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MULTI-PARAMETRIC METABOLIC ASSESSMENT OF CELLS UNDER STRESS CONDITIONS

A THESIS SUBMITTED TO THE NATIONAL UNIVERSITY OF IRELAND, CORK IN THE FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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January 2015

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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.

__________________________  January 2014

Alicia Waters
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-2α stabilisation. These results indicate that both supply and utilisation of key metabolic substrates can affect the pattern of hypoxia inducible factor-1/2α 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 was analysed in human colon cancer cells HCT116 wild type and SCO2\(^{-/-}\) (deficient in complex IV of the respiratory chain) grown for 1 week under 20.9 % and 3 % 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 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$_2^\cdot$ - 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
RPMI – 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. α-KG is then formed using isocitrate dehydrogenase forming NADH; α-KG dehydrogenase then converts α-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, α-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 α-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 α-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 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 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, converts ADP into ATP using the proton motive force generated by Complexes I, III and IV.
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 Q 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 (Antoniello et al., 2014). It works by adjusting the $\text{Ca}^{2+}$ 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 $\text{O}_2$, forms superoxide anion ($\text{O}_2$·$^-$) (Balaban et al., 2005, Bandyopadhyay et al., 1999). This is usually metabolised into hydrogen peroxide ($\text{H}_2\text{O}_2$) by superoxide dismutase (SOD). If $\text{H}_2\text{O}_2$ 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 F_o 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 O_2·− 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 ΔΨₘ 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 $\Delta\Psi_m$ 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 $\Delta\Psi_m$, 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>
</tr>
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</table>
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-bisphophate (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. Phosphoinosmitide 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 dihydrolipoyl acetyltransferase (E2), a dihydrolipoyl 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 phosphastase (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 Mg²⁺ 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 Ca²⁺, with maximal rate of PDP1 activity achieved at a mitochondrial Ca²⁺ 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 antiporter, 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 $\Delta \Psi_m$ 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\textsuperscript{2+} signalling and transporters. Voltage-gated Ca\textsuperscript{2+} channels (vGCC), N-methyl-D-aspartate (NMDA) receptors, nicotinic acetylcholine (n-Ach) receptors, and Orai are involved in Ca\textsuperscript{2+} entry into the cell. The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) and the plasma membrane Ca\textsuperscript{2+} ATPase (PMCA) are responsible for Ca\textsuperscript{2+} efflux. Ca\textsuperscript{2+} is stored in the endoplasmic reticulum (ER) and can enter via stromal interaction molecule (Stim) interacting with Orai forming a Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channel or sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA). Ca\textsuperscript{2+} is released from the ER via the inositol triphosphate (IP\textsubscript{3}) or ryanodine (Ry) receptors. The mitochondrial Ca\textsuperscript{2+} uniporter (MCU) is responsible for mitochondrial Ca\textsuperscript{2+} influx and the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger for mitochondrial Ca\textsuperscript{2+} efflux. Ca\textsuperscript{2+} is bound to Ca\textsuperscript{2+} binding proteins in the cytosol.
Rapid Ca\textsuperscript{2+} transmission is of great importance, mitochondria-associated membranes allow rapid transfer of Ca\textsuperscript{2+} from the endoplasmic reticulum (ER) to the mitochondria. Activation of inositol triphosphate (IP\textsubscript{3}) or ryanodine receptors on the ER results in Ca\textsuperscript{2+} release into the mitochondria, which is taken up by Ca\textsuperscript{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\textsuperscript{2+} plays a crucial role in excitable cells. In neurons Ca\textsuperscript{2+} release triggers neurotransmitter release and synaptic plasticity, in myocytes within skeletal and cardiac tissue Ca\textsuperscript{2+} is necessary for muscle contraction (Holbro et al., 2009). If the mitochondria become overloaded with free Ca\textsuperscript{2+} this can be the start of the intrinsic apoptotic pathway initiating programmed cell death (Fulda and Debatin, 0000). Increased mitochondrial Ca\textsuperscript{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\textsuperscript{2+} can regulate metabolism, increases in intracellular Ca\textsuperscript{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\textsuperscript{2+} has also been shown to regulate key cell survival pathways. Hypoxia inducible factor 1α (HIF-1α), can be regulated in an O\textsubscript{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\textsuperscript{1}-acetyltransferase 1, heat-shock protein 90 and the E3 ligase complex to promote HIF-1α degradation (Hawley et al., 2005). However calcineurin, a Ca\textsuperscript{2+} and calmodulin dependent phosphatase activated by Ca\textsuperscript{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 sarcoplasmic/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-excitible 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-1α in the presence of spermidine/spermine-N$^1$-acetyltransferase 1, heat-shock protein 90 and the E3 ligase complex to promote HIF-1α 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-1α 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$^+/\text{Ca}^{2+}$ exchanger and plasma membrane Ca$^{2+}$ ATP-ase (Grienberger and Konnerth). Calcium can then be either transported into the ER via ryanodine or inositol triphosphate 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 ∆Ψ_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 ∆Ψ_m of -200 mV was 99% whereas a reduced ∆Ψ_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 ∆Ψ_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$^{2+}$ are of great importance during ischemia/reperfusion, when cytosolic Ca$^{2+}$ increases the mitochondria uptake the excess Ca$^{2+}$ 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-1α 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 permeabilisation, 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 O\textsubscript{2} 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. Hydroxylation 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 in 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 O\textsubscript{2} 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.
Figure 1.5 Schematic of HIF signalling under normoxia and hypoxia. Under normoxia HIF constantly undergoes proteosomal-mediated degradation. In the presence of O$_2$ 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 O$_2$ dependent 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, $\Delta \Psi_m$, $\text{Ca}^{2+}$ 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 ∆Ψᵢₒ, ∆Ψᵢₘ 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 nigericin 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.

**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\textsubscript{2} measurement in differing O\textsubscript{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\textsuperscript{2+}, ATP, pH or ∆Ψ\textsubscript{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\textsubscript{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\textsubscript{2}, so as the O\textsubscript{2} levels in the sample are depleted by cellular respiration probe signal increases.

\[ \tau = \frac{(t_2 - t_1)}{\ln(F_2/F_1)} \]  

(Eq. 1.1)

For the intracellular O\textsubscript{2} probe MitoXpress-Intra\textsuperscript{®}, loading occurs as shown in Fig. 1.7. O\textsubscript{2} is calculated using eq. 1.2 which was obtained from calibrations performed in the laboratory. O\textsubscript{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.

\[ O_2 = 18576 \exp(-LT/6.8794) \quad (r^2 = 0.9863) \]  

(Dmitriev et al., 2012)  

(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 O₂ probe (OCR measurement), loading occurs as shown in Fig.1.8 and O₂ is calculated using:

\[ O_2 = 4455.46 \exp(-LT/7.482\,84) \quad (r^2 = 0.993\,25) \quad (\text{Dmitriev et al., 2012}) \]

(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 O₂ diffusion.
Figure 1.9. Processing OCR data. 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 $O_2$ value. LT Values are then converted into B. Extracellular $O_2$ 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 $O_2$ 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 $O_2$ allows assessment of the oxygen consumption rate (OCR) whereas measuring intracellular $O_2$ provides accurate quantitative real-time intracellular $O_2$ values, whilst allowing transient changes to be observed.
(Dmitriev et al., 2012, Fercher et al., 2011, O’Riordan et al., 2007, Zhdanov et al., 2011b).

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 CO$_2$ from the Krebs cycle and as a result of lactate production from glycolysis is measured. It should be noted that small amounts of CO$_2$ are also produced from the pentose phosphate pathway (Wamelink et al., 2008). L-ECA performed without oil allows CO$_2$ 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.

\[
\text{pH} = \frac{(1893.4 - \text{LT})}{227.54} \quad \text{(Eq. 1.4)}
\]

**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 CO$_2$ diffusion allowing measurement of lactate and CO$_2$.

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

O$_2$-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 (PMPI) 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 Ca$^{2+}$ 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>96 well (cells/well)</td>
<td>12 well (cells/well)</td>
<td>6 well (cells/well)</td>
<td>1 cm² dish (cells/dish)</td>
</tr>
<tr>
<td>PC12</td>
<td>5*10⁴</td>
<td>5*10⁵</td>
<td>n/a</td>
<td>2.5*10⁴</td>
</tr>
<tr>
<td>MEF</td>
<td>4*10⁴</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<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*10⁵</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  2  3  4  5  6  7  8  9  10  11  12</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>
<tr>
<td></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>
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<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+})</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>
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<tr>
<td>PMPI</td>
<td>20 nM</td>
<td>Stains ΔΨp</td>
<td>Add 20 nM 30 min prior to measurement</td>
<td>543nm, 555-600nm</td>
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<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>
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### Table 2.4 Antibodies used for Western blotting

<table>
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<th>Antibody</th>
<th>M.W. (kDa)</th>
<th>Secondary</th>
<th>Company</th>
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</thead>
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<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>
</tr>
<tr>
<td>PDH E1-α subunit</td>
<td>43</td>
<td>Mouse</td>
<td>Abcam</td>
</tr>
<tr>
<td>PDK1</td>
<td>60</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
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<tr>
<td>LDHa</td>
<td>37</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>p-AMPK (TH172)</td>
<td>62</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>AMPKα</td>
<td>62</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>LC3A/B</td>
<td>14, 16</td>
<td>Rabbit</td>
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<tr>
<td>PARP</td>
<td>89, 116</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
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<tr>
<td>Cleaved caspase 3</td>
<td>17, 19</td>
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<tr>
<td>mTOR</td>
<td>289</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
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<td>p-mTOR (Ser2448)</td>
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<td>p-Akt (TH308)</td>
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<td>Cell Signaling</td>
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<tr>
<td>p-Akt (Ser473)</td>
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<td>Cell Signaling</td>
</tr>
<tr>
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</tr>
<tr>
<td>VDAC</td>
<td>31</td>
<td>Goat</td>
<td>Santa Cruz</td>
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<td>Mouse</td>
<td>Sigma</td>
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### Table 2.5 Immunostaining Antibodies

<table>
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<tr>
<td>HIF-2α</td>
<td>Alexa-555</td>
<td>R &amp; D</td>
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Table 2.6 Primer pairs used for q-PCR

<table>
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<th>Antibody</th>
<th>M.W. (kDa)</th>
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<th>Company</th>
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<td>HIF-1α</td>
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<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>
</tr>
<tr>
<td>PDH E1-α subunit</td>
<td>43</td>
<td>Mouse</td>
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<td>60</td>
<td>Rabbit</td>
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<td>LDHa</td>
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<td>p-AMPK (TH172)</td>
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<td>14, 16</td>
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<tr>
<td>PARP</td>
<td>89, 116</td>
<td>Rabbit</td>
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</tr>
<tr>
<td>Cleaved caspase 3</td>
<td>17, 19</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
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<tr>
<td>mTOR</td>
<td>289</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
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<td>p-mTOR (Ser2448)</td>
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<tr>
<td>p-Akt (TH308)</td>
<td>80</td>
<td>Rabbit</td>
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<td>p-Akt (Ser473)</td>
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<td>Rabbit</td>
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<tr>
<td>A-tubulin</td>
<td>55</td>
<td>Mouse</td>
<td>Sigma</td>
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</table>
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<sup>-/-</sup>, 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<sup>-/-</sup> cells were created using targeting vectors which were delivered by recombinant adeno-associated virus (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- 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<sup>-/-</sup> 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% CO<sub>2</sub> 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*10<sup>4</sup> cells /well, PC12 cells were seeded at 5*10<sup>4</sup> cells /well, HCT116 and HCT116 SCO2<sup>-/-</sup> cells were seeded at 2.5-4.0*10<sup>4</sup> cells /well. For protein isolation of PC12 cells were seeded in a 12 well plates at a density of 5*10<sup>5</sup> PC12 cells / well. HCT116 cells were seeded in 6 well plates at a density of 1.2*10<sup>5</sup> cells / well at 20.9% O<sub>2</sub> and a density of 3*10<sup>5</sup> when grown under 3% O<sub>2</sub>, HCT116 SCO2<sup>-/-</sup> cells were seeded at 2*10<sup>5</sup> cells / well under 20.9% O<sub>2</sub> and 3*10<sup>5</sup> cells / well at 3% O<sub>2</sub> (different densities to account for differences in growth under different O<sub>2</sub> 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 \(\mu\)M 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 (\(\Delta\Psi_m\) stain). To assess \(\Delta\Psi_m\) and \(\Delta\Psi_p\), 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 60x oil immersion objective in two planes using 0.5 \(\mu\)m 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-1\(\alpha\) (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-1\(\alpha\) 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 \(\mu\)m 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 \( \tau = (t_2-t_1)/\ln(F_1/F_2) \), \( t_1 \) and \( t_2 \) relate to the two delay times and \( F_1 \) and \( F_2 \) the intensity signals at those corresponding times, \( \tau \) 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-Diethylidithiocarbamic 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 Trypsin 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 \( \text{IO}_2 \), 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 iO$_2$, TMRM, Mitochondrial Ca$^{2+}$ (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, iO$_2$ 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-2α 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-2α levels reached maximum in 3 H and then gradually decreased. At the same atmospheric O$_2$, only minor HIF-2α 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 O₂ 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, iO₂, 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 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.

