number of hours. The data are complementary to the analysis of phosphorylated protein levels shown in Fig. 3.13A-E and Fig. 3.15A-D.
Availability of the key metabolic substrates dictates the respiratory response of cancer cells to the mitochondrial uncoupling

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ABSTRACT

Active glycolysis and glutaminolysis provide biosynthetic energy for cancer cells to proliferate. To study the effects of mitochondrial uncoupling by FCCP on the regulation of respiratory responses in cells, we used cancer cells and observed that FCCP induced a rapid increase in respiration rate and oxygen consumption. We found that this effect was highly dependent on the presence of the respiratory chain, which is consistent with previous observations. Our results suggest that mitochondrial uncoupling by FCCP can be used as a tool to modulate the respiratory response of cancer cells.

1. Introduction

A role of the mitochondria is that of a “power plant” of the eukaryotic cell. The electron transport chain (ETC) in mitochondria generates the proton motive force (PMF) which is used by ATP synthase (complex V) to produce ATP through the oxidative phosphorylation (OXPHOS). The efficiency of OXPHOS is tightly regulated by the amount of inorganic phosphate (Pi) utilized for ATP production per amount of O2 consumed, including the level of uncoupling between inward mitochondrial H+ current and ATP synthesis. Indeed, a certain proportion of H+ is always translocated to the matrix bypassing complex V, thus decreasing the mitochondrial membrane potential (ΔΨm). This so-called “cytotoxic” uncoupling can be achieved through the activation of uncoupling proteins, a non-specific ΔΨm-dependent process, which allows oxygen transport and carbonic anhydrase [3]. The weak acid protonophore FCCP (carboxylamide 4-trifluoromethoxyphenylphosphonic acid) [4], which provides reversible uncoupling [5] and dissipation of PMF in a concentration-dependent manner, is commonly used to study the effects of mitochondrial uncoupling in cancer cells.

Isolated mitochondria are a simple and well-established model as they are accessible to the metabolic substrates and pharmacological compounds, and independent of complex inter-compartmental transport of intramitochondrial, cytoplasmic metabolites and ions [5]. The substrates for isolation of mitochondrial oxidases and complexes of FCCP compounds are used for analysis of the functional activity of mitochondrial oxidases, and complexes of FCCP compounds are used for the study of the respiratory control ratio;
and in vitro toxicity of the new pharmacological compounds. The O2 consumption rate (OCR) in isolated mitochondria may be set to a different level by simple addition of the mitochondrial substrates and drugs affecting respiration. Thus, maximal mitochondrial respiration can be achieved by addition of FCCP to the mitochondria in state III [5]. However, the processes taking place in isolated mitochondria may be significantly different from that taking place in intact cells, which have undisturbed cellular networks and environments and represent more physiological relevant model for experiments on bioenergetics and metabolism [6]. Mitochondrial respiration in cells is regulated by many factors and the results of respiration under physiological conditions are not easy to interpret. Changes in the transport of metabolites and loss across the plasma membrane, a decrease in mitochondrial mass and in intracellular Ca2%, all strongly affect respiratory responses to FCCP, and even careful control of FCCP concentration for each cell type [5] may not eliminate indirect effects of this drug on cellular respiration and function in general. Thus, disruption 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 evoke energetic stress. Thus, uncoupling becomes life-threatening when glucose (Glc) is replaced with galactose (Gal), as glycogenolytic capacity no longer main in steady ATP levels.

In cancer cells, Glc supply becomes essential, since glycogenolysis produces large amounts of ATP regardless of high availability of O2 (Warburg effect) [11]. In turn, most of the pyruvate (Pyr), instead of conversion into Acetyl-CoA and utilization in the Krebs cycle [12], is converted to lactate and esterified from the cell. Some intermediates of glycogenolysis (e.g. phosphoenolpyruvate) are also re-directed to aerobic processes generating ATP for actively proliferating cancer cells [13-15]. To further accelerate anaerobic metabolic reactions and ATP production, cancer cells additionally utilize glutamine (Gln), and more than half of ATP as a by product of Glc oxidation (Glu)[1]. As a result, Glc-driven mitochondrial respiration in many cancer cells is active even at high Glc levels [20], and in cancer further upregulation of Glc with Gal.

Considering the complexity of the bioenergetic network, one can anticipate that regulation of cyclically supplied with different substrates may not inform correctly on their ability to respond to mitochondrial uncoupling in a physiological, i.e. by presence and sustained increase in respiration. Here, using zebrafish embryogenesis PCC2 cell and other cell lines, we studied how the availability and utilization of major substrates modulate the respiratory response of cancer cells to mitochondrial uncoupling.

2. Experimental procedures

2.1 Materials

O2-sensitive probe Mitotracker-XTRA [21], Mitotracker-XTRA NanO2 [22] and pH-sensitive probe pH-XTRA [23] were from Enzo Biochem Inc. (Carle, Ireland). Glutamine inhibitor, BPTES (Nε,Nε-(p-phenylenediamidine-1,4-bis(butoxyethyl))] was kindly provided by Dr. Takami Tsuchimoto (John Hopkins University, MD). Mitochondrial membrane potential indicators Lorotrimeric rhodamine methyl ester (L-MRM) [24] and Rhodamine 123 (Rhodam). Mitochondrial membrane potential indicator (TEMP) [25] was from Molecular Devices (Sunnyvale, CA). EGFP Western blotting reagent was from Cell Signaling Life Sciences (Woburn, MA), pre-made ankyrin-rich gels, and transfer buffers were from GenScript (Piscataway, NJ). IAC-™ Protein Assay Kit was from Thermo Fisher Scientific (Rockford, IL). The mitochondrial-targeted Ca2+ biosensor, nEosGFP2 [26] was from Evrogen JSC (Moscow, Russia). Tissueimer-GOR AAT Assay was from Promega (Madison, WI). Mineral oil (type 37) was from Carelift Laboratories (Coral Grove, FL). Lucifer Modified Eagle’s medium (DMEM) and Fetal Bovine Serum (FBS) media, 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 PCC2 cells, human colon cancer HCT116 cells and mouse embryonic fibroblasts (MEFs) were from American Type Culture Collection (ATCC, Manassas, VA). PCC2 cells were maintained in suspension in RPMI 1640 medium supplemented with 10 mM HEKES (pH 7.2), 2 mM L-Glu, 1 mM bovine serum (FBS), 100 U/ml penicillin 100 yagi units streptokinase (5%) in humidified atmosphere of 5% CO2 and 95% AIR at 37°C. HCT116 and MEFs were maintained in the same conditions in DMEM medium supplemented with HEKES, 1-Glu, 1% FBS and F5.x.

PCC2 cells were collected as described previously [26]. Briefly, for experiments with O2, EC and pH, cells were seeded at 5 x 10⁴ cells/cm² on 24-well plates (Corning Inc. One Pestell Line, Germany) coated with 0.01% collagen IV, and differentiated for 5-6 days in RPMI medium supplemented with NaHCO3, L-Glu, 1% bovine serum, 5% and 100 mg/ml NEA for live cell confocal imaging cells were seeded at 2.5 x 10⁴ cells per -1 cm² dish on glass bottom 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 x 10⁴ cells per well and differentiated for 5 days on 12-well plates (Corning Life Sciences, NY) coated with collagen IV or poly-D-lysine (0.003%) for protein analysis cells were seeded at 2.5 x 10⁴ cells per cm² dish on 96-well plates (Corning) coated with 0.01% collagen IV, shown for 2 days prior to analysis.

Washing media (WM) were prepared as follows. Powdered DMEM (Sigma, Cat. No. X980) was reconstituted in deionized water and filtered-stripped. From this plain DMEM, 12 different WM were composed by addition of 100 mM HEPES, 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±0.2 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% CO2 at 37°C for 2 h. To inhibit glutaminase, BPTES (10 µM) was applied to the samples 1 h prior to and kept during the experiment. To test the cell cycle regulation, cells were treated with 1 µM FCCP, optimal for all cell lines, as determined in separate experiment (Supplemental Fig. S1).

2.3. O2 consumption rate (OCR) assay

Measurement of OCR and O2 (see Section 2.5) was performed using a well-established phosphorescence quenching technique [27,28]. Developed for the assessment of O2 consumption by biological specimens on a conventional fluorometer spectrophotometer or polarimeter [21], a water-soluble phosphorescent O2-sensitive probe Mitotracker-XTRA was validated [29,30] and used in a number of studies [31-36]. These works demonstrate that the phosphorescence quenching is a
single, non invasive and versatile quantitative approach which allows for direct, high-throughput, real-time analysis of OCR and provides physiological relevant data on cell metabolism in a broad range of test systems, from isolated mitochondria [31-32] to small organs [38-39]. In this study, growth or differentiation media were replaced with VM and cells were incubated for 2 h (BIPES was added, as required). OCR measurements were conducted in a 100 µl of al-confluent WM supplemented with 200 mM NaCl (pH 7.4) (as described [36]), in the presence of mitochondrial uncoupler FCCP (1 µM) or rotenone (DMSO), which were added to the cells immediately prior to the measurement. Sample wells were quickly sealed with 150 µl of mineral oil pre-warmed to 37°C and the plate was monitored on a TR-FRET reader (Victor 2; PerkinElmer Life Sciences) at 37°C with excitation and emission at 530 and 440 nm, respectively. Each sample was measured in triplicate with a 2-min interval. The intensity signals were converted into photon counts (photons) with the values as follows: $T = (T_1 - T_0) / (I_0 - I_1)$, where $T_1$ and $T_0$ are the TRF intensity signals at delay times of 150 and 70 ns, respectively, and $T_0$ is the average of the samples calculated from four values [50]. Then the OCR measurement was calculated as $O_2$ consumed by cells in 1 min per 1 mg of total soluble protein (manufacturer's mg protein) [28]. Photocurrents were measured using BCA Protein Assay Kit.

2.4. Locate specific extracellular signal detection assay (ELISA)

The ELISA was measured as described [25]. Briefly, the grown or differentiated media were replaced with 150 µl of WM containing 10 µM HRPES and put into CO₂-free conditions at 37°C for 2 h to release absorbed CO₂. Then the media was replaced with unbuffered VM (without HEPES) and put back into CO₂-free conditions for 3 h incubation with BIPES was performed as necessary. After that, 100 µl of unbuffered VM containing 1 µM of the probe and the standards (FCCP or DMSO) were added to experimental wells and the plate was measured on the Victor 2 plate reader at 37°C for 60-90 min in the TE4 mode with excitation emission at 495/530 nm. In general, the intensity signals were calculated as the ratio (nmol of CO₂/ min) of the samples calculated from four values [54]. The extracellular lifetime $r = 0$ was calculated as described for the OCR and converted in pM values [23]. The latter were used to calculate an amount of protein extruded by cells in 1 min per 1 mg of total soluble protein (manufacturer's mg protein). Protein concentration was measured using BCA Protein Assay Kit.