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
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 µM 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 µM 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 ΔiO$_2$ 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. Basel OCR levels (numbers above bars oxygenation values). B. Intracellular O\textsubscript{2} 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 iO\textsubscript{2} 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\textsubscript{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\textsubscript{2} levels upon uncoupling. Cells in WM containing no substrates or Gal alone demonstrated similar patterns in deoxygenation, in cells with coupled mitochondrial O\textsubscript{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\textsubscript{2} levels were similar to those in Glc/Pyr; however when the cells were treated with 1 µM FCCP O\textsubscript{2} levels began to rise after approximately 25-30 min. In cells supplied with just Gln upon uncoupling there was no change in O\textsubscript{2} levels (Fig. 3.4C).
Figure 3.4. Differential O₂ 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 p44 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 $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$) which were dissipated after 1 $\mu$M FCCP addition, in a similar manner in all WM except no substrates or Gal. Bar represents 50 $\mu$m. C. TMRM intensity upon 1 $\mu$M 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 $\mu$M FCCP treatment for 4 hours. Data is quantified in bar chart, protein phosphorylation levels were normalised to $\alpha$-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>
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<td>Resting</td>
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<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>
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<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. \( iO_2 \) profiles for \( iO_2 \) 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. \( iO_2 \) 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 is 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 to -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 O_2 conditions. PC12 cells were incubated with different metabolic substrates for 2 hours and then maintained at 21% O_2 or moved to 4% for 2-4 hours or 0% O_2 for 2 hours, proteins were then isolated. **A.** Western blot of HIF-2α in cells exposed to different WM at 4% O_2 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% O_2 using the CellTiter-Glo assay, n = 4.

The effect of GLS1 inhibition with BPTES on respiration was studied under hypoxia, an increase in iO_2 was observed in cells supplied with Gln at 4% O_2 showing a reduction in the respiration rate (Fig. 3.12A). Significant reductions in ATP levels in cells with Gln at 4% O_2 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% O₂) were similar in different WM. However, after 4 hours exposure to 4% O₂ 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 O₂ 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 % O₂, which decreased after 4 hours at 4% O₂ 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% O₂, 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% O₂, 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 O₂ conditions. However when the medium was switched to Gal/Gln/Pyr and the cells exposed to 0% O₂ 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% O₂ 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). Akt
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).
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. A-KG acts by causing transcriptional repression of the glucose sensitive thioredoxin-interacting protein (TXNIP), which is normally induced is response to high levels of Glc, providing a negative feedback loop (Fig. 3.17). A-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 ∆Ψm and dissipates ∆pH, 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 Ca2+ 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 O$_2$ 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% O$_2$ and dropped dramatically at 0% O$_2$ (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 O$_2$-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 iO$_2$ 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 O$_2$ 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.
CHAPTER 4. REGULATION OF PYRUVATE DEHYDROGENASE COMPLEX BY HYPOXIA, PYRUVATE AND CALCIUM
4.1 Abstract

Pyruvate dehydrogenase (PDH) is a mitochondrial enzyme that converts pyruvate formed by glycolysis into acetyl-CoA, thus feeding the Krebs cycle. PDH can be inactivated by phosphorylation via pyruvate dehydrogenase kinase (PDK1), which is activated under hypoxic conditions. The levels of PDH (total and phosphorylated protein), PDK1 and HIF-1α were analysed in human colon cancer cells HCT116 wild type (WT) and SCO2−/− (deficient in complex IV of the respiratory chain) grown for 1 week under 20.9 % or 3 % O₂. Surprisingly, under normoxia the levels of active non-phosphorylated PDH were substantially higher in the low respiring HCT116 SCO2−/− cells. Under continuous hypoxia WT cells exhibited lower intracellular O₂ coupled to the significant elevation of HIF-α and PDK1 protein levels. A proportion of the inactive phospho-PDH increased in both cell lines. Interestingly, in SCO2−/− cells the level of phospho-PDH were elevated compared to WT cells. Evidence for HIF-1/PDK1 axis independent PDH phosphorylation was provided by siRNA PDK1 KD in HCT116 WT cells, which were still able to phosphorylate PDH without PDK1.

Our data on PDH phosphorylation state indicate that regulation of the enzyme activity under normoxia and hypoxia can occur in a manner independent of the HIF-1/PDK1 axis, mitochondrial respiration and the demand of the Krebs cycle in acetyl-CoA.
4.2 INTRODUCTION

4.2.1 Regulation of Pyruvate Dehydrogenase Phosphorylation

Glycolysis is known to be up-regulated in cancer cells. Pyruvate produced via glycolysis can be converted into acetyl-CoA by pyruvate dehydrogenase (PDH), the key enzyme which commits the cells to utilise the Krebs cycle and OXPHOS. The major components of the PDH complex are: the catalytic PDH subunit (E1), the dihydrolipoyl acetyltransferase (E2), the dihydrolipoyl dehydrogenase (E3) and the E3-binding protein (E3BP) (Patel and Roche, 1990).

The PDH complex is highly regulated via phosphorylation / dephosphorylation of three serine residues on the E1α subunit, Ser-232, Ser-293 and Ser-300 (Rardin et al., 2009). Activation of the PDH complex occurs via two PDH phosphatases (PDP). Two isoforms exist, PDP1 and PDP2 (Huang et al., 1998), both of which fully bind to the complex when activated, which requires Mg$^{2+}$, although PDP1 can also be activated by Ca$^{2+}$ (Denton, 2009).

Inactivation of the PDH complex is controlled by four PDH kinases (PDK1-4), all isoforms are shown to be increased in cancer cells (Board et al., 1990, Bowker-Kinley et al., 1998). PDKs are, in turn, up-regulated either under hypoxia via the hypoxia inducible factor (HIF) pathway (PDK1 and PDK3 protein levels) or upon metabolic and hormone deficiencies (PDK2 and PDK4 expression levels) (Lu et al., 2008, Maj et al., 2006). All PDKs are controlled by metabolic by-products of PDH activity and can be inhibited by high levels of ADP and NAD$^+$ or activated upon elevation of ATP, NADH and acetyl-CoA. PDKs can also be inhibited by the PDH complex substrate, pyruvate, which occurs in an uncompetitive manner by binding to the E2 causing a decrease in PDH phosphorylation (Bao et al., 2004). The Pyruvate analogue dichloroacetate, is able to inhibit PDK in the same manner (Pratt and Roche, 1979). Pyruvate is transported into the mitochondria by the mitochondrial pyruvate carrier (MPC), which is comprised of one MPC1 core with several MPC2 subunits attached (Bricker et al., 2012, Herzig et al., 2012).
There is much research on the hypoxia-dependent PDH phosphorylation via hypoxia inducible factor 1 (HIF-1) which activates PDK1. In the presence of O$_2$, HIF-α is constantly hydroxylated by prolyl hydroxylases (PHD1-3). Once hydroxylated HIF-α undergoes proteasome-mediated degradation facilitated by pVHL ubiquitin E3 ligase which tags HIF-α with ubiquitin marking it for degradation (Pioli and Rigby, 2001). This process is dependent on O$_2$; under hypoxic conditions HIFs get stabilised and activate many genes in the HIF-signalling pathway. HIF-1α transcriptionally activates PDK and lactate dehydrogenase (LDH), causing an increase in lactate and a decrease in acetyl-CoA production. Control of the PDH complex is of relevance to those studying diabetes, heart disease and cancer (Huang et al., 2003, Michelakis et al., 2008). In these diseases, increase PDK levels cause decreased PDH activity and a decrease in mitochondrial biogenesis is observed. Activation of the PDH complex by inhibition of PDKs with drugs is at the forefront of this research area allowing glucose oxidation and preventing pyruvate conversion into lactate (Dugan et al., 2013, Michelakis et al., 2008, Piao et al., 2010).

Another area of research is on patients with Alzheimer’s disease, studied using patient samples or transgenic mouse models which have shown that decreased mitochondrial respiration is associated with decreased PDH activity (Yao et al., 2009, Naik et al., 2012). To the best of our knowledge there is no current literature on the effect of mitochondrial polarisation on the PDH complex.

To help to elucidate the interconnections between respiratory activity and PDH complex activity, two cell lines were used, HCT116 human colon cancer cells and a mutant HCT116 SCO2$^{-/-}$ in which the cytochrome c oxidase assembly (SCO2) is absent and there is no functional complex IV of the electron transport chain rendering the cells devoid of mitochondrial respiration.

The aim was to investigate the regulation of the pyruvate dehydrogenase complex which can occur in a manner independent of HIF-signalling, mitochondrial respiration and the demand for acetyl-CoA in the Krebs cycle.
4.2.2 Role of hypoxia in mitochondrial Ca\(^{2+}\) turnover

Calcium plays an important role in metabolism; increases in mitochondrial Ca\(^{2+}\) levels are known to activate certain dehydrogenases; PDH and the Krebs cycle dehydrogenases, isocitrate dehydrogenase (IDH) and \(\alpha\)-ketoglutarate (\(\alpha\)-KG). Activation of these increases NADH production and therefore provides more reducing intermediates for OXPHOS.

There are two main inner mitochondrial membrane transporters involved in mitochondrial Ca\(^{2+}\) movement, the MCU in charge of mitochondrial Ca\(^{2+}\) influx and the NCLX, responsible for mitochondrial Ca\(^{2+}\) efflux (Baughman et al., 2011, Drago et al., 2011, Palty et al., 2010).

The MCU has low affinity for Ca\(^{2+}\) and its function depends on a large electrochemical gradient across the mitochondrial membrane. Kirichok et al demonstrated that the probability of the MCU 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). Low \(\Delta \Psi_m\) can lead to low mitochondrial Ca\(^{2+}\) levels by this mechanism. The MCU is regulated by MICU1 and MICU2. MICU1 is involved in MCU opening in response to increases in extra-mitochondrial 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 NCLX has low affinity and high capacity for Ca\(^{2+}\). The NCLX pumps Na\(^+\) and Ca\(^{2+}\) in a 3:1 ratio (Dash and Beard, 2008). NCLX expression is linked to mitochondrial Ca\(^{2+}\) efflux; it can be inhibited by CGP-37157. The NCLX is electrogenic, it is able to switch to reverse mode, pumping Ca\(^{2+}\) into the mitochondria and Na\(^+\) out, depending on the \(\Delta \Psi_m\) and cytosolic levels of Na\(^+\) (Kim and Matsuoka, 2008, Smets et al., 2004). This was demonstrated by increases in mitochondrial Ca\(^{2+}\) levels in the presence of a metabolic inhibitor with a reduced \(\Delta \Psi_m\) making the NCLX non-functional. The phenomenon was prevented with reduced Na\(^+\) or in the presence of the NCLX inhibitor CGP-37157 (Smets et al., 2004).
Ca\(^{2+}\) can enter the ER via SERCA, or stored operated channels which function through co-ordinated operation of STIM and CRAC channel protein (Orai). STIM activation can occur by: depletion of ER Ca\(^{2+}\) stores, oxidant stress and small increases in temperature, all which induce STIM translocation into junctions which then couple and open Orai channels. Under hypoxia a rapid inactivation of Ca\(^{2+}\) entry into the ER the STIM/Orai is observed caused by a decline in cytosolic pH blocking the coupling between STIM and Orai.

During hypoxia, intracellular Ca\(^{2+}\) rises which can cause activation of calmodulin-dependant protein kinase kinase (CaMKK) which can phosphorylate and activate AMPK stimulating glucose uptake and glycolytic enzymes (Mungai et al., 2011). This hypoxia induced intracellular Ca\(^{2+}\) increase is dependent on ROS, illustrated by treatment with N-acetyl cysteine blunting the response (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 (SSAT1), heat-shock protein 90 and the E3 ligase complex to promote HIF-1\(\alpha\) degradation. 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).

### 4.2.3 Aims

Apply the CEB platform to investigate the effect of mitochondrial function and hypoxia on the regulation of the PDH complex.
4.3 RESULTS

4.3.1 An increase in PDH E1α phosphorylation under hypoxia may not require activation of HIF-1α /PDK1 axis

Analysing total protein extracts isolated from both HCT116 cell lines, it was found that under normoxic conditions the phosphorylation levels of PDH E1-α subunit at Ser293 (pPDH E1-α) in SCO2−/− cells were significantly lower than in WT (p<0.01) (Fig. 4.1A-B). Total PDH E1-α levels were similar in both cell lines under these conditions (Fig. 4.1 A, C). It is known that, under hypoxic conditions HIF-1α activates PDK1, which in turn phosphorylates PDH. Indeed, in the hypoxic WT cells grown at 3% atmospheric O₂ for 1 week we observed largely elevated pPDH E1-α protein levels, associated with an increase in HIF-1α and PDK1 protein levels (Fig. 4.1A). SCO2−/− cells had a much slower growth rate than the WT cells, with a doubling time 0.5 fold slower under normoxic conditions. Under hypoxic conditions the doubling time of WT cells decreases to that of SCO2−/− cells. Intracellular oxygen levels were measured at 3% O₂ in SCO2−/− and WT cells showed no respiration in the SCO2−/− cells (30 µM), whereas WT cells showed iO₂ to decrease to around 15 µM (Fig. 4.1A).

Intriguingly, in the hypoxic SCO2−/− cells, a significantly more substantial increase in pPDH was observed, although PDK1 and HIF-1α protein levels increased to a lesser extent. PDK1 mRNA levels displayed a similar pattern to that observed in protein levels (Fig. 4.1E). An increase in total PDH E1-α levels, also seen in both cell lines under hypoxia, was more pronounced in SCO2−/− (14% difference) (Fig. 4.1 A,C). However, the considerable increase in pPDH E1-α levels (over 17 fold) in hypoxic SCO2−/− cells indicated that activity of cellular PDH E1-α protein was most likely decreased under hypoxia (Fig. 4.1A). Overall, these data suggest that an increase in PDH E1-α phosphorylation may occur in a manner, independent of HIF-1/PDK1 axis.

However the role of HIF signalling in PDH E1-α phosphorylation in SCO2−/− cells cannot be ruled out. The reason for that was a 3-fold increase in mRNA for HIF-inducible PDK3, observed in SCO2−/− cells subjected to hypoxia (Fig. 4.1 E). Only 50% increase in PDK3 mRNA was observed in WT cells under the same conditions. PDK3
normoxic values were similar in both cell lines. Interestingly, under normoxia mRNA levels for HIF-independent PDK in SCO2/− cells were significantly higher for PDK2 and lower for PDK4, than in WT cells. Hypoxia did not substantially affect PDK2 mRNA levels in either of two cell lines, while PDK4 mRNA levels increased in WT cells over 5 fold (Fig. 4.1 E).
Figure 4.1. Effect of SCO2<sup>−/−</sup> knockdown and hypoxia on PDH E1-α complex regulation. A. Western blotting analysis of pyruvate dehydrogenase E1-α (PDH E1-α), phosphorylated PDH E1-α (pPDH E1-α), PDH kinase 1 (PDK1) and hypoxia inducible factors 1/2α (HIF-1/2α) protein levels in HCT116 and SCO2<sup>−/−</sup> cells after exposure to 20.9% (normoxia) or 3% O<sub>2</sub> for 1 week, representative α-tubulin levels shown. Protein levels of PDH E1-α B. and PDH E1-α (no statistically significant differences) C. obtained from western blotting were quantified, n of at least 3. Values corrected to α-tubulin and normalised to normoxic WT. D. iO<sub>2</sub> levels (µM) for WT and SCO2<sup>−/−</sup> grown under 3% O<sub>2</sub> for 1 week and measured at 3% O<sub>2</sub>. E. mRNA analysis of PDK1-4 from RNA extracted from HCT116 and SCO2<sup>−/−</sup> after 1 week in 20.9% or 3%, values were corrected to β-actin and normalised to normoxic WT cells. * p<0.05, **p<0.01, ***P<0.005,
Resting SCO2⁻/⁻ cells have a reduced ΔΨₘ (Fig. 4.6 A), and we sought to mimic these conditions in the WT cells by continuous exposure to FCCP. After 7 days of treatment, we saw an increase in PDH E1-α phosphorylation in WT (0.5 fold) and SCO2⁻/⁻ cells (4-fold) (Fig. 4.2 A-B). Interestingly, FCCP treatment caused a substantial (4.6 fold, p<0.05) increase in PDK1 protein levels in WT cells, with no effect in SCO2⁻/⁻ cells (Fig. 4.2 A and C).

**Figure 4.2. Phosphorylation status of PDH E1-α controlled by Ca²⁺ levels.** HCT116 and SCO2⁻/⁻ cells were grown in DMSO, 1 µM FCCP or 1 µM antimycin A (AA) for 1 week and proteins isolated. **A.** Western blot analysis of pyruvate dehydrogenase E1-α (PDH E1-α), phosphorylated PDH E1-α (pPDH E1-α), PDH kinase 1 (PDK1), α-tubulin levels shown for loading. Levels of pPDH E1-α (B) and PDK1 (C) were quantified. pPDH E1-α was corrected to PDH E1-α levels, PDH E1-α was corrected to α-tubulin, data were normalised to normoxic WT sample. Error bars indicate standard deviation * - p<0.05 (n=3). If not indicated results are not statistically significant.
To investigate whether PDK1 is necessary for PDH E1-α phosphorylation in WT cells, they were transfected with siRNA against PDK1. Knock down (KD) efficiency was confirmed by qPCR and western blotting analysis, which showed PDH E1-α phosphorylation can occur independent of PDK1 in WT cells under both normoxia and hypoxia (Fig. 4.3 A). To establish whether there was a compensatory increase in other PDKs in response to the PDK1 knock-down, mRNA levels of PDK1, PDK2, PDK3 and PDK4 were assessed in the PDK1 KD cells. No changes in PDK2-3 expression levels were observed under normoxia and hypoxia. However a drop in PDK4 mRNA levels was observed in the PDK1 KD cells under normoxic but not hypoxic conditions (Fig.4.3 B-E).
Figure 4.3. Effects of PDK1 knockdown on PDH E1-α phosphorylation and mRNA expression of PDKs. HCT116 cells were grown in either 20.9 % or 3 % O₂ for 1 week. Cells were transfected with lipofectamine control, siRNA control or PDK1 siRNA and incubated for the final 72 hours. 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. A. Western Blot of pyruvate dehydrogenase E1-α (PDH E1-α), phosphorylated PDH E1-α (pPDH E1-α), PDH kinase 1 (PDK1) and hypoxia inducible factors 1α (HIF-1α) protein levels in HCT116 cells under 20.9 or 3 % O₂ with and without PDK1 siRNA. B-E. Collated mRNA data for PDK1-4, * p<0.05, n=3. If not indicated results are not statistically significant.
4.3.2 Putative roles of PDP1, Ca\(^{2+}\) and pyruvate in PDH E1-\(\alpha\) activity

There are several import regulators of the PDH E1-\(\alpha\) complex which include; Ca\(^{2+}\), PDH phosphatase 1 (PDP1) and pyruvate. Ca\(^{2+}\) is involved in PDP1 activation which is responsible for the dephosphorylation/activation of the PDH complex. Half the maximal rate of PDH phosphatase 1 (PDP1) activity is achieved at a mitochondrial Ca\(^{2+}\) concentration of approximately 3 µM (Turkan et al., 2004). Pyruvate can also regulate the PDH complex; it is transported into the mitochondria by the MPC and at high levels can inhibit PDK1-4 causing a decrease in PDH E1-\(\alpha\) phosphorylation.

4.3.3 Increased PDP1 mRNA levels in SCO2\(^{-/-}\) cells during hypoxia

The PDH complex is regulated by phosphorylation and dephosphorylation reactions. Therefore the expression of PDH phosphatases (PDPs) was analysed next. It was found that under normoxia SCO2\(^{-/-}\) cells had a 36 % reduction in PDP1 mRNA levels compared with WT cells (Fig. 4.4 A). After hypoxic exposure PDP1 mRNA levels increased by 37 % in the SCO2\(^{-/-}\) cells to the level seen in normoxic WT cells, while levels slightly decreased in WT cells. Under normoxia, a 30 % reduction in PDP2 mRNA levels in SCO2\(^{-/-}\) compared with WT and under hypoxia was seen, SCO2\(^{-/-}\) cells levels remained constant, while WT cells dropped to a similar level as the SCO2\(^{-/-}\) cells (Fig. 4.4 A).

4.3.4 Mitochondrial pyruvate transport is regulated by \(\Delta\Psi_m\) dependent MPC activity

As pyruvate is an important physiological inhibitor of PDKs we investigated possible differences in its transport into the mitochondrial between the cell lines. Under normoxic conditions, it was found that MPC2 mRNA levels were similar in both cell lines, while MPC1 mRNA levels in SCO2\(^{-/-}\) cells were higher than in WT cells by 73 % (Fig. 4.4 B). It is known that MPC activity is linked to the \(\Delta\Psi_m\). Our data suggest that: i) pyruvate transport in the SCO2\(^{-/-}\) cells is increased or/and, more likely ii) MPC mRNA levels are increased to compensate for the reduction in pyruvate transport into partially depolarised mitochondria, reported for SCO2\(^{-/-}\) cells (Kim et
al., 2006). Both MPC mRNA levels remained almost unchanged during hypoxia (Fig. 4.4 B).

**Figure 4.4** Effect of SCO2^{-/-} knockdown on mRNA expression levels of key proteins involved in PDH complex regulation. WT and SCO2^{-/-} cells were grown at 20.9 % (N) or 3 % O_2 for 1 week and then the RNA was extracted; mRNA expression levels are shown for A. Pyruvate dehydrogenase phosphatase 1(PDP1) and PDP2, (no statistical significance). B. Mitochondrial pyruvate carrier1 / 2 (MPC1/2) responsible for mitochondrial pyruvate entry is increased in SCO2^{-/-} cells ***P<0.005 n = 3. If not indicated results are not statistically significant.

### 4.3.5 Extracellular acidification is dependent on O_2 levels in WT and SCO2^{-/-} cells

As the transportation into the mitochondria is only one of the possible ways of pyruvate utilisation, pyruvate-to-lactate conversion by lactate dehydrogenase (LDHa) was studied next. LDHa mRNA levels were similar in both cell lines under normoxia, with similar increases upon hypoxia (Fig. 4.5 D). Lactate extrusion was determined by measuring extracellular acidification at different levels of atmospheric O_2. SCO2^{-/-} cells had much higher lactate levels compared with WT cells (Fig. 4.5A). Results showed that in WT cells, extracellular lactate levels increased with a decrease of O_2 availability (r = -0.76). Interestingly, the opposite was observed in SCO2^{-/-}, giving a positive correlation (r = 0.95) (Fig.4B). The L-
ECA_{SCO2^-}/L-ECA_{WT} ratio decreased with decreasing $O_2$, dropping from 4 (under normoxia) to <1 (at 1 % $O_2$) (Fig. 4.5 C).

**Figure 4.5.** Extracellular acidification is dependent on $O_2$ levels in WT and SCO2^- cells. A. L-ECA plot of HCT116 WT and SCO2^- cells over 110 min, at 20.9 % $O_2$ the rate of acidification is much faster in SCO2^- cells compared with WT cells ([H+]min^-1 ml^-1*10^6 cells). B. L-ECA rates in WT and SCO2^- cells grown in 20.9 %, 3 % or 1 % $O_2$ for 1 week and L-ECA measured under the same $O_2$ condition. C. L-ECA ratio of SCO2^-/WT under different $O_2$ values. D. LDHa mRNA levels. Error bars show standard deviation, n = 3. *- $p < 0.05$, **- $p < 0.001$. If not indicated results are not statistically significant.
4.3.6 A decrease in mitochondrial Ca\textsuperscript{2+} levels in SCO2\textsuperscript{-/-} cells is associated with changes in Ca\textsuperscript{2+} transporters expression

Next Ca\textsuperscript{2+} levels in both cell lines were studied. For that, cells were either transfected with a plasmid encoding the 5 ng/ml \textit{mitoCase12} protein (mitochondrial Ca\textsuperscript{2+}) or loaded with 2.5 μM Fluo-4 probe (cytosolic Ca\textsuperscript{2+}). To estimate basal Ca\textsuperscript{2+} levels and mitochondrial capacity as a Ca\textsuperscript{2+} depot, cells were stimulated with FCCP, known to increase outward and decrease inward mitochondrial Ca\textsuperscript{2+} fluxes (Fig. 4.6 A). The decrease in \textit{mitoCase12} fluorescence upon FCCP addition was significantly smaller in SCO2\textsuperscript{-/-} cells (by 30 % compared with 45 % in WT cells, p<0.001), indicating a lower concentration of mitochondrial Ca\textsuperscript{2+} (Fig. 4.6 B). In contrast, cytosolic Ca\textsuperscript{2+} concentrations in SCO2\textsuperscript{-/-} cells were significantly higher than in control (Fig. 4.6 C).
Figure 4.6. Differences in Ca\(^{2+}\) fluxes and transporter expression in HCT116 and SCO2\(^{-/-}\) cells. 

A. Z-stacked images of HCT116 and SCO2\(^{-/-}\) cells transfected with 5 ng/ml mitocase12 using lipofectamine 2000 (mitochondrial Ca\(^{2+}\)) or 2.5 µM Fluo-4, 1 hour staining, wash leave for 30 minutes then measure (cytosolic Ca\(^{2+}\)) with 20 nM TMRM, 30 minutes staining and then maintained at a 20 nM (∆Ψ\(_m\)), 2 µM FCCP was added. 