2.5. Intracellular $O_2$ measurement

Developed and validated for the monitoring of $O_2$ concentration within the cell’s large molecules [30,40-41] intracellular $O_2$ probes were further improved and currently represent cell-penetrating small molecule [44-45] and nano-particle [22-24] based structures. The probes allow for precise quantification of the real-time measurement of cell oxygenation levels at different conditions [44-45], and rapid transient changes in respiration upon cell treatment [24-25]. Here we describe, in detail and validated in [22-24], MicroSens (i.e., MicroSens) probe, a small molecule that represents a non-toxic, non-invasive sensor, working with high throughput, reproducibility and efficiency in plate reader and fluorescence microscope platforms, as reviewed in [37-39]. Most recently, the probe was effectively applied for hyposia research [34-44]. The method was established in the method containing 10 µg/mL NaCoO₂ probe for 18-24 h at 37°C [22], then was DMSO supplemented with 18 HS (supplied with NCI for PC12 cells) and then VM (150 µl). Then the cells were replaced by a fresh aliquot (200 µl), and the cells were incubated at 37°C (BIPES was added when needed). Two hours later VM was replaced with a fresh aliquot (200 µl), and plate was read after 24 h. Uptake of NAD(P) or in a hypotensive chamber (Coy Laboratory Products, Grant Lake, MI) pre- to 4.58 O₂. Monitoring of CO₂ was performed similarly to OCR measurement at 37°C with 430 nm excitation and 660 nm emission spectra. The CO₂ concentrations and cell deoxygenation rates were calculated as described [22].

Briefly, the plate was incubated in the reader for 10 min and then monitored for ~20 min to achieve steady-state oxygenation of resting cells, then the plate was quickly withdrawn from the reader, and then F (CA) and pCO₂ were added to the cells (30 µl of 10x stock solution) and monitoring was resumed for further 10 min.

2.6. Monitoring of GFAP, SNR and mitochondrial Ca²⁺

PC12 cells were seeded 2.5 × 10⁵ cells per cm² and differentiated for 4-5 days with NGF containing basic fibroblast growth factor (BFGF) (20 ng/mL) and platelet-derived growth factor (PDGF) (20 ng/mL). Transfection with miR-375 plasmid was carried out using (profection 2000 and OptiMEM I medium) with miR-375 concentration adjusted to 2 µM. During the transfection, miR and miRNA and Father medium were added to the cells 20 min and 1,000 µl/24-well, respectively.

Confocal laser fluorescence imaging was carried out on a Zeiss Axiovert 200M/S confocal laser scanning microscope with 488 nm Ar laser wavelength and 514 nm HeNe laser. The miR375 probe was excited at 488 nm (20% of maximal power) with emission collected at 500-540 nm. ToF and FPI measurements were excited at 540 nm (2.5x and 1%, respectively) collecting emission at a 525-560 nm filter. The probe was most highly used individually, although in some experiments, ToF and miRNA were used simultaneously. Since miR-375 can decrease the miRNA binding (when expressed at very high levels), only the cells with untransfected miRNA signal were selected for analysis. Acquisition of each signal was done in sequential laser mode with emission gates adjusted to avoid overlaps.

All images were acquired with DeltaVision 4D (Olympus) and analyzed using ImageJ (NIH, USA) and ImageJ plug-ins for 3D visualization and Western blot analysis.

The cells were differentiated for 12-well plate and pre-incubated for 2 h in different VM. The media were replaced with 10 µg/mL NAD(P)H for 2 h well, and the cells were incubated at 37°C for 4 h in 5% VM (see Results section) with or without 1 µM FCCP. Whole cell lysate protein preparations were prepared as described [41]. Briefly, cells were washed twice with PBS containing phosphate buffer and lysed for 15 min on ice with lysis buffer, containing 150 µM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM sodium vanadate (PMSF = 7.5%), and protein inhibitors (Hoechst, Iodine). Afterly, lysates by centrifugation for 10 min at 16000 g and 4°C, protein concentrations were measured using BCA Protein Assay kit with a high throughput. Protein concentration was determined using BCA Protein Assay kit, quantified by absorbance at 562 nm using a Shimadzu UV-1601 PC spectrophotometer. The protein was separated by 6% and 4-20% polyacrylamide gel electrophoresis (Biorad, USA) and transferred to a 0.2 µm nitrocellulose membrane. Proteins were detected using 6% for free mill or 5% Iodine in TBS overnight at 4°C (primary) and 2 h at room temperature (secondary). Membrane was visualized and analyzed with NIH Image software.
Quantitative image analysis was performed with Imagej program using co-tabilis signals for normalization. Images were processed with ImagePro, Photoshop, and Illustrator programs.

To normalize the results of metabolic assay for total protein content in different samples, proteins were prepared as above and measured using BCA TM Protein Assay kit.

2.6 ATP measurement

Analysis of total cellular ATP was performed using CellTiter-Glo kit as described in Methods. Total protein in the samples was determined as described above.

2.7 Statistics

Statistical analysis was performed using the results of 3-6 independent experiments. Confidence levels of 0.05 and 0.01 were deemed as statistically significant. To ensure data accuracy and fidelity, the majority of the experiments were performed in 3 replicates.

The levels of OCR, ECA, 10A, and ATP were normalized to the total protein content in the samples. ATP levels were normalized to the total protein content in the samples. The difference between the means was evaluated using two-tailed Student’s t-test (equality of variance in the samples was first examined using Levene's test). The difference between the means was evaluated using Mann-Whitney U-test.

3. Results

3.1 Effect of metabolic substrate composition on the respiratory response to FCCP

A highly metabolically active cell line with equally well developed glycolytic and mitochondrial ATP sources [29, 30], Rb cell were commonly used for studies on bioenergetics of cancer and neuronal cells [31-52]. Mitochondrial uncoupling with FCCP continuously increases respiration, and activates glycolysis in PO2 cells supplied with Glc, Pyruvate, and C12. Here, we pre-incubated different F12 cell lines in 12 different working media (WM, Table 1) for 2 h to achieve metabolic adaptation, and then treated them with 1 μM FCCP.

Cell oxygenation is inversely related to their respiratory activity, and changes in intracellular O2 concentration on the magnitude of the respiratory response to stimulation [36, 37]. We found that the highest O2 demand was provided by C12 combined with Glc (with or without Glc). Consuming glucose at a rate of 5 μM/min per 1 μg of total intracellular protein, the cells most effectively achieved O2 demand (10 min, Fig. 1A). Cells utilizing Glc plus Glcy or of Glc also exhibited high respiratory activity (over 4 μM/min per 1 μg of protein) and were strongly desensitized. The other WM were scored according to cells respiration as follows: Glc > Pyruvate, Glc, Glcy, Glc > Glc, no substrates. WM containing Pyruvate, C12, and Glc, addition of Glc decreased respiration.

Upon addition of FCCP, we observed four types of respiratory responses, classified according to the changes in cell oxygenation ΔO2 = O2f − O2i, where O2f and O2i are the O2 concentrations in the cell monolayer before and at any time point after FCCP addition, respectively. Changes in ΔO2 agreed with the data on OCR (Table 2), calculated for the initial linear phase of the experiments (Supplemental Fig. S2).

The first type was characterized for the cells supplied with Glc combined with either C12 or Pyruvate (Fig. 1B, C). Cell deoxygenation was deep and continuous (over 1 h), suggesting steady increase in mitochondrial respiration. Equally large ΔO2 were observed in the cells exhibiting high (Glc/Pyr/C12, Pyruvate/Glc) and moderate (Glc/G12, Glcy/Pyr/Glc) OCR at rest.

The second type, a significant inhibition of respiration characterized by progressive oxygen consumption, was seen in the cells supplied with Glc or Glcy/Glc and moderate OCR at rest. Initially, the decrease in respiration in the cells supplied with Glc or Glcy/Glc was particularly surprising, since ‘resting’ OCR in these cells was high (Fig. 1A).

The third type, a moderate transient drop in OCR, was observed in the cells supplied with Glc or Glcy/Glc and the low-respiring cells deprived of all substrates or supplied with Glc.

Strong decrease in oxygen consumption was observed in the cells supplied with Glc/Glc or Glcy/Glc suggested possible inhibition of cellular function and cell death. Using microscopy and Western blotting analysis, we found that treatment with FCCP for 1 h caused partial detachment of cells from collagen-coated surface and active apoptosis, as indicated by PARP degradation [33] (Fig. 1D). LUCI/VEGFR2 ratio [54] was decreased in all cells treated with FCCP, pointing to an activation of autophagy.

3.2 Mitochondrial substrate regulates basal activity between respiratory parameters: a search for hemi-energetic parameters and pathways upon uncoupling with FCCP

To explain the differing effects on the respiratory response to FCCP, we subjected effects of metabolic substrate and the use of hemi-energetic parameters and pathways upon uncoupling with FCCP.

We found that significant differences in resting ATP levels exist for the cells deprived of all substrates or supplied with Glc alone (Fig. 2A) only a minor decrease in ATP level was observed within 10 min after the addition of FCCP to the cells supplemented with Glc, in any combination (Fig. 2B). In contrast, without Glc, ATP flux, cellular ATP decreased dramatically within 10 min after the addition of FCCP. Detectable by E-LISA analysis only in the cells supplied with Glc, glycolytic activity was inversely related to OCR (Fig. 2C and 3A). Upon uncoupling, 12CA could not be measured in the samples containing Glc/Glc or Glcy/Pyr/Glc, which produced a strong continuous respiratory response to FCCP (Fig. 1B, C).

No correlation between ATP levels and ΔO2 was seen upon uncoupling (10 min, r = 0.13, p = 0.12; 60 min, r = 0.08, p = 0.46), suggesting that ATP does not regulate the respiratory response to FCCP. Indeed, equally strong responses were observed in the cells with the highest (ECA and the lowest (Pyruvate/Glic) ATP levels, while in the presence of Glc (high ATP) or Glc alone (low ATP) the responses were very low.

Likewise, changes in respiration did not depend on ΔN1 and mitochondrial GCF levels, which were proved by IMM and immunoGCF (28) staining respectively. Strong mitochondrial wave imaging, observed similar level in ΔN1 polarization in all setting cells except for the cells supplied with only Glc or deprived of all substrates (Fig. 3A). With the use of 5-nitro-5-iodo-2',3'-dideoxyuridine, ΔN1 and mitochondrial GCF1 drastically decreased in a similar manner (Fig. 3B and Supplemental Table 3). Plasma membrane potential (ΔΨm), profiled by PMH1, was not affected by FCCP. It should be noted that in cells deprived of substrates, the ΔΨm was substantially depolarized at rest (Supplemental Fig. S3).

Substantial changes in Glcy and perturbed ATP and ion turnover could affect metabolic pathways involved in hypoxia signaling and energy metabolism. We performed Western blotting analysis of AMPKα and E2 (p44/42) phosphorylation in the samples treated with FCCP for 4 h (Fig. 2E) and found that this pathway was strongly affected by mitochondrial uncoupling. The levels of p42 E2, pSer296 and p44 E2, (Thr202) and Thr204 phosphorylation were
significantly decreased in the cells supplied with GalCh (particularly with GalCh/Glc and Glc/GalCh), and increased in the cells fed with GalCh/Pyr/Gln (except for m-TOR). AMPK phosphorylation in all samples treated with FCCP was slightly lower than in non-treated controls. In the cells supplied with Glc/GalCh, phosphorylation of the aforementioned protein was not observed.

3.3. Role of glutaminolysis in the respiratory response to FCCP

In agreement with a dominating role of Glc in bioenergetics of cancer cells [18,19], we observed the most prominent respiratory response to FCCP only in the cells supplied with Glc. To further address the mixed glutaminolysis in the cellular response to uncoupling, we used BPTES, a specific inhibitor of triacylglycerol synthase (GLS1). An inhibition of GLS1 was achieved by incubation of the cells with 10 μM BPTES for 1 h prior to and during the experiments. This concentration was shown to inhibit glutaminolysis by 60% in isolated extracts of rat kidney liver mitochondria [20].