B. Mitochondrial Ca\(^{2+}\) (mitoCase12) ∆% values for WT and SCO2\(^{-/-}\) cells (data normalised to WT). 

C. Cytosolic Ca\(^{2+}\) fluorescence (Fluo-4) values baseline and 10 min after FCCP addition. Qualitative data were obtained by analysing 5–20 cells in 3 independent experiments. *** - p<0.001, ** - p<0.01, * - p<0.05 (n = 4). Error bars show SEM. Bar represents 40 µm. If not indicated results are not statistically significant.
Having found these differences in Ca\(^{2+}\) levels, we next analysed factors, involved in regulation of Ca\(^{2+}\) exchange between cytosol and mitochondria. For that, the mRNA levels of the MCU and the NCLX, conducting mitochondrial Ca\(^{2+}\) influx and efflux, respectively, were analysed (Fig. 4.7 D). Increased MCU (by 38 %, p<0.01) and decreased NCLX mRNA (by 25 %, p<0.05) levels were observed in SCO2\(^{-/-}\) cells.

Considering lower Ca\(^{2+}\) levels in the mitochondria of SCO2\(^{-/-}\) cells, the difference in the expression of the Ca\(^{2+}\) transporters may be a compensatory attempt to increase influx and decrease efflux of mitochondrial calcium.

Under hypoxic conditions MCU mRNA levels increased in WT cells (by 28 %) to a similar level as that observed in SCO2\(^{-/-}\) cells under normoxic conditions (Fig. 4.6 E), though in SCO2\(^{-/-}\) cells MCU expression did not change. At 3% O\(_2\), NCLX expression increased two-fold in SCO2\(^{-/-}\) cells compared to normoxic values. No change was observed in NCLX mRNA expression in hypoxic WT cells (Fig. 4.7 D).

It is known that Ca\(^{2+}\) levels within the mitochondria can modulate energy production by activation of key dehydrogenases. pPDH levels in cells treated with MCU and NCLX inhibitors were studied. Inhibition of the NCLX with CGP-37157 had no effect on pPDH or ATP levels (Fig. 4.7A). However when the MCU was blocked, decreasing mitochondrial Ca\(^{2+}\) levels, there was an increase in phosphorylated PDH E1-\(\alpha\), with no changes in total PDH E1-\(\alpha\) levels, suggesting a reduction in active levels of PDH E1-\(\alpha\) (Fig. 4.7A-B).
Figure 4.7. Changes in pPDH E1-α and ATP levels with inhibited mitochondrial Ca^{2+} transporters in HCT116 and SCO2^{-/-} cells. A. Western blot of protein isolated from WT and SCO2^{-/-}, grown in either DMSO (control), 20 µM CGP-37157 (mitochondrial Na^{+}/Ca^{2+}exchanger (NCLX) inhibitor) or 10 µM ruthenium red (mitochondrial Ca^{2+} uniporter (MCU) inhibitor) at 20.9% O_{2} for 7 days. B. Quantitative analysis of pPDH E1-α protein levels. C. Blocking mitochondrial Ca^{2+} entry causes a reduction in ATP levels in SCO2^{-/-} cells. D. HCT116 and SCO2^{-/} were grown in 20.9 or 3% O_{2} for 7 days and RNA isolated, mitochondrial Ca^{2+} uniporter (MCU) and Na^{+}/Ca^{2+} exchanger (NCLX) expression was analysed. *** p<0.001, ** p<0.01, * p<0.05 (n=4). Error bars show standard deviation. If not indicated results are not statistically significant.
4.4 Discussion

The effects of hypoxia, respiration and mitochondrial function on the key proteins involved in the regulation of the PDH complex in two HCT116 cell lines, WT and SCO2 knockdown was studied. Under normoxia, very low levels of PDH E1-α phosphorylation in the non-respiring SCO2−/− cells was observed (Fig. 4.1 A-B) despite the fact that conversion of pyruvate into acetyl-CoA is not required for ATP production via OxPhos in these cells. With no obvious metabolic requirement, under hypoxia (3 % O₂) phosphorylation of PDH E1-α in SCO2−/− cells dramatically increased, in striking contrast to WT, which exhibited only minor elevation of p-PDH E1-α (Fig.4.1 A-B). Intriguingly, PDK1 protein levels in WT cells increased to a much greater degree, in agreement with their deeper deoxygenation (Fig. 4.1 C). Below is a schematic of the regulation of PDH E1-α along with regulators of key proteins involved (Fig. 4.8).
These findings implicated that the regulation of the PDH E1-α can occur in a manner independent of the HIF-1/PDK1 axis. PDH activity was shown to be independent of hypoxia in osteoclasts, due to no hypoxia associated increases in PDK1 protein level despite increases in PDK1 mRNA levels (Morten et al., 2013). However, HIF signalling cannot be ruled out as a regulator of the PDH E1-α in WT or SCO2−/− cells, as increased mRNA levels of the HIF inducible PDK3 was seen in both cell lines (1.5-
fold and 3-fold increase in WT and SCO2⁻/⁻ cells respectively), which may be responsible for the increase in pPDH E1-α levels under hypoxia in these cells (Fig. 4.1 D). It is known that PDK2 and PDK4 are activated in times of metabolic deficiencies (Lu et al., 2008). Increased levels of PDK2 were observed in SCO2⁻/⁻ cells under normoxia compared with HCT116, possibly as a result of increased ROS in these cells inhibiting PDK2 activity and causing a compensatory increase (Hurd et al., 2012). Further evidence for HIF-1/PDK1 axis independent PDH phosphorylation was provided by siRNA PDK1 KD in HCT116 WT cells, which were still able to phosphorylate PDH without PDK1 (Fig. 4.3A). PDK2-4 mRNA levels were analysed in PDK1 KD WT cells; no compensatory increase was observed (Fig. 4.3 B-E).

The PDH complex can also be regulated by PDPs, which lead to dephosphorylation and activation of the complex. PDP1 and PDP2 mRNA levels were lower in SCO2⁻/⁻ under 20.9 % O₂ (Fig. 4.4 A). PDP1 can be activated under conditions of increased mitochondrial Ca²⁺ levels. Given lower mitochondrial Ca²⁺ levels observed in SCO2⁻/⁻ cells (Fig. calcium) one can expect a decrease in PDP1 activity and, consequently, an increase in PDH E1-α phosphorylation. Under hypoxia, PDP1 mRNA levels decreased slightly in WT cells but remained at a similar level in SCO2⁻/⁻ cells. PDP2 mRNA levels were reduced in WT cells (30 %) but were unchanged in SCO2⁻/⁻ cells (Fig. 4.4 A).

Differences observed in cytosolic (higher) and mitochondrial (lower) Ca²⁺ levels in SCO2⁻/⁻ cells were associated with changes in mitochondrial Ca²⁺ transportation. Increased MCU (Ca²⁺ influx) and decreased NCLX (Ca²⁺ efflux) mRNA levels were shown, together with a decrease in ΔΨₘ in SCO2⁻/⁻ cells (Fig. 4.7 D). This may be a compensatory measure to increase mitochondrial Ca²⁺ levels in these cells. Under hypoxic conditions MCU mRNA levels increase in WT cells to a similar level to that seen in SCO2⁻/⁻ cells. It is known that low ΔΨₘ can lead to low mitochondrial Ca²⁺ levels (Nicholls and Budd, 2000b). The mitochondrial Ca²⁺ uniporter (MCU) depends on a large electrochemical gradient to function. It was shown that the probability of the Ca²⁺ channel being open in cells with a ΔΨₘ of -200 mV was 99% whereas a reduced ΔΨₘ of -80 mV had only 11 % probability of the MCU allowing Ca²⁺ entry into the mitochondria (Kirichok et al., 2004, Hoffman et al., 2014).
possible that the increases in MCU mRNA levels in both SCO2\(^{-/-}\) and hypoxic WT cells are to adjust for decreased function of the \(\Delta\psi_m\) dependent \(Ca^{2+}\) transporter.

NCLX expression levels were increased in SCO2\(^{-/-}\) cells after exposure to 3 % O\(_2\) for 1 week. 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\(^+\) and Ca\(^{2+}\) (Kim and Matsuoka, 2008, Smets et al., 2004). Under certain physiological conditions such as ischemia/reperfusion, when intracellular Ca\(^{2+}\) levels are high or Na\(^+\) levels are low, reverse mode of the NCLX can occur (Boyman et al., 2013). Therefore under conditions where the \(\Delta\psi_m\) is reduced increasing cytosolic Ca\(^{2+}\) levels, the NCLX may not aid in mitochondrial Ca\(^{2+}\) efflux. Possibly increased cytosolic Ca\(^{2+}\) levels are causing the NCLX to work in reverse mode.

Previous studies have shown that changes in mitochondrial Ca\(^{2+}\) can activate PDH (Denton, 2009). Changes in PDH E1-\(\alpha\) phosphorylation when cells were treated with MCU or NCLX inhibitors was observed. Inhibition of the NCLX, raising mitochondrial Ca\(^{2+}\) levels caused a slight increase in pPDH E1-\(\alpha\) protein levels in WT cells, whereas blocking the MCU decreasing mitochondrial Ca\(^{2+}\) levels gave a significant increase in levels of PDH E1-\(\alpha\) phosphorylation in WT cells (p<0.05) (Fig. 4.7 A, B). Changes observed in SCO2\(^{-/-}\) cells were minimal. ATP measurements with ruthenium red treatment which decreased mitochondrial Ca\(^{2+}\) showed a significant decrease in ATP in WT cells (p<0.001) (Fig. 4.7 C). However inhibition of the MCU did not have such a dramatic effect in SCO2\(^{-/-}\) which we have also showed has increased expression of the uniporter and lower mitochondrial Ca\(^{2+}\) levels (Fig. 4.6 E).

An increase in pPDH E1-\(\alpha\) protein levels was observed in FCCP treated cells, which could be caused by a reduction in PDP1 activation with decreased mitochondrial Ca\(^{2+}\) (Fig. 4.2). A large (7-fold) increase in PDK1 protein levels was observed in WT cells treated with FCCP (Fig. 4.2 A, C). FCCP can decrease the levels of antioxidants GSH and superoxide dismutase, leading to increased levels of reactive oxygen species (ROS) after 48 hour treatment. Increased levels of ROS have been shown to
inhibit prolyl hydroxylases which hydroxylate HIFα which then undergo proteasome mediated degradation (Chandel et al., 2000, Pan et al., 2007). In these conditions, there would be increased levels of HIF-1, possibly increasing PDK1 levels as observed (Fig. 2).

SCO2⁻/⁻ cells have high levels of glycolysis, which cause increased pyruvate production, as well as high levels of NADH and O₂, which cause increased levels of ROS (Sung et al., 2010). The increased NADH levels are utilised in the conversion of pyruvate into lactate forming NAD⁺, an essential co-enzyme for the glycolytic conversion of glycerolaldehyde-3-phosphate into 1,3-phosphoglycerate reforming NADH (Sung et al., 2010). Increased lactate levels in normoxic SCO2⁻/⁻ compared with WT cells were demonstrated (Fig. 4.5). This increase in lactate levels maybe a protective measure to maintain low levels of pyruvate. Pyruvate has been shown to inhibit histone deacetylases (HDAC1 and 3), which are highly expressed in colon cancer cells, inhibition of HDACs can induce cell death by apoptosis (Wang et al., 2007). Research by Thangaraju has shown colon cancer cells keep pyruvate levels low as a protective measure, preventing HDAC1/3 inhibition which can lead to cell death (Thangaraju et al., 2009). Another reason for increased lactate levels in normoxic SCO2⁻/⁻ cells maybe to protect against increased superoxide (O₂⁻) and ROS levels under these conditions. Both O₂⁻ and ROS levels decrease with decreasing O₂ concentrations, which would connect with decreased lactate levels observed.

SCO2⁻/⁻ cells have increased glycolytic flux, therefore producing high levels of pyruvate, increased MPC1 mRNA levels in SCO2⁻/⁻ cells may be explained by increased pyruvate entry into the mitochondria (Fig. 4.4 B). However MPC1 is proton-linked and so the decreased ∆Ψᵟ present in the SCO2⁻/⁻ may lead to decreased functionality of this protein, meaning mRNA levels maybe up-regulated to compensate for the decreased activity (Herzig et al., 2012). This would also affect FCCP treated WT cells with a decreased ∆Ψᵟ.

Both cell lines have high levels of glutaminolysis, this was demonstrated (data not shown) when cells were grown with BPTES, a glutaminase 1 (GLS) inhibitor. BPTES inhibits the conversion of glutamine to glutamate which can then be converted into
α-KG and fed into the Krebs cycle forming malate, which can be transformed into pyruvate and then lactate. WT cells treated with BPTES were still able to grow but at a slower rate for at least 7 days, whereas, SCO2−/− were unable to survive after 3 days. BPTES substantially attenuates cell growth after 48 hour treatment in SCO2−/− cells, with a 50 % increase compared to 30 % in WT cells (Fig. 8 A, B).