BPTES was able to have no major effect on cellular ATP measuring cells (Table 2), demonstrating that residual GLS1 activity maintained sufficient energy levels in the cells deprived of Glc. Upon uncoupling, GLS1 inhibition leads to a factor decrease in ATP in these cells (see also Fig. 2B), highlighting the importance of glutaminolysis for energy balance.

In the VM containing Glc, treatment with BPTES only slightly affected ‘resting’ levels of OCR and O2 (Fig. 4A and C), Table 4). Upon uncoupling the effect of BPTES was more clearly evident. The response of the first type was almost completely abolished (see also Fig. 1). Moreover, in the cells supplied with GalCh/Pyr/Gln or Pyr/Gln (Fig. 4B, C), uncoupling was observed, which was similar to the second type response in VM containing GalCh or Pyr, respectively (Fig. 4D). In the media without Glc, respiration was not affected by BPTES treatment, demonstrating high specificity of the drug.

Analyzing the effect of GLS1 inhibition on the rate of glycolysis in the media supplemented with Glc, we found that treatment with BPTES increased 1-EGA rate in resting cells supplied with Glc/Gln/Pyr and Glc/GalCh (2.6-fold and 1.5-fold, respectively), suggesting significant activation of glycolysis (Fig. 4D). 1-EGA rate in uncoupled cells was almost unaffected.

Table 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>OCR (pM)</th>
<th>O2 (pM)</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>Glc</td>
<td>51</td>
<td>11</td>
<td>N/A</td>
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<tr>
<td>Glc</td>
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<td>Glc</td>
<td>51</td>
<td>11</td>
<td>N/A</td>
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*GlS1 not very low.*
3.4. Cells specificity of the responses to RCP

In human colon cancer HCT116 cells, a common model for studies on cancer cell metabolism, we anticipated similar to PC12, Gln-dependent respiratory responses to RCP. In contrast, in non-cancer MEFs immortalized with N40 large T antigen [15][16], glutaminolysis can be strongly activated by Myc overexpression [17], suggesting relatively low rates of Glu utilization in resting MEFs. Based on these observations, we expected a specific pattern of the response to uncoupling in MEFs, distinct from that observed in cancer cells.

Indeed, HCT116 cells exhibited high overall reduction with PC12 cells in OCR and oxygenation levels at rest as well as upon uncoupling (Fig. 5A). The most pronounced decrease in OCR upon RCP treatment was observed in the cells supplied with Glu with either Pyr or Glc (with or without Gal), however, in the presence of Glu, this effect was less prominent than in PC12 cells (Fig. 5A, C). Aids to PC12 cells, HCT116 cells could not withstand uncoupling when supplied with Glu or Ca/Glu; although Glc levels were low at rest, they noticeably increased upon addition of RCP, suggesting a decrease in OCR. Treatment with BFTS caused a large reduction of the responses in all cells supplied with Glu and normally strongly increasing respiration upon uncoupling. However, HCT116 exhibited lower sensitivity to BFTS than PC12 cells.

In MEFs, the response to uncoupling was generally more rapid and transient (Fig. 5B, C). Although Glu remained important for generating pronounced responses to RCP, they were quite specific and could be classified as: 1) a non-rapid increase in respiration, followed by partial or complete restoration of resting OCR levels (Glu in all combinations with Glc and Pyr, as well as Glc/Gly/Pyr); 2) type two decrease in respiration and cell respiration (no substrate, Glc and Pyr alone or in combination with Gal); and 3) type three—no significant changes in respiration and Glc or Glc/ Gal alone. Unlike PC12 and HCT116 cells, the most pronounced response to RCP was observed in MEFs supplied with Glc/Gly/Pyr (Glcn down to 20 in Glc), and Glu inhibition only partially decreased the response to uncoupling. Moreover, MEFs deprived of Glc and supplied with Glc/Gly/Pyr were capable of generating continuous positive response to RCP (Fig. 5E).

4. Discussion

The contribution of a metabolic substrate to mitochondrial respiration is determined by its transport across cell membranes and efficiency of its utilization by corresponding pathway(s).

Cells generate ATP mainly through glycolysis and OXPHOS, the absence of Glc can be compensated by increased OXPHOS flux and mitochondrial respiration, provided that Pyr and Glc are available. Glc is a key metabolite required for energy production and anaplerotic reactions in cancer cells [18]. In many cell types it is efficiently utilized through glycolysis, giving rise to over 90% of cellular ATP and maintaining respiration at high levels [19]. In agreement with this, we observed highest OCRs in the cells supplied with Glc and Glc/Pyr (Fig. 1A, B). In all media containing Pyr or Glc, respiration was decreased in the presence of Glc because ATP production was partially shifted from OXPHOS to glycolysis. Without Pyr or Glc added to the medium, respiration was supported by Glc oxidation. Sustained H+ transporters in the inner mitochondrial membrane by RCP rapidly and sustainably dissipates the ΔΨm and ΔpH [19] in these conditions. ATP is no longer produced by the mitochondria, instead, mitochondrial complex V converts ATP working in reverse mode on the restoration of ΔpH. As a result, glycolysis must be activated to compensate for the loss of OXPHOS flux and to supply complex V with ATP.
Fig. 3. Effects of substrate composition on the ATP depletion and mitochondrial protein phosphorylation upon mitochondrial uncoupling. A, Quantitative analysis of TMEM localization at rest shown a significant decrease in the ATPi only in the cells supplied with no substrates (labeled Gal). A Representative Western blot of PFP1 and COX1 in the cells treated with TMEM and mitochondria protein (respectively). B, Higher panel demonstrates protein localization in 5 min after FCCP addition. C, The profiles of ATP depletion upon treatment of the cells with FCCP. D, Western blotting was performed to detect TFI (Pyr/C12), p38 MAPK (Tyr180), and p-PKA (Ser40) phosphorylation upon FCCP addition (5 min, 5 μM). The data demonstrate phosphorylation of the selected proteins upon treatment with FCCP. Phospho-specific difference in phosphorylation of p38 (p-p38 and p-p38, 10 μM and 5 μM). In all cases, the levels of the peptides phosphorylated were normalized to the total protein content, which remained almost unchanged (not shown). Bar in (A) represents 50 μM. Error bars indicate 95% of the protein intensities within a cell population (90–100%, n = 4).

Analysis of total ATP revealed striking differences in the levels of energy stress experienced by the cells upon uncoupling. When deprived of Glc, PC12 cells rapidly lost ATP (Fig. 3), while in the presence of Glc, ATP levels remained almost unchanged, although expected, demonstrates that respiratory performance of uncoupled mitochondria surprisingly does not depend on cellular ATP, which is often used as a marker of cell viability. Indeed, no correlation was found between ATP levels and the magnitude of the respiratory responses to FCCP, and even after dramatic decreases in ATP (by up to 85% within 10 min) in cells supplied with Glc plus Pyr were capable of maintaining a high respiratory rate for more than 1 h.

The most pronounced responses were observed in cells containing Glc combined with Glc or Pyr, which agrees with the Glc addiction of cancer cells (Figs. 1, 3, 5). Supplemental Fig. S2). However, considering the dominant role of glutaminolysis in cancer metabolism, a decrease in respiration upon uncoupling of the cells supplied with Glc or Gal.

Table 3: Effects of Glc addition on ATP levels in cell mitochondria upon uncoupling

| Glc/Glu | Glc/Pyr | Glc/He | Glc/Py/He | Glc/Pyr/He | Glc/Py/Glu | Glc/Py/Glu | Glc/Pyr/Glu | Glc/Py/Glu |
|---------|---------|--------|-----------|------------|------------|------------|------------|------------|------------|
| 95       | 106     | 124    | 1.32      | 0.86       | 0.93       | 0.97       | 0.89       | 0.93       | 0.86       |

Data are shown as ATP(+) / ATP(−) ratio (in arbitrary units (a.u.)).
was unexpected. Although resting O2 consumption rate in these cells is very high, FCCP not only inhibits mitochondrial function, but also induces apoptosis (Fig. 1D).

To explain this phenomenon, we reviewed Glu transport and utilization pathways. As shown schematically (Supplemental Fig. S4), Glu is transported into the cytosol via a Glu transporter SNT1 and amino acid transporter AAT1/AAT2, all of which involve Na+/K+ co-transport and may potentially be affected by the diphosphorylase inhibitor. Since the uncoupling effect on the isolated cells was seen, specific effects of FCCP on Glu transport across plasma membrane can be ruled out (Supplemental Fig. S5).

Mitochondrial transport of Glu and its derivatives is versatile. Since differentiated PC12 cells exhibit similarities with neuronal cells, we report via mitochondrial glutamate carrier, typical for the brain tissue [57], is particularly relevant (Fig. 6). Although electroneutral, Glu uptake by the mitochondria in neurons depends on the ATP, and therefore can be suppressed by inhibiting (reserpine) and uncoupling (RCP) the mitochondrial [58]. Glu can also be converted by cytosolic glutaminase into glutamate (Glu), which then enters the mitochondria through either Glu carriers in Glu-Phos or, as the latter are a part of the substrate-transporter (ATP) shuttle [59]. Moreover, it is directed to the translocation of the resting equilibrium (NADH) from the cytosol, where they are produced mainly through glycolysis and Pyruvate dehydrogenation, to the mitochondria across the mitochondrial membrane, which is impermeable for NADH Glu/Pyr-linked dehydrogenases of the transport of cytosolic Glu derivatives through the Mal-App shuttle is inhibited due to a deficiency in cytosolic NADH. Moreover, Glu-App carriers and transporters are electrogenic and acquire electrochemical potential across the mitochondrial membrane, which is impermeable to uncoupled cells. In norm, it is carriers [60] that transport Glu into the mitochondrial matrix in a cytosol with H+, and therefore this pathway is also inhibited upon disruption of ATP with FCCP. Taken together, without Glc and Pyr supply when the Mal-App shuttle activity is decreased, uncoupling can partially activate mitochondrial Glu and Glc carriers and decrease respiration, as demonstrated in our experiments.