Previous work has shown BPTES does not have an effect on ATP levels (Table 3.1). However in SCO2−/− cells a dramatic effect was observed with BPTES treatment. It is suspected that SCO2−/− cells undergo reductive carboxylation which can provide the cell with the major pathways for citrate formation as well as generating acetyl-CoA for fatty acid formation. This pathway has been shown to occur in tumour cells with mitochondrial defects (Dang et al., 2010, Mullen et al., 2012, Gameiro et al., 2013, Losman and Kaelin, 2013).
4.5 **Conclusions**

Taking these results together, it can be concluded that PDH E1-α phosphorylation can occur in a manner independent of the HIF-1/PDK1 axis, mitochondrial respiration and demand for acetyl-CoA in the Krebs cycle. This is of particular relevance for those studying possible treatments with PDK1 inhibitors for diabetes, heart disease or cancer.
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 O$_2$ 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 iO$_2$ 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 O$_2$ 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/− under normoxia needs to be investigated. PDH levels should be studied in a larger range of O₂ 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/− cells is connected with the hypothesis that SCO2/− 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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**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 increased proton demand to cancer cells in physiological conditions. Under hypoxic, metabolic and mitochondrial stress, or pharmacological treatment, a lack of key metabolic substrates may become life-threatening to cancer cells. We studied the effects of mitochondrial uncoupling by FCCP on the respiration of cells isolated from human cell lines (A549, HeLa, HCT116). We found that the respiratory response to FCCP was dependent on the metabolic state of cells. FCCP-induced uncoupling led to a decrease in oxygen consumption in cancer cells with a high mitochondrial membrane potential (ΔΨm), but had no effect on cells with a low ΔΨm. These findings suggest that mitochondrial uncoupling has a potential for substrate-specific inhibition of cell function and can be explored for anticancer treatment.

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1. Introduction

A key role of the mitochondria is that of a “power plant” of the eukaryotic cell. The electron transport chain (ETC) conducts a cascade of Redox reactions and generates proton motive force (PMF), which is utilised by ATP synthase (complex V) to produce ATP through the oxidative phosphorylation (OXPHOS). Being the most efficient way of ATP production, OXPHOS is tightly regulated. The efficiency of OXPHOS, defined by the amount of inorganic phosphate (Pi) utilized for ATP production per amount of O2 consumed [1], may be affected by a number of factors, including the level of uncoupling between inward mitochondrial ΔΨm current and ATP synthesis. Indeed, a certain proportion of O2 is always translocated to the matrix by the cytochrome complex, thus decreasing the mitochondrial membrane potential (ΔΨm). This so-called “leak” uncoupling can be accelerated through the activation of uncoupling proteins, which sequester ΔΨm-dependent protons, leading to decreased oxygen uptake and chemical uncoupling [3]. The weak acid protonophore FCCP (carboxyl semicarbazone-4-trifluoromethylphosphonic acid) [4], which provides reversible uncoupling [5] and dissipates PMF in a concentration-dependent manner, is commonly used in the experiments with isolated mitochondria and whole cells.

Isolated mitochondria are a simple and well-established model as they are accessible to the metabolic substrates and pharmacological compounds, and independent of complex inter-compartmental transport of substrates, cytoplasmic and mitochondrial ion fluxes [5]. The substrates feeding the Krebs cycle and complex I strongly affect respiration of isolated mitochondria [6]. Different combinations of the substrates and co-factors are used for analysis of the functional activity of mitochondrial enzymes, and control of FCCP, complex IV, the state (II and state IV) respiration and respiratory control ratio;
and its totality of the new pharmacological compounds. The O₂ consumption rate (OCR) in isolated mitochondria may be set to different levels by single additions of the mitochondrial substrates and drugs affecting respiration. Thus, the mitochondrial respiratory capacities can be achieved by addition of FCCP to the mitochondria in state III [8]. However, the processes other than isolated mitochondria may be significantly different from that taking place in intact cells, which have undisturbed cellular networks and environments and represent a more physiological model for experiments on bioenergetics and metabolism [8]. Mitochondrial respiration in cells is regulated by many factors and the results of respirometry under physiological conditions are not easy to interpret. Changes in the transport of metabolites and ions across the plasma membrane, a decrease in mitochondrial size and increase in cytosol, Ca²⁺-activation of glycolysis, cytoplasmic and extracellular acidification, can all strongly affect respiratory responses to FCCP, and even careful optimization of FCCP concentration for each cell type [8] 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 elevate energy stress. Thus, uncoupling becomes life-threatening when glucose (Glc) is replaced with galactose (Gal), as glycogen can no longer maintain steady ATP levels.

In cancer cells Glc supply becomes essential, since glycolysis produces large amounts of ATP regardless of high availability of O₂ (Warburg effect) [11]. In turn, most of the pyruvate (Py), instead of converting into Acetyl-CoA and utilization in the Krebs cycle [12], is converted to lactate and excreted from the cell. Some intermediates of glycolysis (e.g. phosphoglcyeroxy) are also re-distributed to adenosine nucleotide production for actively proliferating cancer cells [12–15]. To further accelerate anaerobic metabolic reactions and ATP production, cancer cells actively utilize glutamine (Gln), and more than half of ATP as a basis for Glc synthesis [16]. As a result, Glc-driven mitochondrial respiration in many cancer cells is active even at high Glc levels [29], and it is easier than upon replacement of Glc with Gal.

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

2. Experimental procedures

2.1. Materials

O₂-sensitive probe Mitofluor-Xtra [21], Mitofluor-Xtra NanoO₂ [22] and pH-sensitive probe pH-XTra [23] were from Enztec Bioscience (Cork, Ireland). Glutamine inhibitors, BPTES (Nε2-(Glu-2)-prolyl-L-glutamic acid-1,4-butanediyl ether sulfone) [24] was kindly provided by Dr. Takako Tsuchimoto (John Hopkins University, MD), mitochondrial membrane potential indicator Intramitochondrial membrane potential indicator (MMP) [25] was from Molecular Devices (Sunnyvale, CA). Free Western blotting reagents were from Cell Signaling Technology (Danvers, MA). IAP-β-luciferase assay kit was from Promega (Madison, WI). Mineral oil (type 37) was from Caragile Laboratories (Coral Gables, FL). Dibucaine Miledge’s medium (DMEM) and Roswell Park Memorial Institute (RPMI) media, nerve growth factor (NGF), collagen I, FCCP, D-glucose, D-galactose, L-glutamine, sodium pyruvate and other reagents were from Sigma-Aldrich.

2.2. Composition of the media and experimental conditions

Rat pheochromocytoma PC12 cells, human colon cancer HCT16 cells and mouse embryonic fibroblasts (MEFs) were from American Type Culture Collection (ATCC, Manassas, VA). PC12 cells were maintained in suspension in RPMI 1640 medium supplemented with 10 mM HEPES (pH 7.4), 2 mM L-Glu, 100 mM L- arginine (L-Arg), 5% low glucose serum (FBS), 100 units/ml 1000 units/ml penicillin/streptomycin (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 HEPES, L-Glu, 10% FBS and F. PC12 cells were differentiated as described previously [26]. Briefly, for experiments with O₂, ECA and O₂, cells were seeded at 5 × 10⁴ cells in 96-well plates (Corning Inc. Corning, NY) and suspended in DMEM supplemented with NaHCO₃, L-Glu, 10% horse serum, 9% and 10% L-glutamine for low cell (1000 cells/well) and high cell (5000 cells/well) conditions in 96-well plates, respectively. For protein analysis cells were seeded at 5 × 10⁵ cells per well and differentiated for 5 days on 12-well plates (Corning Life Sciences, NY) coated with a mixture of collagen I (0.0075%) and poly-D-lysine (0.033%). For O₂ analysis cells were seeded at 2.5 × 10⁶ cells per well and differentiated for 2 days prior to analysis.

Working media (WM) were prepared as follows. Powder DMEM (Sigma, Cat. No. X230) was reconstituted in deionized water and filtered sterilized. From this plain DMEM 12 different WM were composed by addition of 100 mM L-Arg, 10 mM L-Glu, 10 mM Gal, 2 mM L-Gln and 1 mM Pyr as shown in Table 1. No serum was added. All WM contained 20 mM HEPES, pH 7.2, except for O₂ 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 experiments. To test for p-hydroxybenzoic acid, cells were treated with 1 mM L-Arg (pH 7.2), 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 phosphate quenching technique [27,28]. Developed for the assessment of O₂ consumption by biological specimens on a conventional fluorometer spectrophotometer or plate reader [28], a water-soluble phosphate O₂-sensitive probe Mitofluor-Xtra was validated [29,30] and used in a number of studies [31–36]. These works demonstrate that the phosphate quenching is a

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* + / + + indicates the presence of an excitatory amino acid or L-glutamine, respectively, in the corresponding concentration.
single, non-invasive and versatile quantitative approach which allows for direct, high-throughput, real-time analysis of OCR and provides physiologically relevant data on cell metabolism in a broad range of test samples, from isolated mitochondria [31,32] to small organisms [33,34].

In this study, growth or differentiation media were replaced with W3M, and cells were incubated for 2 h (BFTES was added, as required). OCR measurements were conducted in a 100 ml of 10 mM acetate-buffered W3M supplemented 100 mM HEPES (pH 7.4) as described [35], in the presence of mitochondrial non-superoxide FOP (1 mM), or mitochondrial (DMSO), which were added to the cells immediately prior to the measurement. Sample wells were quickly sealed with 1/2 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 300 and 428 nm, respectively. Each sample well was measured repetitively every 1-5 min over 60-90 min by taking two intensity readings at emission of 510 and 520 nm at a gain of 100%. The intensity signals were converted into phosphorescence lifetime (τ) values as follows: 

\[ \tau = (t_1 - t_2) / (F_{11} - F_{22}) \]

where \( F_{11} \) and \( F_{22} \) are the TRF Intensity signals at delay times of 1 and 2, respectively. Average \( \tau \) levels across the samples were calculated from 10 τ values and then the OCR for each working medium was calculated as OCR measured by cell in 1 min per 1 mg of total soluble protein (ms/mg protein) [36,37]. Protein concentrations were measured using BCA Protein Assay Kit.

2.4. Locate specific extracellular lipid deposition assay (LCLA)

The OCR was measured as described [38]; finally, the growth or differentiation media were replaced with 150 µM W3M containing 10% HEPES and put into CO2-free conditions at 37 °C for 2 h to release absorbed CO2. Then the media was replaced with unbuffered W3M (without HEPES) and put back into CO2 free conditions for 1 h (incubation with BFTES was performed as necessary). After that, 100 µM of unbuffered W3M containing 1 µM mito-Fura red and the substrates (FOPC 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 TR-FRET mode with excitation at 485 nm. Then TR-FRET intensity signals were measured at emission of 510 and 520 nm and a measurement window of 30 ms. The emission lifetime τ was calculated as described for the OCR and converted in pH values [39]. The latter were used to calculate an amount of protein extracted by cells in 1 min per 1 mg of total soluble protein (ms/mg protein). Protein concentrations were measured using BCA Protein Assay Kit.

2.5. Intracellular GSH measurement

Developed and validated for the monitoring of GSH concentration within the cell. The intracellular 10 mM HEPES (pH 7.4) buffer was used as a pretreatment (2 h per cell) and the cells were incubated at 37 °C for 4 h in W3M (see Results section) with or without 1 µM FCCC. Whole cell lysate proteins were extracted and solubilized with 1% SDS containing bovine serum albumin (BSA) and lyced for 15 min in ice with buffer containing 150 µM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 50 mM HEPES (pH 7.4), and protease inhibitors (Roche, Basel). After lyse centrifugation for 10 min at 16,000 g and 4 °C, protein concentrations were measured using BCA Protein Assay Kit. Protein concentrations were separated by SDS-PAGE on an electrophoresis gel and transferred to a nitrocellulose membrane. Membranes were then blocked with 5% and 5-10% polyacrylamide gel electrophoresis (GeneScript, NJ) and Bio-Rad, respectively, transferred onto a 0.2 µm (Immun-Star Western Blotting Membrane), and probed with primary antibody. After incubation with secondary antibody at room temperature, the blotted membranes were washed with PBS and incubated with secondary antibody, and then developed with chemiluminescent substrate (M-PER Crime Membrane, Thermo Scientific). The blots were visualized using a low-light digital camera (Fluoir 800 and 6200, Fujifilm, Tokyo, Japan) and Image Reader LAS-3000 2.0 software.
Quantitative image analysis was performed with ImageJ program using co-tailed signals for normalization. Images were processed with Fiji, 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™ Protein Assay kit.

2.6 ATP measurement

Analysis of total cellular ATP was performed using CellTiter-Glo® Luminometric Cell Viability Assay (Promega). Cells were counted and treated as described in Sections 2.2 and 2.4 and were lysed with CellTiterGlo® agent. After initial shaking for 5 min, the samples were transferred to a 96-well plate, and read on a VICTOR® 2 (PerkinElmer) plate reader under standard luminescence settings.