RCP can also affect the pathways recruiting Glc and Pyr in PC12 cells. Transported into the cell through GLUT-1 and GLUT-4, Glc is converted to Pyr, which can be also delivered into the cell via the H+-linked monoglucopyruvate transporter (MCT). Since in our experiments FCCP did not affect the GlcPyr transport of Glc and Pyr across the plasma membrane, should not change much. From the cytosol Pyr enters the mitochondria by means of H+-coupled transport through the mitochondrial pyruvate carriers (MPC) (Fig. 6) (50,51), and is converted to acetyl-CoA and -ketoglutarate (a-KG) by pyruvate dehydrogenase and -ketoglutarate dehydrogenase, respectively. The rate of Pyr transport into the mitochondria through MPC is PMF-dependent and decreases upon its

Table 4: Effect of FCCP inhibition on OCR in the cells at rest and upon ATP removal

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glu</th>
<th>Glc</th>
<th>Glu</th>
<th>Glc</th>
<th>Glu</th>
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<th>Glu</th>
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<tbody>
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<td>Control</td>
<td>1.00</td>
<td>0.96</td>
<td>1.00</td>
<td>0.96</td>
<td>1.00</td>
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<td>1.00</td>
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<td>1.00</td>
<td>0.96</td>
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</tr>
<tr>
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<td>1.10</td>
<td>1.12</td>
<td>1.10</td>
<td>1.12</td>
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<td>1.12</td>
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</table>

Data are shown as BHT (± SEM), *p < 0.05, N/A.
Fig. 3. Differential regulation of the respiratory response to FCCP in HCT116 and MEFs by metabolic substrate availability and inhibition of glutaminolysis. A, in HCT116, unlike the C, pyruvate dehydrogenase kinase is phosphocarboxylase isophosphatase (PDK) the most observed response change is in the cells supplied with Glu/Gly/Asp. Additionally, in cells treated with 2-deoxy-D-glucose (2-DG), the levels of Glu were increased. B, in MEFs, with Glu/Gly/Asp the levels of Glu were increased.

dissipation [63], which occurs in all the media upon uncoupling (Fig. 3). This may explain why no significant increase in mitochondrial respiration is observed upon addition of FCCP in the cells supplied with Glu, Pyruvate, or Glu/Pyr. A detailed quantitative comparison analysis of activity of the transporter involved in Glu and Pyruvate uptake in intact and uncoupled mitochondria would be very interesting.

However, other factors can also contribute to the lack of classical respiratory response to FCCP in the cells supplied with Glu and deprived of Glu. For example, the presence of the Glu transporter can be activated by α-ketoglutarate (produced through glutaminolysis and the Krebs cycle), which can activate transcriptional regulation of the membrane-associated protein (NDP) through interaction with the MEK protein. Upon Glu deprivation or inhibition of glutaminolysis, the levels of α-ketoglutarate in the cell are decreased, and therefore the MEK protein can activate the NDP expression, leading to a decrease in Glu uptake. Similarly, the mechanism is based on a G0-dependent nuclear accumulation of the NDP complex, which is known as transcriptional regulation of NDP.

Additionally, a dramatic decrease in mitochondrial respiratory capacity (Fig. 3) can lead to the partial inhibition of the Krebs cycle enzymes pyruvate, oxaloacetate, and α-ketoglutarate dehydrogenases [64], thus decreasing the amount of the reducing equivalents but increasing the electron transport chain, ultimately down-regulating ATP production. Cell toxicity of FCCP, related to its ability to interact with mitochondrial thiol and redox systems (cytochrome c, proton transfer chain), can also be differently modulated by metabolic substrates. Similarly, substrate composition may be an important factor regulating FCCP-
induced ROS dependent apoptosis in cancer cells, since Gin is one of the precursors of an antioxidant GSH [9]. FCCP has been shown to decrease the levels of GSH and the activity of the mitochondrial superoxide dismutase in a number of cancer cell lines, causing an increase in ROS levels after 48 h of treatment, which was associated with cell death [1,7,22].

A decrease in NADPH levels and ROS production at early stages of FCP treatment [7,34] changes in ATP and Ca2+ turnover—all can mutually modulate activity of the major pathways involved in cell metabolism and energy production. Upon FCP treatment we found significant decrease in the phosphorylation of Akt (Ser473), AMPK (Thr172) and mTOR (Ser2448) in all cells treated with Gin in combination with Gln and Pyr (Fig. 3D). A decrease in both Akt and mTOR phosphorylation was more pronounced in the cells supplied with both Gin and Gln, capable of producing a strong respiratory response. We cannot explain these effects, however, most probably they are ATP-independent, because ATP levels in the presence of Gin were not affected by uncoupling. On the other hand, an increase in NADPH/NADP+ ratio and cytoplasmic Ca2+, as well as a decrease in ROS production may largely affect phosphorylation of these proteins. Surprisingly, in cells supplied with Gln/Gln/Pyr we observed a decrease in AMPK phosphorylation, which is known to increase with an elevation of AMP/ATP ratio; ATP and ROS levels [3,35]. We believe that, once in these cells FCCP reduced both ATP and ROS, a decrease in the latter might have compensated for a decrease in the former. In contrast, E2k and particularly Akt phosphorylation in the medium containing Gln/Gln/Pyr was increased. We propose that such a strong elevation of phospho-Akt suggests an attempt to increase glycolysis (though not feasible without Gin), which is regulated by Akt in cancer cells [71]. In the cells supplied with Gln/Gln and treated with FCCP, protein phosphorylation was detectable, which can be related to a relative increase in glucose. Activity of the major pathways and respiration upon uncoupling require further investigation.

Overall, our results demonstrate that for significant response to uncoupling to be achieved both Gln and Pyr (including Pyr produced from Gln) are required, as FCCP can strongly decrease influx of these metabolites into the mitochondrial matrix.

In agreement with the concept of "shared" contribution of the Ox/Res and glycolysis in the maintenance of cellular ATP pool, L-ECG analysis demonstrated that glycolytic activity was inversely related to OCR. Thus, the rate of lactate extraction from the cells supplied with Gln/Gln/Pyr was lower, since Ox/Res in these cells was the highest among the cell groups in the presence of Gln (Fig. 7). Upon uncoupling, the most prominent increase in glycolysis was observed in the cells supplied with Gln, which may be associated with an increased Glu transport in these cells (through Monocar). On the other hand, these cells exhibited strong sustained response to FCCP, which may require additional ATP production through glycolysis. However, this is doubtful, because the cells depleted of Gln with extremely low ATP levels are able to produce even stronger respiratory responses to uncoupling (Fig. 1A).

Although in the presence of Gln or Glu, Glu respiration dropped upon uncoupling, Glu metabolism is the major contributor to the response to FCCP in non-cancer cell lines (OCI12 and HCT116). This was confirmed in the experiments with BPTES, which decreased glucose metabolism through specific inhibition of Glu5. Interestingly, in the presence of BPTES, respiration was only slightly decreased, while the response to FCCP was almost completely abolished. This suggests that residual Glu5 activity (~20% of the maximal level) is not sufficient to maintain respiration but the maximal respiration rate. Moreover, respiration in OCI12 cells supplied with Gln and treated with BPTES dropped below the resting levels upon uncoupling. Glycolytic activity in resveratrol-sustained cells supplied with Gln/Gln/Gln or Gln/Gln was elevated totalizing ATP levels upon Gln inhibition (Fig. 4, Table 3). Upon FCCP treatment, L-ECG increased only in the cells supplied with Gln/Gln/Pyr. This could be credited to increased glycolysis and increased Pyr resulting in overall increase in Pyr which is converted into lactate thereby contributing to L-ECG rate. The respiratory response of OCI12 cells to uncoupling was similar to that of OCI12 with a smaller effect seen when Glu5 was inhibited. The respiration decreased but not blunted like in OCI12. This could be due to relatively low activity of mitochondrial electron transport complexes (HAD and COX) due to high expression of Glu5 [79], a pre-existing, liver type mitochondrial, which is resistant to BPTES [79].

Resting and uncoupled respiration of MDRs were also substrate-dependent. However, the response to FCCP differed significantly from the control cell lines. In the optimal media (Fig. 5), FCCP induced less sustained and pronounced increase in respiration, than in the cancer
anis. Although Gls remained very important for active respiration, the contribution of Glr to the respiratory response was more substantial than in PC12 and HTC16 cells. Illustrating this phenomenon, the most pronounced decrease in oxygen was observed in the cells supplied with Glc/Byr/Glu, similar to the response in PC12 and HTC16 cells supplied with Glc/Byr/Glu (Figs. 1B, 4A). Highlighting the decreased contribution of glutamine to the uncoupling response in these cells, MTS supplied with Glc/Byr produced positive response to FCCP (Fig. 2C). Finally, an inhibition of GCS did not only partial decrease in free energy to uncouple, indicating that MTSs were capable of generating a significant portion of energy via mitochondrial uncoupling and non-mitochondrial pathways.

Takeda et al. [11] used a mitochondrial permeability transition (MPT) assay to measure mitochondrial membrane potential (ΔΨm) in the presence of glutamine. The results showed that glutamine treatment caused a significant decrease in ΔΨm, indicating that glutamine can act as an uncoupler and induce mitochondrial dysfunction.

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Differential contribution of key metabolic substrates and cellular oxygen in HIF signalling

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Abstract
Changes in availability and utilisation of O2 and metabolic substrates are common in ischemic and cancer. We examined effects of substrate deprivation on HIF signalling in PC2 cells exposed to different atmospheres: 5% O2; 5% O2 + 15% CO2 (normal); 1% O2 + 15% CO2 (hypoxic); 0.1% O2 + 15% CO2 (extreme hypoxic). HIF-1α protein levels were determined by the availability of glutaminolysis and glucose, a source for NADPH, for cell survival. Cell viability, proliferation, and glucose consumption were higher in the presence of glucose compared to glutamine. In addition, the glucose concentration was inversely related to HIF-1α levels. In the presence of glutamine, the glucose concentration was inversely related to HIF-1α levels. In the presence of glutamine, the glucose concentration was inversely related to HIF-1α levels.

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Introduction

In the vast network of metabolic and signaling cascades, hypoxia inducible factors (HIF) are particularly important for the adaptation of aerobic organisms to low O2. HIF-1 and HIF-2 mediate transcription of several hundred genes [1] involved in the regulation of glucose and iron metabolism, erythropoiesis and their sequelae. The latter include cell cycle progression, proliferation and inflammation, maintenance of stem cell niches and embryogenesis, ischemia preconditioning and cancer development [2-7].

To start acting as transcriptional factors, HIF-1 and HIF-2 must be assembled from α and β subunits and activated by interacting with α-knobs/CBP protein. Biology of HIF and HIF-1/2 differs; gene expression is described elsewhere [1,4] importantly for HIF signaling in cellular levels of α subunits are tightly controlled. Transcription is regulated by NF-κB [3] and numerous epigenetic factors [9, including micro-RNA-125b (m-RNA-125b) [3,7], miRNA-192 (m-RNA-192) [8], miRNA-2-3p and miRNA-30a-3p [8]. Translation is up-regulated through the Met and stk pathways [9], and inhibited by low-regulatory proteins RhoP [11,13] and small regulatory molecule [11], interacting with the low regulatory elements in 5’UTR of HIF-2α mRNA. AMPK decreases HIF-α production [14], and also orchestrates cellular energy stress by acting in a NF-κB-independent manner [15,16]. Akt, P38 and MAPK pathways are interlinked as they conduct their HIF-regulating activities through the mTOR complex 1 (mTORC1), a master regulator of protein synthesis and cell proliferation [17].

When intracellular O2 (O2) concentrations are sufficient for enzymatic hydroxylation of dedicated prolyl and asparagyl residues in HIF-α subunits (Pro402, Pro409, Asp406 in humans), they undergo rapid prolyl hydroxylation or transcriptional inactivation [19]. Highly dynamic process of HIF-1α hydroxylation [18], monitored by E2-dependent HIF-1α and HIF-2α, it is determined by the rates of glucose and glutamine oxidation, along with the efficiency of the sensor and the cells [20-22]. On the other hand, metabolism of glucose and glutamine strongly affects HIF hydroxylation by regulating the levels of cellular α-ketoglutarate (α-KG), reducing equivalents, ROS and metabolites of the Krebs cycle [3].