2.5 Statistics

Statistical analysis was performed using the results of 3-6 independent experiments. Confidence levels of 0.01 and 0.001 were deemed as statistically significant. To ensure data accuracy and fidelity, the majority of the experiments were performed in triplicates.

The levels of OCR, ECAR, and ATP were normalized to the total protein content in the samples. ATP levels were normalized to ATP present in untreated cells in GC-3/Tu cell medium, which was defined as 1.0. Furthermore, intensities on the confocal images (PMRA™, Thermo, miniGluC) were examined in three independent mice models analyzing 5-10 cells in 3 independent experiments. The difference between the mean values was evaluated using two-tailed Student’s t-test (equality of variance in the samples was first examined using Law reins test). The differences in 

3. Results

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

A highly metabolically active cell line with equally well developed glycolytic and mitochondrial ATP pools [59, 62]. FCCP cells are commonly used for studies on bioenergetics of cancer and neuronal cells [60-52]. Mitochondrial uncoupling with FCCP continuously increases respiration and activates glycolysis in FCCP cells, which is used to elicit metabolic 'adaptation' and treat them with 1 μM FCCP.

Cell oxygenation is inversely linked to their respiratory activity, and changes in intracellular O2 (iO2) report on the magnitude of the respiratory response to stimulation [59, 61]. We found that the highest local OCR was provided by Glu combined with Pyr (with or without Gal). Consuming Glu at a rate of 5 μM/min per 1 μg of total cell protein, the cells maintain mitochondrial OCR around 55-60% of normal (Fig. 1A). Culturing Glu plus Great Y or Gal also exhibited high respiration activity (over 4 μM/min per 1 μg of protein) and were strongly desegregated.

The other NMR were scored according to cells respiration as follows: Glu > Pyr > Pyr+Gal > Glu + Gal, no substrates in VM containing Pyr or Glu, addition of Gal 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 O2 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 cells supplied with Glu combined with either Cr or Pyr (Fig. 1B, C). Cell respiration was deep and continuous (over 1 h), suggesting steady increase in mitochondrial respiration. Equally large ΔO2 were observed in the cells exhibiting high (Gal/Pyr+Glu, Pyr+Glu) and moderate (Glu/Cit, Glu/Pyr+Glu) OCR rates.

The second type, a significant inhibition of respiration characterized by progressive inactivation, was seen in the cells supplied with Pyr or Glu (with or without Gal). Initial minor decrease in ΔO2 could be observed. A decrease in respiration in the cells supplied with Glu or Glu/Glu was particularly surprising, since "resting" OCR in these cells was high (Fig. 1A).

The third type, a moderate transient drop in ΔO2, was observed in the cells supplied with Glu and in the low-energy-consuming deprived of all substrates or supplied with Gal.

Strong desegregation is observed in cells supplied with Glu/Glu or Glu suggested possible inhibition of cellular function and cell death. Under microscopy and Western blotting analysis, we found that treatment with FCCP for 4 h caused partial detachment of cell from collagen-coated surface and active apoptosis, as indicated by PARP degradation [53] (Fig. 1D). OCR/UC ratio [54] was decreased in all cells treated with FCCP, pointing to a possible apoptosis.

3.2 Metabolic substrate regulates interplay between respiration and mitochondrial bioenergetic parameters and pathways upon uncoupling with FCCP

To explain the differing effects in the respiratory response to FCCP, we subjected the cells as a response to varying mitochondrial bioenergetic parameters, such as ATP levels, glycolytic activity, Σm and mitochondrial Ca2+.

We did not find significant differences in resting ATP levels except for the cells depleted of all substrates or supplied with Gal alone (Fig. 2A). Only a minor decrease in ATP level was observed within 90 min after the addition of FCCP to the cells supplemented with Glu, Cit, and in any combination (Fig. 2B). In contrast, without glucose, FCCP flux allowed ATP turnover to be maintained as well as ATP levels, and the cells supplied with Glu, Cit, and in any combination showed higher ATP levels as compared to the cells supplemented with Glu and in the low-energy-consuming deprived of all substrates or supplied with Gal.

No correlation between ATP levels and ΔO2 was seen upon uncoupling (10 min: r = -0.13; p = 0.26; 60 min: r = -0.08; p = 0.64), suggesting that ATP does not regulate the respiratory response to FCCP. Indeed, equally strong responses were observed in the cells with the highest (Cit and Glu) and the lowest (Pyruvate) ATP levels, while the presence of Glu (high ATP) or Gal alone (low ATP) the responses were very low.

Likewise, changes in respiration did not depend on Σm and mitochondrial Ca2+ levels, which were proved by "MitoB" and miniGluC (Fig. 2C) staining respectively. However, low-level cell imaging, we observed similar levels in FCCP in all setting cells, except for the cells supplied with only Glu or deprived of all substrates (Fig. 2A). With the exception of FCCP, mitochondria and mitochondrial Ca2+ levels decreased in a similar manner (Fig. 3B and Supplemental Table S1). Plasma membrane potential (ΔΨp), proved by PMH, was not affected by FCCP. It should be noted, that in cells deprived of substrates, the ΔΨ was substantially depleted at rest (Supplemental Fig. S3).

Substantial changes in Glu and perturbed ATP and ion turnover could affect major pathways involved in bioenergetic coupling and energy metabolism. We performed Western blotting analysis of AMPK and TCA/FAD (p48/p72) phosphorylation in the samples treated with FCCP for 4 h (Fig. 2E) and found that ATP pathway was strongly affected by FCCP-induced mitochondrial uncoupling. The levels of p42 Erk (Thr202/Tyr204) (Fig. 2E), Akt, Ser473) and mTOR (Ser2448) phosphorylation were
significantly decreased in the cells supplied with Glc (particularly with Glc/Glu/Pyr and Glc/Glu), and increased in the cells fed with Gal/Glu/Pyr/Gln (except for m-TOR; AMPK phosphorilation in all samples treated with FCCP was slightly lower than in non-treated controls). In the cells supplied with Gal/Glu, 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 the cellular response to uncoupling was assessed by the specific inhibitor of trisacylglycerol (GSI). An inhibition of GSI 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 80% in heat-stressed extracts of rat kidney cell mitochondria [20].

BPTES was selected as having no major effect on cellular ATP content (Table 2), demonstrating that residual GSI activity maintained sufficient energy levels in the cells deprived of Glc. Upon uncoupling, GSI inhibition leads to a faster 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 the logarithm levels of OCR and GlcOx (Fig. 4A and C, Table 4). Upon uncoupling, the effect of BPTES was seen clearly; the response of the first type was almost completely abolished (see also Fig. 1). Moreover, in the cells supplied with Gal/Glu/Pyr or Prx/Glu (Fig. 4B, C), re-oxygenation was observed, which was similar to the second type response in VM containing Gal/Glu/Pyr or Prx, respectively (Fig. 4E). In the media without Glc, respiration was not affected by BPTES treatment, demonstrating high specificity of the drug.

Analyzing the effect of GSI inhibition on the rate of glycolysis in the media supplemented with Glc, we found that treatment with BPTES increased l-EGC rate in resting cells supplied with Glc/Glu/Pyr and Glc/Glu (2.6-fold and 1.5-fold, respectively) suggesting significant activation of glycolysis (Fig. 4D). l-EGC rate in uncoupled cells was almost unaffected.
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, Glu-dependent respiratory responses to RCP. In contrast, in non-cancer MEFs, unexposed to 50% large tumor antigens [55,56], gliosynthesis can be strongly activated by Myc oncogene [16], suggesting relatively lower rate 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 respiration with PC12 cells in OCR and oxygenation levels at rest and upon uncoupling (Fig. 3). 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. 3A, C). Adding to PC12 cells, HCT116 cells could not unmask uncoupling when supplied with Glu or Ca/Glc; although Glu levels were low at rest, they suddenly increased upon addition of RCP, suggesting a decrease in OCR. Treatment with IFES 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 IFES than PC12 cells.

In MEFs, the response to uncoupling was generally more rapid and transient (Fig. 3B, C). Although Glu remained important for generating pronounced responses to RCP, they were quite specific and could be classified as: 1) a decrease in OCR; 2) an 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/Pyr); 3) type two decrease in respiration and cell survival rate (no substrate, Glc and Pyr alone or in combination with Gal); and 4) type three--no significant changes in respiration and D2 (Glc or Gal alone). Unlike PC12 and HCT116 cells, the most pronounced response to RCP was observed in MEFs supplied with Glc/Pyr/Gln (down to 20 μM), and Glu inhibition only partially decreased the response to uncoupling. Moreover, MEFs deprived of Glu and supplied with Glc/Pyr were capable of generating continuous positive response to PFP (Fig. 3C).

4. Discussion

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

Cells generate ATP mainly through glycolysis and OxPhos. The absence of Glu can be compensated by increased OxPhos flux and mitochondrial respiration, provided that Pyr and Gln are available. Glu is a key metabolic substrate for energy production and anaplerotic reactions in cancer cells [16]. In many cell types it is efficiently utilized through glutaminolysis, giving rise to over 50% of cellular ATP and maintaining respiration at high levels [16].

In agreement with this, we observed highest OCRs in the cells supplied with Glu and Glc/Pyr (Fig. 1A). 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. Starved on Glc supply, the inner mitochondrial membrane by FCCP rapidly and substantially decreases the ΔΨm and ΔnGTP [15] in these conditions ATP is no longer produced by the mitochondrial complex V anymore ATP working in reverse mode on the restoration of ΔΨm. 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 concentration on the ATP depletion and ATP phosphorylation upon mitochondrial uncoupling. A: Quantiative analysis of TIM41 translocation as not shown a significant decrease in the ATP only in the cells supplied with no transportable substrates. B: Representative images of PCCP treatment on the A549 and mitochondria of Ca²⁺-stained with TMRM and individual substrate (respectively) at 8 min. C: Mitochondrial membrane potential measurement at 16 min after PCCP treatment. D: The profile of ATP depletion and ATP phosphorylation of the cells with PCCP and substrate treatment with of Glc or Glc. Western blotting analysis of p-AMPAK (Thr197) and p-GSK-3β in the cytosol of A549 (left) and PCCP-treated (right) cell extracts after PCCP treatment (for 1 h). Total levels of AMPK (Thr197) and GSK-3β in the whole cell lysate are presented in panels A and C, respectively. p-AMPAK (Thr197) and p-GSK-3β were determined by densitometry for quantitation. Data are presented as mean ± SD. *p < 0.05 compared with the control. For in (D), representative Western Blot analysis of the protein expression within a cell population (50-60% of the total). 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, PCC2 cells rapidly lost ATP (Fig. 3), while in the presence of Glc, ATP levels remained almost unchanged. This, 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 response to FCCP, and even after dramatic decreases in ATP (by up to 85% within 10 min) cells supplied with Glc plus Pyr were capable of maintaining a high respiratory rate for more than 1 h.

The most pronounced responses were obtained in A549 cells containing Glc, which induced a higher mitochondrial ATP content compared with Glc or Pyr, which agrees with the Glc addiction of cancer cells (Figs. 1, 4, 5, Supplemental Fig. S1). However, considering the dominant role of glutaminolysis in cancer metabolism, a decrease in respiration upon uncoupling of the cells supplied with Glc or Glc.

Table 3

<table>
<thead>
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<th>Treatment</th>
<th>Glc/Pyr/Glc</th>
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<td>125</td>
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<td>125</td>
<td>100</td>
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<tr>
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<td>125</td>
<td>150</td>
<td>125</td>
<td>100</td>
<td>150</td>
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<tr>
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<td>150</td>
<td>125</td>
<td>150</td>
<td>125</td>
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<td>150</td>
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Data are shown as BTR/5 (± SEM) cells in arbitrary units (AU).
was unexpected. Although resting O$_2$ consumption rate is high in these cells, FCCP only inhibits mitochondrial function, but also induces apoptosis (Fig. 1D).