High rates of glycolysis and glutaminolysis assist rapid cancer cell growth and tumour expansion even in deep hypoxia [23,24]. Metabolic addiction makes these pathways attractive targets for cancer therapy, generally via reduction of the transport and utilization of glutamine and glucose [25-28]. Often therapeutic approaches are directed to the inhibition of glycolysis through down-regulation of HIF signaling [29,30]. In turn, pharmacological modulation of glycolysis was proposed as an approach to target HIF-1α for cancer therapy [31,32].

The importance of glucose and glutamine for HIF-1α signaling in hypoxia has been shown in a number of publications, including studies on different cancer cell lines, myocytes and stem cells [31-37]. The negative effect of glucose and glutamine deprivation on HIF-1α levels was demonstrated in human fibroblasts and pancreatic cancer cells and related to a decrease in protein stabilization [31,33,34]. Wu and others have also shown that the synthesis of HIF-1α protein is abrogated in anoxia of O2 and glucose deprivation (GC) and proposed that under ischemia/HIF signaling can be down-regulated on the translational level through depletion of cellular ATP [36,37]. However, specific pathways and mechanisms involved in substrate-dependent regulation of HIF-1α levels are still only understood vaguely and their elucidation can substantially increase the precision, feasibility and successful outcome in therapy of cancer, ischemia and other diseases.

In this work we use metabolically active differentiated PC12 cells [41] to systematically study precise links between the different factors involved in activation of HIF-1 protein, notably (a) the availability of key metabolic substrates and duration of metabolic deprivation; (b) oxygen consumption rate (OCR), (c) concentrations of glutamine/α-KG, glucose/α-KG and oxygen/α-KG; (d) nuclear factor (NF)κB and Akt activation; (e) activation of AMPK, Akt and Erk; (f) cellular ATP levels; (g) transcription of HIF-α genes and (h) miR levels.

Materials and methods

Materials

O2-sensitive probe, MiniOrangeXc (Mox4) [42], Mox4, Mox4-ia, Mox4 -ia, Nanoua [42] and phospho-activation probe p-Akt [43] were from Cellvivo Biosciences (Ghent, Belgium), GLI1 inhibitor BTV45 (bic-2 (5-glycosyl-aminobicyclo[2.2.2]octan-2-yl) methyl sulfone) [44] was kindly donated by Dr. C. A. S. de Felipe. Dimethylaminobutyrol [DABCO] and dimethylamine [DMAM] were from Tocris Bioscience (Bristol, UK). Amestamp™ DCD™ Prime reagent was from GE Healthcare (Waukesha, WI). Phospho-Phosphorylated Phosphatase Inhibitor Cocktail Tablets were from Roche (Basel, Switzerland); pre-made acrylamide gels, running and transfer buffers were from GeneScript (Piscataway, NJ); phosphatase inhibitors were from Active Motif (Carlsbad, CA). BCA Protein Assay, Kit was from BioRad Laboratories (Richmond, CA). 4,6-Diamidino-2-phenylindole (DAPI) was from Molecular Probes (Eugene, OR). 4,6-Diamidino-2-phenylindole (DAPI) was from Molecular Probes (Eugene, OR). 4,6-Diamidino-2-phenylindole (DAPI) was from Molecular Probes (Eugene, OR).

Cell culture, media and experimental conditions

Cell culture, media and experimental conditions

Derived from an adrenal medulla, pheochromocytoma PC12 are actively regulating and highly glycolytic cells commonly used as a model in hypoxia and cancer research [39-42]. PC12 cells, purchased from ATCC (Manassas, VA) were maintained in suspension in high serum RPMI 1640 medium supplemented with N2B27, 10 nmol/FL EPES, 2 mmol/l-glutamine (Glu), 10% horse serum (HS), 5% fetal bovine serum (FBS), 100 unit penicillin and 100 µg/ml streptomycin (100, in 5% CO2 and 100% humidified atmosphere at 37oC.

For cell adhesion, plastic or glass surfaces were pre-coated with 0.02% collagen IV or a mixture of collagen IV (0.0075%) and poly-D-lysine (0.0002%). Each well was seeded at 5 x 10^6 cells/well on 96-well plates (Greiner Bio One) and differentiated for 3-5 days in RPMI supplemented with NaHCO3, EPES, 2 mmol/l Glu, 10% HS, FBS, and 100 ng/ml NGF [45]. For protein and mRNA analysis of mRNA were seeded at 5 x 10^3 and 1 x 10^4 cells/well on 6-well plates (Corning), and differentiated for 5 days. For immunofluorescence cells were seeding at 75 x 10^3 cells/well in 96-well chamber and
differentiated as described [21]. In hypoxia experiments, cells were grown consistently in adherent state in high serum RPMI 1640 medium on collagen IV coated flasks in a dedicated hypoxia workstation (Coy Laboratory Products, Casa Lake, MI) at 37 °C, atmospheric O2 for up to 21 days. Powdered DERM (Sigma, cat. no. SD002) resuspended in deionized water and filter-sterilized was used to prepare 12 different working media (WM) by adding 10 mM D-glucose (Glc), 10 mM D-glucamine (Glu), 2 mM Glic, 1 mM pyruvate (Pyruv) and 1 mM MOPS (MES), respectively [40]. Except for ECA rate analysis, all media contained 20 mM HEPES buffer (pH 7.2).

In all experiments, the differentiation RPMI medium was replaced with WM and the cells were conditioned for 2 h at 37 °C. Experiments with hypoxia were performed using hypoxia workstation (Coy) and hypoxia chambers (Coy and BHH type-Beteromatic). To inhibit GSH, 10 μM EPIK was added to WM 1 h before the measurements. AMO was inhibited by addition of DM (2 μM) at different time points of the experiments. See more details in “Materials” section. Ponceau inhibitor MGl12 (50 μM) was added immediately prior to the experiments.

Protein isolation and Western blotting analysis

Cells were washed twice with PBS and lysed for 20 min on ice with lysis buffer containing 50 mM NaCl, 1 mM EDTA, 5 mM EGTA, 20 mM Glucose, 10% Gulin buffer, 1% Triton X-100, and 0.1% SDS. After lysis, lysis was centrifuged for 10 min at 16,000g and 4 °C. Protein concentrations were measured using BCA™ Protein Assay kit and equalized. Protein (30 μg per lane) were separated by reducing denaturing 6% and 4–20% anyseal gel electrophoresis (GeneScript, NJ and BioRad, CA), transferred onto a 0.2 μm polyvinylidene difluoride (PVDF) membrane (Sigma) using wet media transfer system (Biorad) TE 220 blotter, CO2, and blocked with corresponding antibodies in 1% skimmed milk or 5% BSA for phosphorylated proteins in TBS buffered with 0.02% Tween 20 overnight at 4 °C (primary) and 2 h at room temperature (HRP-conjugated secondary). Blots were analyzed using ECL™ reagents. LAS-3000 Imager and software (FujiFilm, Japan). Ponceau Phos was used as a protein MW marker. Quantitative analysis was performed with ImageJ program using NIH Image or nonphosphorylated protein signals for normalization. Images were processed with Picasa (Google, Mountain View, CA). Photographs and illuminant program (both Adobe, San Jose, CA).

intracellular O2 (P02) measurement

After cell differentiation for 4 days, 10 μg/mL of Prox22 probes were added to EPIK medium and cells were incubated for further 18–24 h at 37 °C. Cells were washed with WM (150 μL), then 100 μl of WM was added and incubation was continued for 2 h at 37 °C. Finally, WM was replaced with fresh medium (300 μL), the plate was transferred to a TR F reader Victor 2 (PerkinElmer Life Sciences) present at 37 °C and measured at 23665 (on the bench), 660 or 355 nm wavelength (i.e. with the reader placed in the hypoxia chamber). When plate with cells was transferred from normoxia to hypoxia, O2 content decreases until new steady state O2 levels were reached. Anoxia, O2 levels in the cell did not affect the increase/decrease of the intensity of the emission. Each sample well was measured repetitively every 3–5 min over 6 h, taking two intensity readings at delay times of 50 and 70 μs and gate time 100 μs (excitation/emission: 340/640 nm). These intensity signals were converted into phophorobe signal using emission of O2, O2, T2 and T1. Experiments were performed by 3 samples of cell monolayer were measured from each of the samples. 

O2 consumption rate (OCR) assay

Measurements were conducted in 100 μL of air-saturated WM which contained 200 mM TrisHCl–333 bar probe [31]. Experimental medium was quickly scaled with 150 μL of NaHCO3, warmed to 37 °C and pH was 7.3. The plate was monitored on a TR F reader Victor 2 at 37 °C (340 nm excitation and 642 nm emission filters).

In each sample well was measured repetitively every 1.5 min over 60–90 min, taking two intensity readings at delay times of 30 and 70 μs and gate time 100 μs. The intensity signals were converted into r values. Average O2 levels across the samples were calculated from r values [30,31]. From the resulting O2 probe, OCR was calculated as n mole/min×106 cells.

Lactate-specific extracellular addition (ECA) assay

The ECA was measured as described in [35]. Each plate was included in incubated CO2-free conditions at 37 °C for 3 h, to release CO2 absorbed by the plate [43]. This time included conditioning in WM, 30-60 min prior to the experiment, cells were washed with buffered WM in 9X or 3X HEPES buffer, 30 mL of fresh WM was added. The sample was then measured on the Victor 2 plate reader at 37 °C for 60–90 min in the TR F mode (340 nm excitation and 384 ± 8.5 nm emission filters). Two TR F intensity signals were measured at delay times of 300 and 300 μs at a measurement window of 50 μs. The emission lifetime τ was calculated as described for O2 and converted to pM value [46]. The resulting pM values were used to calculate the rate of proton extrusion, moles [106] pM × 106 cells.

ATP and LDH release measurement

Analysis of total cellular ATP was performed using CellTiter-Glo® assay. Following the manufacturer’s protocol, briefly, cells were seeded, differentiated and treated as described in “Experimental Procedures” and “Results” and then quickly lysed with CellTiter-Glo® reagent. After intensive shaking for 2 min, the samples were transferred into wells of white 96-well platelet (Greiner Bio-One) and read on a Victor 2 plate reader under standard luminescence setting. LDH release was measured according to the manufacturer’s protocol for 30 well plate, recording absorbance at 450 nm.

Isolation of RNA and RT–PCR analysis of gene expression

Isolation of total RNA and reverse transcription (RT) reaction were performed using Promega kits as per manufacturer’s protocols. Each RT reaction was performed at 40 °C for 3 h using 2 μg of total extracted (RNA-free RNA in 90 μL of RT buffer containing oligo(dT)12-18, primers, dNTPs, RNase inhibitor and reverse transcriptase. A protocol for the preparation of genome DNA-free reaction was performed without RT. Standard PCR was performed on a Mastercycler (Eppendorf, Germany). Genes and primers are shown in Supplemental Table 51. lower cycle numbers (25–30 cycles) linear phase of amplification.
we used to catch the differences in cDNA between the samples. PCR products were separated on 1%–2% agarose gels, visualised using GelView DNA stain and analysed using ImageJ program.

**Immunofluorescence**

Cells were seeded, differentiated and treated in 100 μl chambers as described in [21], then washed with PBS. Fixed with 4% PFA in PBS, quenched with 50 mM NH4Cl, permabilised with 0.3% TTX-100, blocked with 5% BSA, incubated with anti-HIF-1α primary and fluorescent secondary antibodies, counterstained with DAPI, mounted with Prolong® Gold and air-dried. The intensity and localisation of HIF-1α was analysed on an Olympus FYX000 confocal laser scanning microscope. DAPI and Alexa 555 images were collected with a 60X oil immersion objective in eight planes with 0.5 μm steps in sequential mode, using standard excitation

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**Fig 1** - Effects of key metabolic substrates on cell oxygenation, ATP levels and O2-dependent HIF-2α stabilisation. (A) O2 profiles in VM with Glc and Gal (left and right panels) recorded at 0% and 4% O2, respectively. (A) O2 levels remain close to ambient. (B) Initial rates of cell oxygenation, calculated as the slope from (A) for the first 40 min (shown by dashed line), depend on substrate composition and are proportional to OCR (r = 0.96, p = 0.0038). (C) Western blotting analysis of HIF-2α levels at 0% O2 (2–4 h) and in anoxia (2 h); typical example of α-tubulin is shown. (D) Quantitative analysis of HIF-2α levels (shown in (C)) and corresponding cellular ATP. In the cells deprived of Glc, ATP continuously decreases in hypoxia and drops dramatically in anoxia. (E) Changes in PHD2 and PDK1 mRNA levels upon incubation for 4 h under reduced O2 levels versus corresponding value in cells supplied with Glc/Gln/Pyr under normoxia. Asterisks indicate significant difference from normoxia. The number of independent experiments (n) = 4, error bars represent SD.
and emission wavelengths. The resulting single plane DIC and z-stacked fluorescent images were analysed using Fiji (developed by National Institutes of Health, USA) and Image J software. Western blotting and mRNA expression were detected using a Bio-Rad Chemi Doc XRS+ system and Image Lab software (version 5.2.1), respectively. Protein phosphorylation levels were normalised to the total content of the corresponding protein (Supplemental Fig. S5).

Data normalisation and statistical analysis

Statistical analysis was performed using the results of 3-4 independent experiments, and the numbers of independent experiments, and p-values were determined using Student’s t-test (SigmaPlot software, SPSS Inc., USA). P values of ≤0.05 were considered statistically significant. To ensure the accuracy and fidelity of the data, the experiments were performed in 3-8 replicates.

Table 1 - Correlation between the level of HIF-2α and O2/ATP content in the cells supplied with different metabolic substrates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3 h 4% O2</th>
<th>3 h 4% O2</th>
<th>6 h 4% O2</th>
<th>3 h SBSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-2α and ATP</td>
<td>0.02±0.01</td>
<td>0.02±0.01</td>
<td>0.01±0.01</td>
<td>0.00±0.01</td>
</tr>
<tr>
<td>HIF-2α and O2</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

Fig 2 - Effect of C/EBP inhibition on respiration, oxygenation and ATP. (A) Treatment with 10 μM BPTES reduces respiration and oxygen consumption in cells supplied with Glc and exogenously added 4% atmospheric O2 (SBSO was used as mock control). Respiration rates calculated as slopes for the initial 30 min of hypoxia were significantly decreased by BPTES in all Glc (+) media examined (p<0.05). Grey lines indicate levels in cells treated with BPTES. (B) Effect of BPTES on ATP levels at different atmospheric O2 and substrate composition. Data are shown as mean ± SD. Asterisks indicate significant differences from control cells (supplied with Glc and not treated with BPTES, p<0.05). Each data point shows significant difference in ATP between BPTES (+) and BPTES (-) cells (p<0.05). N=3, error bars represent SD.
Results

Metabolic substrates regulate differently HIF-2α accumulation under hypoxia and anoxia

For hypoxic research, O₂ concentrations within the cells, rather than in the atmosphere or surrounding medium become particularly informative, providing an insight into HIF signaling and other O₂-dependent cellular processes. In this work, O₂ was accurately monitored using cell-penetrating phosphorescent nanoparticle-based NanO2 probe [42].

The use of O₂ consumption, dictated by availability, and utilization of metabolic substrates [5, 6] strongly affect HIF levels, particularly when atmospheric O₂ is reduced [25]. When PC12 cells supplied with different combinations of Glc, Gal, Gld, and Pyl were transferred from...
HIF-1α to moderate hypoxia (4% O₂). Initial rate of cell senescence correlated well with OCR (r = 0.82, p = 10⁻³) [45]. Accordingly, in all cells, maintaining Glc O₂ reached 10 μM threshold at different time points within 30–60 min interval (Fig. IA–B). Cells deprived of Glc were unable to achieve deep hypoxia. In cells supplied with no substrates or with Cal alone, steady-state Glc O₂ levels at 4% O₂ were similar to that in cells treated with an inhibitor of mitochondrial complex III (antimycin A). Considering these results, we anticipated striking effects of substrate composition on HIF-1α levels, particularly HIF-2α, which is highly expressed in PC22 cells and strongly associated with development of proangiogenic traits [33].

Concurrent with cell deoxygenation, HIF-2α levels rapidly increased in cells supplied with Glc in combination with Cal and/or Pyr (Fig. IC–D). Specifically, co-supplementation of Glc/Cal/Pyr/HIF-reached its maximum after 2 h of hypoxia, whereas the low respiring cells remained almost unchanged. Within 2–4 h course of hypoxia, HIF-1α and HIF-2α showed a strong inverse correlation (r = –0.65, p < 10⁻³, Table 1). Importantly, ATP content progressively decreased in cells deprived of Glc (Fig. ID).

Upon exposure to 2h anoxia, i.e., when all the cells were equally deoxygenated, HIF-2α levels appeared to be drastically different (Fig. IC–D). High HIF-2α levels were observed only in cells supplied with Glc + CaM with Glc but without Glc, HIF-2α levels at 4% O₂ were considerably lower than at 10% HIF-1α levels under anoxia correlated with cellular ATP (r = 0.25, p = 10⁻²), which was strongly decreased in both WM without glycolytic flux (Fig. ID, Table 1, Supplemental Fig. S1).

H2O2 levels in Glc-treated anoxia were negligible (not shown).

Tak engages these results show that Glc and CaM aid HIF-2α accumulation under moderate hypoxia, maintaining high respiratory activity and energetic stability of cells. Under short-term (hours) anoxia, the availability of Glc as the only source of ATP is most likely the major factor limiting HIF signaling.

AKT, ERK and AMPK pathways play distinct substrate-dependent roles in accumulation of HIF-1α under hypoxia and anoxia

Using different WM and atmospheric O₂ levels, we next analyzed the effects of key metabolic substrates on phosphorylation of protein regulating the neo production of HIF. To better understand the role of protein phosphorylation in HIF signaling, we inhibited GSK3 with BPTES so that the cells could take up but not effectively utilize Glc [44]. BPTES decreased OCR in all WM with Glc, thereby delaying deep hypoxia of cells under hypoxia (Fig. 2A). Moreover, in all WM without Glc, inhibition of GSK3 caused a decrease in ATP levels (Fig. 2B).

In WM with Glc/Gln/Pyr, first 2 h of hypoxia caused a substantial increase in phosphorylation of HIF-activating proteins p44/p42 ERK 1/2, Thr202/Thr204 and Akt (Thr308 and Ser473) (Fig. 3A, Supplemental Fig. S3). In the 2 h this effect almost disappeared, while HIF-2α levels progressively increased (Fig. 3D). A similar pattern of protein phosphorylation was observed in WM with Glc/Pyr (Fig. 3C) and D, supporting that CaM/HIF accumulation was partly dependent. Under anoxia (2 h), a significant elevation of HIF-α levels in both WM containing CaM was accompanied by only a moderate increase in phosphorylated Akt (p-AKT). Treatment with BPTES caused a major change in both HIFs and biophotons and protein phosphorylation levels (Fig. 3A, C).

In WM with Gla/Pyr, the pattern of protein phosphorylation was different (Fig. 3B, Supplemental Fig. S5). Akt phosphorylation was more sustained than in WM with Glc, with only a minor increase in p-AKT levels. Under anoxia, phosphorylation of HIF-activating proteins was generally decreased, while p-AMPK increased markedly elevated. Inhibiting p44/p42 ERK, notably increased HIF-2α and Akt phosphorylation, but strongly reduced HIF-1α levels (Fig. 3E, F). Curiously, treatment of hypoxic cells with BPTES caused a reduction in p-AMPK.

No correlation was found between mTOR phosphorylation (Ser2448) and other parameters tested.

Data shown in Fig. IA–D and Fig. IA–D suggest that under moderate hypoxia HIF-α subunits were produced in all media containing either Glc or Gln, but could only be stabilized in actively respiring cells. At the same time, lack of production de novo, rather than active degradation seemed to be the reason why HIF-1α and HIF-2α levels did not increase in anoxic cell deprived of Glc. To test this, we treated cells with a potent proteasome inhibitor MG132, exposed them to hypoxia and anoxia and then examined levels of HIF-2α protein. Levels of HIF-2α results confirmed our hypothesis: cells supplied with Glc/Pyrs were the only sample where HIF-2α content did not increase upon inhibition of protease activity (Fig. 3F).

Immunofluorescence analysis revealed strong correlation between nuclear (Fig. 1G and H) and total HIF-1α levels in the cells (r = 0.88, p = 0.001), suggesting effective translocation of HIF-1α to the target DNA. Importantly, in all the samples, chosen in Fig. 3, changes in PHD2 and PHD3 levels were negligible.
(Supplemental Fig. S4) and could not contribute significantly to the degradation of HIF-2α. 

Computing cell death by Py, we found a dramatic difference between WM containing Glc/Gal and Gal/Glc (Fig. 4A, B). Despite a pronounced increase in p-Akt and deep deacetylation of histone 3 without Glc (Fig. S4A), HIF-2α levels were lower than in cells supplied with Glc. Under anaemia, Glc phosphorylation abolished HIF-2α signalling and caused divergent changes in AMPK and Akt (Ser473) phosphorylation, which were strongly increased and decreased, respectively, compared to hypoxic samples. Further analysis revealed the general nature of this phosphorylation signature. Similar changes in p-Akt and p-AMPKα levels were observed in all Glc - media under anaemia (Fig. 4C, E), indicating that such a specific protein phosphorylation pattern may be required for (or coupled with) a down-regulation of HIF signalling in (OG) conditions.

In an attempt to modulate HIF-2α levels via AMPK pathway we treated cells with an AMPK inhibitor DM (compound C) [34] and exposed cells to anaemia for 3 h in WM with and without Glc (2 μM DM was applied 2 h prior to the onset anaemia). No effects of DM on HIF-2α, p-AMPKα or ATP levels were observed (Fig. 4E, F). However, in anaemic cells deprived of Glc the levels of extracellular lactate increased upon treatment with DM, indicating a reduction of cell viability (Fig. 4G). In cells supplied with Glc/Ci, this effect was significant (p < 0.01). To address such a difference in ATP and L-lactate release in these cells, we performed a time course ATP analysis and found that upon DM treatment cellular ATP levels decrease faster than in the controls (Fig. 4H). More continuous energy crisis in DM (+) cells led to an activation of apoptosis, even at a decrease in the level of ATP for 4 h anaemia followed by 2 h reperfusion (Fig. 4I).

Overall, these data demonstrate that an increase in p-AMPKα levels in PC12 cells is always associated with reduced production of HIF-2α upon OG, whereas Akt/Tk phosphorylation and Gsp-dependent accumulation of HIF-2α is not tightly linked.