To explain this phenomenon, we reviewed Gln transport and utilization pathways. As shown schematically (Supplemental Fig. S6), Glu is transported into the cytosol via a Glu transporter SN1 and amino acid transporters ASC1/ASC2, of which involve Na$^+$-dependent transport and may potentially be affected by the dPPy disorganization. Since upon uncoupling no effect on the dPPy was seen, non-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 to neuronal cells, it is reported via mitochondrial glutamine carrier, typical for brain neurons [57] is particularly relevant (Fig. 6). Although extra- and intracellular Glu uptake by the mitochondria is dependent on the Glu$_p$ and therefore can be suppressed by inhibiting (extracellular) and uncoupling (dPPy) the mitochondrial Glu$_p$ can also be converted by cytosolic glutaminase into glutamate (Glu), which then enters the mitochondria through either Glu carriers or Glu-Pyruvate, the latter is a pH-dependent mitochondrial (Mit)-Asp_a shuttle [69]. As Glu uptake is directed to the translocation of the resulting phosphorylations (NADP$^+$) from the cytosol, where they are produced mainly through glycolysis and Pyr degradation, into the mitochondria across the mitochondrial membrane, which is impermeable for NADH/Glu-Pyruvate transport. The transport of Glu derivatives through the Mit-Asp_a shuttle is inhibited due to the deficiency in cytosolic NADH. Moreover, Glu-Asp aminotransferase is electrogenic and acquires electrochemical potential across the mitochondrial membrane [90], which is partially uncoupled. In normal, it is a carrier [91] that translocates Glu into the mitochondrial matrix in a symport with H$^+$, and therefore this pathway is also inhibited upon dissipation of ∆ψ with FCCP. Taken together, without Glu and Pyr supply, when the Mit-Asp_a shuttle activity is decreased, uncoupling partially inactivates mitochondrial Glu and Glu carriers and decreases respiration, as demonstrated in our experiments.

FCCP can also affect the pathways recruiting Glu and Pyr in DOPA synthesis. Transports into the cell through GLUT4 and GLUT1, Glu is converted to Pyp, which can be also delivered into the cell via H$^+$-linked monocarboxylate transporter (MCT). Since in our experiments FCCP did not affect the dPPy transport of Glu and Pyr across the plasma membrane, α-amino-glutamate (α-AG) is conserved to α-amino-$\text{COOH}$ and α-ketoglutarate (α-KG) by pyruvate dehydrogenase and pyruvate carboxylase, respectively. The rate of Pyp transport into the mitochondria through MCT is PMF-dependent and decreases upon its

### Table 4

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<th>Glu/Pyruvate Treatment</th>
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<td>0.06</td>
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<td>0.14</td>
<td>0.18</td>
<td>0.13</td>
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<tr>
<td>Glu/Pyruvate</td>
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<td>0.47</td>
<td>0.27</td>
<td>0.30</td>
<td>0.13</td>
<td>0.31</td>
<td>0.12</td>
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Data are shown as BTPSS (± SEM). N.A. = not applicable. (n=5).
Fig. 3. Differential regulation of the respiratory response to FCCP in HCT116 and MEFs by metabolic substrates availability and inhibition of glutaminolysis. A. In HCT116, unlike the O2 flux, demonstrating high metabolic activity is unchanged in MEFs (but typical of the response to FCCP). The most sensitive response change is in the cells supplied with G6P/Glc/Gln, as compared with Glc/Gpy or Glu/Gln. B. A detailed comparison of the activities for the transporters involved in Glc and Gln uptake in intact and uncoupled mitochondria would be very interesting.

However, other factors can also contribute to the “classical” respiratory response to FCCP in the cells supplied with Glc and deprived of Glu (Fig. 3). It has been shown that in the presence of Glc, Glu transport can be activated by α-Glc (produced through glutaminolysis and the Krebs cycle), which causes transcriptional repression of the mitochondrial protein (DNMP) through interactions with MondoA protein. Upon Glu deprivation or inhibition of glutaminolysis, the levels of α-Glc in the cells are decreased, and therefore MondoA activates TNF expression, leading to a decrease in Glu uptake. Similar results are obtained in HEPes with α-β-threonine (α-β-threonine dehydrogenase) of the mechanism is known as transcriptional activation of TNF.

Additionally, a dramatic decrease in mitochondrial Ca2+ upon uncoupling (Fig. 3) can lead to the partial inhibition of the Krebs cycle enzymes pyruvate, and α-Glc-mediated dehydrogenases [38], thus decreasing the amount of Ca2+ required for the electron transport chain and hence causing a decrease in ATP production. The exact mechanism of FCCP, related to its ability to interact with the mitochondrial thylakoid membranes, has not yet been elucidated [38] and can also be differently modulated by metabolic substrates. Similar to substrate supply, substrate composition may be an important factor regulating FCCP-
induced ROS-dependent apoptosis in cancer cells, since Glu is one of the precursors of an antioxidant GSH [9], FCP has been shown to decrease the level of GSH and 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 [7,172].

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 activities of the major pathways involved in cell metabolism and energy production. Upon FCP treatment we found significant decrease in the phosphorylation of Erk (Thr202/Tyr204), Akt (Ser473), AMPK (Thr172) and mTOR (Ser2448) in all cells fitted with Glu in combination with Gln and Pyr (Fig. 3D). A decrease in Erk and Akt phosphorylation was more pronounced in the cells supplied with both Glu and Gln, capable of producing a strong respiratory response. We cannot explain its role effects, however most probably they are ATP-independent because ATP levels in the presence of Gln were not affected by uncoupling. On the other hand, an increase in NADPH/NAD(P)H ratio and cytosolic Ca2+, as well as a decrease in ROS production, may largely affect phosphorylation of these proteins. Surprisingly, in cells supplied with Gal/Glm/Pyr we observed a decrease in AMPK phosphorylation, which is known in increase with an elevation of AMP/ATP ratio, ATP and ROS levels [7,172]. We believe that, once in these cells FCP reduced both ATP and ROS, a decrease in the latter might have compensated for a decrease in the former. In contrast, Erk and particularly Akt phosphorylation in the medium containing Gal/Glm/Pyr was increased. We propose that such a strong elevation of phospho-Akt suggests an attempt to increase glycolysis (though not feasible without Gln), which is regulated by Akt in cancer cells [77]. In the cells supplied with Gal/Glm and treated with FCP, protein phosphorylation was largely detectable, which can be related to a major increase of apoptosis (Fig. 1D). Interplay between metabolic substrates, 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 FCP can strongly decrease influx of these metabolites into the mitochondrial matrix. In agreement with the concept of shared contribution of the OXPHOS 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 Glc/Gln/Pyr was the lowest, since OXPHOS 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 is observed in the cells supplied with Gln, which maybe associated with an increased Gln transport in these cells (through Mon4, path way). On the other hand, these cells exhibited strong sustained responses to FCP, which may require additional ATP production through glycolysis. However, this is difficult, because the cells deprived of Gln with extremely low ATP levels are able to produce even stronger respiratory responses to uncoupling (Fig. 4, 2A).

Although in the presence of Glc or Gal/Gln respiration drops upon uncoupling, Glc metabolism is the major contributor to the response to FCP in both cancer cell lines (PC3 and HT116). This was confirmed in the experiments with BRES, which determined glucose metabolism through specific inhibition of G6Pase. Interestingly, in the presence of BRES testing respiration was only slightly decreased, while the response to FCP was almost completely abolished. This suggests that residual G6Pase activity (~ 20% of the maximal level [24]) is sufficient to maintain respiration but not the maximal respiration rate. Moreover, respiration in PC3 cells supplied with Glc and treated with BRES dropped below the resting levels upon uncoupling. Glycolytic activity in resting cells supplied by Glc/Gln or Glc/Glm was elevated to maximum ATP levels upon Glc inhibition (Fig. 4, Table 3). Upon FCP treatment, L-ECG increased only in the cells supplied with Glc/Glm/Pyr. This could be related 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 HT116 cells to uncoupling were similar to that of PC3, with a smaller effect seen when G6Pase was inhibited. The respiration decreased but not blunted like in PC3 cells. This could be due to relatively low sensitivity of HT116 cells to BPTES treatment (compare Figs. 4A and 5A) due to high expression of G6Pase [78]. A possible lactic acid type glutaminase, which is resistant to BPTES [79].

Resting and uncoupled respiration of MDA were also substantially dependent. However, the responses to FCP differed significantly from the two cancer lines. In the optimal media (Fig. 5C), FCP induced less sustained and pronounced increase in respiration, than in the cancer
Although GLS remained very important for active respiration, the contribution of GLS to the respiratory response was more substantial than in PC2 and HCT116 cells. Illustrating this phenomenon, the most pronounced decrease in O2 was observed in the cells supplied with Glc/Pyruvate similar to the response in PC2 and HCT116 cells supplied with Glc/Pyruvate (Figs. 1A, 5A). Highlighting the decreased contribution of glutathione dithiol to the uncoupling response in these cells, MUs supplied with Glc/Pyruvate produced positive responses to FCCP (Fig. 2C). Finally, an inhibition of GLS caused only partial decrease in the response to uncoupling, indicating that MUs were capable of generating a quinone-dependent response to FCCP stimulation. However, further comparative analysis of oxidative activity of the major metabolic and signaling pathways upon mitochondrial uncoupling in cancer and non-cancer cells is required.

Taken together, these data demonstrate that respiratory response in FCCP and O2 levels in uncoupled cells are strongly modulated by the availability of metabolic substrates. Although difficult to translate directly into in vivo models, the conditions used in this work resemble a number of common pathophysiological conditions including: i) reduced supply of nutrients and O2 during exhaematoxie; ii) age-related decrease in glutathione synthetic activity in atrophy which supply enzymes with Glc (11), iii) hypoxemia in the patients with liver carcinoma (12) and diabetics mellitus (33) and iv) cancer-associated alterations in expression level of the mitochondrial uncoupling proteins (39). Cancer cells have altered metabolism and mitochondrial function (87), and pharmacological uncoupling has particular relevance in some development of drugs for cancer therapy, especially in monoclonal anti-growth therapy (17,73). It was also shown that cancer cells can be distinguished by specific responses to FCCP, which strongly depend on the availability of Glc and O2. Such specificity of the response allows for a potential application of mitochondrial uncoupling for substrate-dependent impairment of cancer development.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bjp.2007.07.003.

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References


Research Article

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 PG2 cells exposed to different atmosphere: 21%, 1% O2, 2–4% moderate hypoxia. HIF-α protein levels were dictated by the availability of glucose and glutamine, a necessity for cell proliferation and glycolytic ATP flux. Nuclear accumulation of HIF-1α dramatically increased upon inhibition of glutaminolysis or glutamine deprivation. Elevation of HIF-2α levels was transcription-independent and associated with the activation of Akt and mTOR2. Upon 2 h recovery, HIF-2α levels strongly correlated with cellular ATP production exclusively via glycolysis. Without glucose, HIF signalling was suppressed, giving way to other regulators of cell adaptation to energy crisis, e.g. AMPK. Consequently, viability of cells deprived of O2 and glucose decreased upon inhibition of AMPK with dorsomorphin. The capacity of cells to accumulate HIF-2α decreased after 24 h glucose deprivation. This effect, associated with increased AMPK phosphorylation, was sensitive to dorsomorphin. In chronically hypoxic cells, glutamine played no major role in HIF-2α accumulation, which became mainly glucose-dependent. Overall, the availability of O2 and metabolic substrates intrinsically regulates HIF signalling by affecting cell proliferation, ATP levels and pathways involved in production of HIF-α.

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Keywords:
Cellular oxygen
HIF signalling
Metabolic substrates
Glycolysis
Glutaminolysis
Cancer cell
Introduction

In the vast network of metabolic and signalling cascades, hypoxia-inducible factor (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 sanguination, angiogenesis, cell cycle progression, proliferation and metastasis, 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 p300/CREB proteins. Biology of HIF and HIF-1α/2α driven gene expression is described elsewhere [1,4] importantly for HIF signaling, cellular levels of α subunits are tightly controlled. Transcription is regulated by NF-E2 [3] and numerous enzymatic factors [6], including microsomal P450s [6], mitogen-activated protein kinases (MAPK), Akt, PKC and AMPK 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 [7].

When intracellular O2 (O2) concentrations are sufficient for enzymatic hydroxylation of dedicated prolyl and aquaporin residues in HIF-α subunits (Pro402, Pro564, Arg580 in humans), they undergo rapid proteasomal degradation or transcriptional inactivation [8]. Highly conserved prolines of HIF-α are hydroxylated [9], monitored by a downstream-dependent HIF-1α and HIF-2α protein, it is determined by the rates of glucose and glutamine oxidation, along with the efficiency of the cells [29-32]. On the other hand, metabolism of glucose and glutamine strongly affects HIF hydrogen supply by regulating the levels of cellular α-ketoglutarate (α-KG), reducing equivalents, ROS and metabolites of the Krebs cycle [8].

High rates of glycolysis and glutaminolysis allow rapid cancer cell growth and tumour expansion even in deep hypoxia [33,24]. Metabolic addiction makes these pathways attractive targets for cancer therapy, generally via induction 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].

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, murine and stem cells [31-37]. The negative effect of glucose and glutamine deprivation on HIF-1α levels was demonstrated in human fibrosarcoma and pancreatic cancer cells and related to a decrease in protein translation [1,13]. We and others have also shown that the synthesis of HIF-1α protein is abrogated in conditions of O2 and glucose deprivation (GD) and proposed that under ischemic HIF signaling can be down-regulated on the translational level through depletion of cellular ATP [38,28]. 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 [42] to systematically study protein levels between the different factors involved in accumulation of HIF-1α protein, thereby investigating the availability of key metabolic substrates and duration of metabolic deprivation; (ii) oxygen consumption rate (OCR), (iii) concentrations of ATP, lactate and ROS, (iv) cell proliferation and cell survival; (v) activation of AMPK, Akt and p38; (vi) cellular ATP levels; (vii) expression of HIF-1α and VEGF levels.