Effects of prolonged Glc and Glu deprivation on HIF-2α accumulation

PC12 cells actively produce ATP through Oxidative and glycolytic [35] and therefore have a high capacity for adaptation to crisis conditions, such as deprivation of Glc and metabolic substrates. HIF-2α signalling in these cells usually dominates, although both HIF-1 and HIF-2 pathways become activated at low O2 [36]. Having found dramatic differences in HIF-2α levels after short adaptation of PC12 cells to specific metabolic environments (Figs. 1–4), we also investigated the effects of mid-term substrate deprivation to estimate contribution of possible differences in O2-uptake and ATP in HIF-2α levels during prolonged incubation of cells in hypoxic conditions, we stabilized HIE-2α under normoxia by treating cells with a PdH inhibitor DMOG (1 mM for 4 h) [36]. First, we grew cells for 24–28 h at DM in different WM (Fig. 5A) and observed a decrease (~20%) in the total protein content in cells deprived of Glc and grown in WM with Ck/Pyr. Upon treatment of these cells with DMOG only a small increase in HIF-2α levels was seen, along with a noticeable reduction of Akt/Ser473 and Akt (Ser473) phosphorylation (Fig. 5B, C). In cells deprived of Glc, treatment with DMOG caused only minor increase in HIF-2α, with a concurrent elevation of p-AMPKα and, intriguingly, a decrease in ATP levels (Fig. 5D). We hypothesized that the capacity of Glc (-) cells to accumulate HIF-2α can be restored by inhibiting AMPK activity. To test it, we pre-incubated with DM for 4 and 24 h prior to and during the treatment with DMOG. Phosphorylation of Akt, a marker of AMPK activity, was significantly decreased in both samples treated with DM (Fig. 5C). However, only minor increase in HIF-2α protein levels (p < 0.05) was observed upon 4 h pre-inhibition of AMPK.

Relatively small differences in HIF-2α mRNA between samples (Fig. 5E) led us to propose that prolonged Glc and Glu deprivation reduces HIF-2α production at novel proximities, at the transcriptional level. This was confirmed in experiments with CM121, which failed to increase stabilization of HIF-2α by DMOG in cells grown for 24 h in WM with Ck/Pyr or Glc/Gal/Pyr (Fig. 5F).

Chronic hypoxia modulate the effects of substrates on HIF stabilization

Continuous exposure to low O2 markedly affects metabolism, biorenergetics and HIF-signaling in PC12 cells [37]. To study the effects of metabolic substrates on HIF-2α accumulation in chronic hypoxic cells, we continuously grew (15 days) and differentiated them (5 days) at a concentration of 20 μM (Fig. 6A). The hypoxic cells exhibited elevated HIF-2α levels and a shift towards glycolytic phenotype with increased ECA rate (Supplemental Fig. S6). However, a capacity of the cells to accumulate HIF-2α upon treatment with DMOG was strongly decreased (Fig. 6B).

Measuring using NanoPro test, O2 levels in chronically hypoxic cells varied in the range of 5–25 μM, depending on the substrate composition (Fig. 5G). Interestingly, when Glc was available, HIF-2α was elevated regardless of the presence of Ck, Glc in cells supplied with Glc/Pyr but deprived of Glc, despite the lower levels of Glc (3–10 μM), HIF-2α was barely detectable and did not increase upon treatment with DMOG or CM12 (Fig. 6D). Remarkably, in these cells both active (Akt and Edk) and negative (AMPK) regulation of HIF-2α production were activated (Fig. 6D, E). Cellular ATP was similar in all samples, therefore low levels of HIF-2α at a factor preventing HIF-2α accumulation without Glc was noted (Fig. 6F). Inhibition of Glut1 with BTP15 did not affect HIF-2α levels upon treatment with DMOG (Supplemental Fig. S7).

Taken together, these results indicate that in chronically hypoxic PC12 cells, glucose metabolism does not play a major role in HIF-2α signalling, which becomes largely dependent on Glc metabolism.

Discussion

The involvement of HIF pathways in cell metabolism as transcription factors makes them major contributors to cellular responses during hypoxic energy crisis and metabolic disorders. In turn, HIF pathway activity is regulated by various factors, including the rate of supply and utilization of O2 and key metabolic substrates [32–34]. Although O2 level is an extremely important parameter for hypoxia research, it is rarely measured, and the published experimental protocols usually report on the atmosphere O2 the cells were exposed to. From this perspective, a direct quantitative monitoring of O2, greatly improves the experiment design and data interpretation. Here we demonstrate that glucose and glutamine can play differential roles in cancer cell deacetylation and...
Fig 4 – Dichotomy in AMPK and Akt phosphorylation and effect of AMPK inhibition on cell viability upon OGD. (A) Western blotting analysis of HIF-2α and phosphorylation of HIF-regulating proteins in Glc/Ch or Ga/Ch media at different atmospheric O₂. (B) Quantitative analysis of (A) normalised to corresponding values in monosomic cells supplied with Glc/Ch. In WM containing Ga/Ch, at 4% O₂, Akt/Erk phosphorylation is increased, however HIF-2α levels are significantly lower, than in WM containing Glc/Ch. Under anoxia, an increase in HIF-2α levels is abolished in cells supplied with Ga/Ch. Strong increase in p-AMPKα and decrease in p-Akt are highlighted by quadrangles. (C) Western blotting analysis shows a large increase in p-AMPKα levels under anoxia in all WM without Clc. (D) Conversely, p-Akt levels decrease. (E) Upon 3 h OGD, treatment with an AMPK inhibitor DM (2 mM for 5 h) does not affect HIF-2α, p-Akt or p-AMPKα levels, but causes a slight decrease in Erk/1/2 phosphorylation; in cells supplied with Glc p-Erk1/2 levels increase. (F) ATP decreases to the same level in DM (-) and DM (+) cells subjected to OGD for 3 h. However, LDH release from cells supplied with Ga/Gln significantly increases upon AMPK inhibition (G), in these cells ATP levels decrease at a higher rate (H), suggesting faster development of the energy crisis than in DM (-) control. (I) After 3 h anoxia followed by 2 h reperfusion of cells supplied with Ga/Gln, the level of PAP (16 kDa band) was reduced upon AMPK inhibition. Data are normalised to the values in WM containing Clc (1 x). Asterisks indicate significant difference. In (B) – between corresponding values in WMs containing Glc/Gln and Ga/Gln; in (C) and (D) – from anoxic Clc (-) sample, in (F) – from Clc (-) sample, in (G) – between DM (+) and DM (-) cells. In (A), (B) and (E), n = 3; in G, n = 4. Error bars represent SD.
Fig. 5 - Changes in HIF-2α levels upon prolonged Glc or Gln deprivation. (A) Layout of the experiment. (B) Western blotting analysis of HIF-2α and phosphorylation of HIF-regulating proteins in cells supplied with Glc/Gln/Pyr, Gln/Glc/Pyr and Glc/Pyr and treated as shown in (A). HIF-2α levels are normalised to corresponding values in DMOG (-) cells supplied with Glc. (C) Quantitative analysis of Akt, Erk1/2 and AMPKα phosphorylation. Data are normalised to the values in WM containing Glc/Gln/Pyr (1 a.u.). (D) Normalised ATP levels in DMOG (-) and DMOG (+) cells pre-incubated in different WM. (E) Levels of HIF-2α mRNA in cells pre-incubated in different WM, normalised to β-actin mRNA. (F) Effect of the proteasome inhibitor MG132 (10 μM) on HIF-2α protein level, shown as fold change relative to mock-treated cells. Asterisks indicate significant differences; * from corresponding values in Glc/Gln/Pyr medium; ** from corresponding values in two other media; *** from corresponding value in mock-treated. N=4, error bars represent SD.

It is known that activity of the major ATP-producing pathways is balanced and mutually regulated. Thus, substitution of Glc with Gln, which weakens glycolysis and yields no ATP, causes a compensatory increase in OXPHOS flux and cell/scale senescence, while Glc deprivation decreases OXPHOS flux, increases K+ and activates glycogen (46).

Recently, Gin was shown to be sufficient to drive the Krebs cycle in Glc-independent manner in hypoxic cells overexpressing M6C oncogene (57). We show here that availability of Glc is one of the main factors necessary for HIF-2α accumulation at moderate hypoxia in actively growing PC12 cells, as Gin determines kinetics and levels of cell senescence (Figs. 1 and 2). HIF-2α levels are known to increase exponentially at O2 below ~40 mmHg (or 30 μM) [4]. In all WM Glc O2 threshold is rapidly passed when cells were transferred from 15% to 4% atmospheric O2 (Fig. 1). However, cells deprived of Glc were not sufficiently deoxygengated for significant HIF-2α stabilisation (Figs. 1 and 3). Importantly, in the absence of Glc, O2 dropped below 10 μM and reached 5 μM (i.e. the optimal O2 level for HIF stabilisation [8]) in WM containing GaI/Gln/Pyr, cells stayed below 10 μM for more than 40 min during the first 2 h of hypoxia, effectively accumulating HIF-2α. On the other hand, deoxygengation of cells supplied with GaI/Gln was also rapid and deep, however an increase in HIF-2α levels was less pronounced than in the presence of Glc/Gln. This result indicated that substrate-dependent factors other than O2 sharply affect HIF levels under hypoxia.

Indeed, substrate availability appears to determine the rate of HIF-2α synthesis, most probably by regulating cellular ATP levels and pathways involved in HIF-1α translation (Fig. 15). Unlimited Glc availability is essential for cancer cells even at high O2 levels (Warburg effect) [58] and becomes particularly important under deep hypoxia/pseudohypoxia, when OXPHOS flux decreases. Here we demonstrate that in PC12 cells deprived of Glc Glc supply is compulsory for HIF-2α accumulation, which does not occur upon OGD (Figs. 1, 3 and 4). Furthermore, this HIF-2α levels strongly correlate with cellular ATP accumulation, when energy is produced exclusively via glycolysis (Table 1 and Supplemental Fig. S1); these findings support the idea that under ischemic/ anoxic HIF signalling might be inhibited or even turned off on a translational level [59], while cell metabolism becomes regulated by other players, e.g. via AMPK phosphorylation.
Fig 6 - Effect of continuous hypoxia on substrate-dependent HIF-2α accumulation. (A) Layout of the experiment. PC12 cells were grown for 15 days and differentiated for 5 days at 3% atmospheric O2 and then pre-incubated with different substrates for 2 h in the same conditions. After addition of DMOG (1 mM) or MGI12 (10 μM) to a part of the samples, cells were further kept at 3% O2 for 4 h prior to protein collection or ATP analysis. (B) Comparative Western blotting analysis of endo and DMOG-stabilized HIF-2α levels in cells grown continuously in high versus RPMI 1640 medium at normoxia (N) or 2% hypoxia (H). (C) In cells chronically exposed to 3% or 19–21% atmospheric O2, HIF-2α levels vary depending on substrate composition; measurement is performed using NanoVue in 50–100% confluent culture. (D) Analysis of HIF-2α levels and phosphorylation of HIF-regulating proteins in cells supplied with metabolic substrates and treated as shown in (A). HIF-2α levels are normalized to the corresponding values in cells supplied with Glc/Glu/Pyr. Effect of DMOG on HIF-2α levels is minor, while MGI12 increases HIF-2α levels in Glc/Glu/Pyr medium. (E) Quantitative analysis of endo, Akt and AMPKα phosphorylation shown in (D). (F) ATP levels in hypoxic cells supplied with different substrates for 4 h, are normalized to the value in Wt with Glc/Glu/Pyr. Asterisks indicate significant difference. All results represent five independent experiments (mean±SD).
[85] Akt phosphorylation, required for activation of TORC1 [177] 
was elevated under hypoxia in all WAT lines (Fig. 3A, C, D), in 
agreement with [166,82]. In anoxic cells (at least at 2 h time po 
lnt), Akt phosphorylation is strongly linked to Glc availability.
Interestingly, HF2 can also activate TSC1 and increase cell 
proliferation under hypoxia by inhibiting expression of the 
aminic acid carrier SLC5A9 [69]. 

In our experiments, levels of p-Snftor (Ser2483) do not corre 
late with Akt, Erk, or AMPKα phosphorylation (Fig. 3, 6). The 
change in Akt phosphorylation may arise from the differ 
ences in the kinetics of (de)phosphorylation events, which 
are regulated by time for these proteins. This difference can also 
explain why PI3K inhibition in HF2 via phosphorylation of 
Akt and Erk phosphorylation was not observed with Glc/oxyo
my, in cells treated with Glc/oxyo, p-Akt levels reached the same 
levels after 2 h and then noticeably decrease, while Erk levels increase 
progressively (Figs. 3, 6). In Glc (-) cells, which are using 
their main metabolite, a decay of Akt and Erk phosphorylation is 
more gradual, than in the presence of Glc. 

Glucosamine, the most abundant free amino acid, is taken up by 
cells through a process mediated by a transporter and is metabolised 
mainly through glycolysis. It is also a precursor of N-glycosylation, 
therefore it can decrease Glc glycolysis or inhibition of Glc with 
AMPKα as a consequence of Glc with AMPKα. All AMPKα 
levels decrease and activity of AMPKα protein may decrease 
progressively [66]. This could potentially lead to a PI3K-dependent 
activation of HK2-receptors in WAT without Glc, which was not 
the case as demonstrated in the experiments with WAT contain 
ing Glc/oxyo, Glc/oxyo, and with cells treated with AMPKα. 
These results confirm our previous findings [170]. On the other 
hand, in low-requiring cells deprived of Glc, Akt, Erk, and 
AMPKα levels can be sufficient for HK2 to hydrolyse HF2 even 
at reduced availability of Glc. Interestingly, an increase in Akt 
levels has been shown to contribute to AMPKα activation by 
interacting with p-Akt, which is a known activator of AMPKα [171]. 
Inhibition of PDK1 by PDK1 can also explain why in hypoxic cells 
with Glc/oxyo Glc is produced via glycolysis. 

AMPKα levels are much higher than in the presence of Glc/Glu (Fig. 4). 

In a simplified manner, proposed effects of short-term (several hours) 
substrate deprivation on HF2 levels are summarized in Figure 7A. 
A possibility that AMPKα accumulation is regulated via a negative feedforward mechanism at the PDK2 transcriptional level 
was also seen in an increase in HF2 transcription in HF2 levels 
without Glc, which is the main component to the cellular 
biogenesis. In turn, in activity requiring cells without Glc, p- 
AMPKα levels can increase in an attempt to activate 
mitochondrial biogenesis through the PGC-1α and 
AMPKα pathway [72] and in response to 
an elevation of ROS production by Glc, demonstrated in mouse brain 
and human impairs [73,74]. It is attractive to propose that 
inhibition of AMPKα can be used for the activation of pro 
 proliferative HF2 signaling and energy consumption, 
which leads to exacerbated energy stress and death of cancer cells. 
Indeed, the capacity to accumulate HF2 was partially restored 
in cells treated with LMI in mid-term substrate deprivation 
experiments. However, we were unable to substantially increase 
HF2 levels by pharmacological inhibition of AMPKα (Fig. 5C). 
Similarly, almost no changes in HF2 were seen in cells treated 
with LMI with short-term Glc and Glc deprivation (Fig. 5G). 

To end this study is worthy to note that DH, although broadly used, 
is rather non-specific inhibitor of AMPKα pathway, shown to cause 
AMPKα-independent inhibition of AMPKα signaling [73,75], 
amplification of autophagy [76] and induce p-eIF2α in breast cancer [61]. 
The latter was actually seen in cells treated with Glc/oxyo [4A, 7C]. 
These observations suggest that more specific strategies for AMPK α 
activation may be necessary to re-activate HF2-signalling and 
that target cancer cells limited with Glc and Glc supply. 
A decrease in cellular ATP levels upon treatment with DMOG 
observed in cell deprived of Glc for over 24 h agrees 
with an activation of AMPKα (Fig. 5D). This result is intriguing and 
requires further analysis of the cell energy budget in these conditions, both 
under normoxia and hypoxia. 

Although most of the in vivo studies with cell lines, including 
PC12 cells, are routinely conducted in 21% O2/95% N2 atmosphere 
O2, these cells must be defined as hypoxic, because normal physiological 
oxygenation of mammalian tissues varies between 1% and 2% O2 
(suspending to partial pressure of 72-85 mmHg or dissolved 
saturation of 36-109 mmHg) [77]. Therefore, to obtain data 
relevant in physiological normoxia we analyzed the effects of 
substrate deprivation on HF2 signaling using PC12 cells continu 
ously maintained at 2% O2 and 95% N2. Recently we showed that 
these conditions of hypoxia cells become similar to OGD conditions 
and that the capacity to accumulate HF2 was partially 
in response to O2 deprivation [69]. Here we demonstrate that in 
chronically hypoxic cells Glc no longer plays a crucial role in HF2 production, 
which becomes distinctly Glc-dependent (Fig. 4). Shown here for the 
first time, this phenomenon is most probably associated with a general 
pre-glycolytic rearrangement of metabolism in PC12 cell under 
hypoxia (Supplemental Fig. 6). Moreover, Glc, as an independ 
ent source of nitrogen [78], remains essential for proliferation of 
hypoxic cells, which depend upon Glc supplementation (data not shown). 
Interestingly, in chronically hypoxic cells without Glc, despite 
their deeper depolarization and higher p-eIF2α and p-p70S6K levels, 
HF2 was noticeably lower in Glc (+) cells (Fig. 6C). Similar 
effects were observed in transiently hypoxic cells supplied with 
Glc/oxyo, Glc/oxyo, and with depletion of [4A, 7C], as well as in cells 
supplied with Glc/oxyo [4A, 2A, 2B and 4A]. Since AMPKα phos 
phorylation pattern was not detectable in transiently and chronically 
hypoxic cells without Glc, we hypothesized that in certain metabolic en 
vironments HF2 accumulation can be inhibited on post 
translational level in a manner independent on Akt, Erk, and 
AMPKα pathways. Transcriptional regulation can be ruled out based 
on the results of mRNA analysis (Fig. 5) [18]. This observation does 
not clarify the mechanism involved, although makes further 
investigation of them attractive.

Finally, we believe that the pattern of HF2 accumulation 
in tissues can be strongly affected by Glc, Glc and Glc supply 
and utilization, which differentially regulate O2 and ATP levels, 
availability of Akt/Erk/AMPKα pathways and other factors deriving HF
Fig 7 - Summarised effects of key metabolic substrates and metabolic deprivation on cellular HIF-α levels. (A) In the short term metabolic deprivation experiments (hours), under moderate hypoxia (0.3-2% atmospheric O₂) Glc driven HIF accumulation through derepression of cell active AKT/Erk pathways and GSK3β inactivation. Pyruvate can also contribute to these factors. Without Glc several conditions required for HIF elevation are not met: (1) HIF, levels are sufficient for HIF protein to induce HIF-α degradation; (2) the levels of p-Akt and p-Erk do not increase; (3) mitochondrial ATP flux decreases. The latter also occurs in the presence of Glc, when cells limited in O₂ partially consume it and become anaerobic. In such “auto-anaerobic” cells as well as in O₂ deprived cells, Glc becomes the major factor maintaining ATP levels and capacity of cells to produce sufficient HIF. Cells deprived of Glc not only rapidly lose ATP, required for translation, but also shut down many energy-consuming processes through the activation of AMPK pathway. If cells are deprived of key metabolic substrates continuously (days), their direct contribution to HIF signalling becomes masked by many additional factors (A, right). In brief, substrate-dependent factors/pathways up-regulating HIF-α accumulation. In red: effects of substrate deprivation. (B) Proposed scenario of the inhibition of HIF-α synthesis upon medium metabolic deprivation. In differentiated PC12 cells grown for 24–28 h without Glc or, a decrease in HIF-α is coupled to and may be regulated via inactivation of AKT/Erk or activation of AMPK pathways, respectively. Inhibition of AMPK pathway can therefore lead to an increase in transcription of HIF-α levels in cells without Glc. To test this hypothesis, cells were grown in different W1 medium and pre-incubated with an AMPK inhibitor (20 μM) for 2 and 24 h and then treated with DMSO (1 μM) in the presence or absence of DMSO. Western blotting analysis demonstrated that HIF-α levels did not change significantly upon AMPK inhibition, however a positive trend was observed in cells supplied with Glc/Glu/Pyr and particularly CaCl₂/Pyr. In contrast, the decrease in p-AKT levels confirms a reduction of AMPK activity in cells deprived of Glc and treated with DM. In (C), n = 3; error bars represent SD.
(ii) hyperglycemia in patients with diabetes mellitus [82] and liver cirrhosis [103]; (iii) reduced glutamine synthase activity in astrocytes [104]. Our data add to the understanding of key metabolic substrates as major contributors to HFE signaling and promising targets for prevention and therapy of cancer and ischemia.

Conflict of interests
DP is an employee of Luru Biosciences Ltd., Cork, Ireland. All the other authors have no conflict of interest.

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Appendix A Supporting information
Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecece.2014.02.006.

REFERENCES
**PROJECT OUTCOMES**

**PAPERS**


Alicia H. C. Waters, Alexander V. Zhdanov, Dmitri B. Papkovsky. Regulation of the PDH complex: contribution of hypoxia and respiration (in preparation).


**CHEBANA MARIE-CURIE MEETINGS**

- **Barcelona, Spain, Jan 2012 (presentation)** - Multi-parametric bioenergetic assessment of PC12 cells under normal and stressed conditions.
- **Mannheim, Germany, Sept 2012 (oral + poster presentation)**: Availability of key metabolic substrates determines metabolic responses, local oxygenation and HIF-2α stabilisation in pheochromocytoma PC12 cells.
- **Biarritz, France, Sept 2013 (oral presentation)** Role of mitochondrial activity and hypoxia in mitochondrial calcium turnover in colon cancer cells.
- **Regensburg, Germany, Sept 2014 (oral presentation and poster)** Multi-parametric metabolic assessment of cells in disease state.
INTERNATIONAL CONFERENCES

- Hypoxia Net – ‘Dealing with Hypoxia’ conference, 8 - 12 June 2013 in Oulu, Finland (poster presentation).
- Experimental Biology conference 2014, in San Diego, USA (poster presentation).

SECONDMENTS

Two secondments were undertaken within the ITN.

- The University of Regensburg, Germany (03/2014) (electroporation and ECIS (electrical cell-substrate impedance sensing) measurements).
- Cranfield University, UK (05/2012) (toxicity testing on probes synthesised in the Piletsky lab).
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