Materials and methods

Materials

O2-sensitive probes: Moi PROX1-oxa [4]. Moi PROX1-nta Nano2 [42] and pre-activation probe J oligo [41] were from Isotec (Bioscience, GbL, Ireland). GLT1 inhibitor BITTS (bis-2-(5-phenoxy-4-oxo-2-butylidene-2H-1,2-dithiol-2-yl) allyl sulfide) [44] was kindly donated by Dr. Takahiro Nose (Osaka University, Osaka, Japan). Prox1/Stau1 promoter luciferase plasmid was from Promega (Madison, WI). DMEM, RPMI medium, exogenous growth factor (EGF), collagen IV, anti-alpha A, M2 and other reagents were from Sigma-Aldrich, VWR, Thermo Fisher Scientific. Cell lines: MCF7, ZR-75-1, T47D, ZR-75-1 (from ICR; Ireland), CB17/Scid/Scid and NOD-SCID (from Jackson Lab, USA). Primary human cell lines were kindly provided by Dr. Dietmar Schütz (DKFZ, Germany) and Dr. Sandrine Massacrier (Université Bordeaux, France). Cells were maintained in high-serum RPMI 1640 medium supplemented with NaCl, 50 nmol/ml FPPS, 2 mmol/L-glutamine (Glu), 10% horse serum (HS), 5% fetal bovine serum (FBS), 1000 U/ml penicillin and 100 μg/ml streptomycin (G40) in 5% CO2 and 100% humidified atmosphere at 37 °C.

For cell adhesion, plates or glass surfaces were pre-coated with 0.025% collagen IV or a mixture of collagen IV (0.0275%) and poly-D-lysine (0.0025%), respectively. For L-DEA, ATP, LUX cell culture and flow cytometry experiments, cells were seeded at 5 × 10^5 cells/well in 96-well plates (Greiner Bio One), and differentiated for 3:5 days in RPMI supplemented with 5%HS, 2 mmol Glu, 10% HS, 5%, and 1000 U/ml pen/strep (G30) for proteins and Western blot analyses of KS were seeded at 5 × 10^4 cells/well in 6-well plates (Corning) and differentiated for 5 days. For immunofluorescence cells were seeded at 75 × 10^4 cells/well in 48-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, Ann Arbor, MI) at an atmospheric O₂ for up to 21 days.

Powder DMSO (Sigma, cat. no. SD0010) reconstituted in deionized water and filter-sterilized was used to prepare 12 different working media (WM) by adding 30 mM D-glucose (Glc), 10 mM D-glucaric acid (Glu, 2 mM Glc, 1 mM aspartate (Asp), and 0.1 mM N-Glut, respectively [48]. Except for ECA rate analysis, all media contained 20 mM HEPS buffer (pH 7.2).

In all experiments, the differentiation RPMI medium was replaced with WMs, 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 Bellco-Brecknell, Rochester, NY). To inhibit GLS, 10 μM IPlPS was added to WM 1 h before the measurements. AMK was inhibited by addition of DM (2 μM) at different time points of the experiments. See more details in “Inference” section. Protamsin inhibitor MG132 (10 μM) was added immediately prior to the experiments.

Protein isolation and Western blotting analysis

Cells were washed twice with PBS and fixed for 20 min on ice with 1% TCA buffer containing 150 mM NaCl, 50 mM EDTA, 1% IEPBS, and 0.1% sodium deoxycholate. After lyase activity by centrifugation at 10 min at 10,000g and 4 °C, proteins were measured using BCA. Protein assays kit and reagents. Protein (30 μg per lane) were separated by reducing denaturating 6% and 4–20% acrylamide gel electrophoresis (Genetrap, NJ) and 8% or 10% SDS–polyacrylamide gel electrophoresis (BD, NJ). X-ray film (Amersham, NJ) or NIH Image software (NIH, Bethesda, MD) was used to read the results. Quantitative analysis was performed with ImageJ software (NIH). Images were processed with Adobe Photoshop (Adobe Systems, San Jose, CA).

Lactate-specific extracellular addition (ECA) assay

The ECA assay was performed as described [53]. Each plate included in basal 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 buffer-free WM with a SOD (sodium ditoxil) probe [43]. The balloon was cut with buffer-free WM and 100 μL of this medium containing 1 μM SOD-bpa probe was added. The plate was then disposed of on a Victor 2 (Labsystems) at 37 °C for 30–60 min. The SOD probe (10 μM excitation and 440 nm emission) was used in the ECA assay. Two IFP intensity signals were measured at delay times of 1000 and 3000 μs and a measurement window of 30 μs. The ECA intensity was calculated as described for CO₂ and converted into pI values [46]. The resulting pI values were used to calculate the ratio of protein emission, where 10 μM = 100 cells.

ATP and LDH release measurement

Analysis of total cellular ATP was performed using CellTracker Glo® assay. According to the manufacturer’s protocol. Briefly, cells were seeded, differentiated, and treated as described in “Experimental Procedures” and “Results” and then quickly lyzed with CellTracker Glo® reagent. After intensive shaking for 2 min, the samples were transferred into wells of white 96-well plates (Greiner Bio One) and read on a Victor 2 plate reader under standard luminescence settings. LDH release was measured according to the manufacturer’s protocol for 200 μL well plate, recording absorbance at 490 nm.

Isolation of RNA and RT-PCR analysis of gene expression

Isolation of total RNA and reverse transcription (RT) reaction were performed using Promega Mini-Axis, as per manufacturer’s protocol. Each RT reaction was performed in 40 μL. For 1 h using 2 μg of total RNA-free RNA in 50 μL of RT buffer containing oligo-dT(15) primers, DNPs, RNA inhibitor and reverse transcriptase. As a control for the presence of genomic DNA the reaction was performed without RT. Standard PCR was performed on a Mastercycler (Eppendorf, Germany). Genes and primers are shown in Supplemental Table S1. L-luciferase activities (25–30 cycles, linear phase of amplification).
we used to check the differences in cDNA between the samples. PCR products were separated on 1.5–2% agarose gels, visualized using GelView DNA stain and analyzed using ImageJ program.

**Immunofluorescence**

Cells were seeded, differentiated, and treated in 10 μl chambers as described in [21], then washed with PBS, fixed with 4% PFA in PBS, quenched with 50 mM NH4Cl, permeabilized with 0.1% Triton X-100, blocked with 5% BSA, incubated with primary and fluorescent secondary antibodies, counterstained with DAPI, mounted with ProLong Gold and air-dried. The intensity and localization of HIF-1α was analyzed on a Zeiss LSM 780 confocal laser scanning microscope. DHFR and Alexa 555 images were collected with a 60× oil immersion objective in eight planes with 0.6 μm steps in sequential mode, using standard excitation wavelengths.
and emission wavelengths. The resulting single plane DIC and z-stacked fluorescent images were analysed using PV3000 Viewer software (Olympus), Adobe Photoshop and Illustrator.

Data normalisation and statistical analysis

Statistical analysis was performed using the results of 3–4 independent experiments, and programs GraphPad Prism (Biomedical, San Diego, CA). P values of < 0.05 were deemed as statistically significant. To ensure the accuracy and fidelity of the data, the experiments on five wells per plate were performed in 3–8 replicates.

ECG, OCR, cell deoxyribonucleic acid, LDH release, and ATP values were normalised for total protein content in the samples, and the LDH release levels were related to the corresponding value in untreated cells in Glc/Pyr/Glu medium, which were defined as 100%. For Western blotting and PCR, the results were normalised to α-tubulin and p-AKT, respectively. Protein phosphorylation levels were normalised to the total content of the corresponding protein (Supplemental Fig. S5).

Unless otherwise stated, protein, mRNA and ATP levels were related to corresponding values in mock-treated cells supplied with Glc/Pyr/Glu at 0 (defined as 100%). The differences (fold changes versus control) were evaluated using non-parametric Mann-Whitney (M-W) test. The differences between mean values were evaluated using non-parametric Student t-test (equality of variances in the samples was first estimated using a variances test). Statistical dependence between HIF-1α and other parameters was evaluated using non-parametric Spearman’s rank-correlation analysis. Graphically, the results are presented exactly as series of mean values in the groups of comparison.

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

<table>
<thead>
<tr>
<th></th>
<th>Normoxic</th>
<th>1 h Glc</th>
<th>3 h Glc</th>
<th>6 h Glc</th>
<th>2 h Glut</th>
<th>2 h Ketone</th>
</tr>
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<tbody>
<tr>
<td>HIF-2α and ATP</td>
<td>NA</td>
<td>0.2±0.05</td>
<td>0.2±0.05</td>
<td>0.2±0.04</td>
<td>0.2±0.02</td>
<td>0.2±0.02</td>
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<tr>
<td>p&lt;0.001</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>HIF-2α and Glc/O2</td>
<td>NA</td>
<td>0.01±0.02</td>
<td>0.01±0.02</td>
<td>0.01±0.02</td>
<td>0.01±0.02</td>
<td>0.01±0.02</td>
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<tr>
<td>p&lt;0.001</td>
<td>0.001</td>
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Fig 2 - Effect of GCS1 inhibition on respiration, oxygenation and ATP. (A) Treatment with 10 μM BPTES reduces respiration and increases Glc/O2 in cells supplied with Glc and exposed to 8% atmospheric O2 (BPTES was used as mock control). Glc-decarboxylation rates calculated as slopes for the initial 30 min of hypoxia were significantly decreased by BPTES in all Glc (+) media examined (p<0.01). Grey lines indicate Glc levels in cells treated with BPTES (solid line) vs. Glc levels in cells treated with Glc (dotted line). (B) Effect of BPTES on ATP levels at different atmospheric O2 and substrate composition. Data are shown as Means ± SD. Asterisks indicate significant difference from control cells (supplied with Glc and not treated with BPTES). p<0.01. Black lines show significant difference in ATP between BPTES (+) vs. BPTES (-) cells (p<0.01). 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 signalling and other O₂-dependent cellular processes. In this work, O₂ was accurately monitored using well-penetrating phosphorescent nanoparticles-based NanoSope [42].

The use of O₂ consumption, dictated by availability and utilization of metabolic substrates [53], strongly affects HIF levels, particularly when anoxic O₂ is induced [28]. When PC12 cells supplied with different combinations of Glc, Gal, Gln and Pyruvate were transferred from
GLs, the metabolic brain fuel, are mainly regulated by the HIF-1α protein, which is activated in response to hypoxia. HIF-1α plays a critical role in the induction of key metabolic enzymes, such as glucose transporters (GLTs) and pyruvate dehydrogenase complex (PDC), which are essential for maintaining energy homeostasis under hypoxic conditions.

In the absence of oxygen, HIF-1α accumulates and activates the expression of genes involved in glucose uptake and metabolism. This results in increased glucose transport and glycolysis, which are crucial for survival under hypoxic conditions. However, in the presence of oxygen, HIF-1α is rapidly degraded, preventing the activation of these genes.

HIF-1α regulates the expression of the NOS3 gene, which encodes for nitric oxide synthase (NOS). NOS catalyzes the production of nitric oxide (NO), which has various biological functions, including vasodilation and anti-inflammatory effects. However, overproduction of NO can lead to oxidative stress and endothelial dysfunction.

In summary, the regulation of HIF-1α expression by hypoxia or oxygen availability is a critical mechanism that enables cells to adapt to changes in oxygen availability. This regulatory mechanism is crucial for the survival and function of many types of cells, including those in the brain.

**Figure 2** - Effects of GLs, Glc and O2 availability on the levels of HIF-1α and phosphorylation of HIF-regulating proteins. (A–C) Western blot analysis of HIF-1α and protein phosphorylation levels in WM containing GLs/Glc/Pyr (A) and WM without Glc (B) or Glc (C). (D) GLS activity was inhibited with 10 μM BPTES (DMSO). (E) Quantitative analysis of the effects of GLs (green) and Glc (blue) depletion on HIF-1α levels; bands are highlighted in corresponding colors in (B) and (C). (F) Data were normalized to the corresponding values in control conditions. (G) O2 levels in WM containing GLs/Glc/Pyr (A) and WM without Glc (B) or Glc (C). (H) Quantitative analysis of the effects of GLs (green) and Glc (blue) depletion on HIF-1α levels; bands are highlighted in corresponding colors in (B) and (C). (I) Quantitative analysis of the effects of GLs (green) and Glc (blue) depletion on HIF-1α levels; bands are highlighted in corresponding colors in (B) and (C). (J) Quantitative analysis of the effects of GLs (green) and Glc (blue) depletion on HIF-1α levels; bands are highlighted in corresponding colors in (B) and (C). (K) Quantitative analysis of the effects of GLs (green) and Glc (blue) depletion on HIF-1α levels; bands are highlighted in corresponding colors in (B) and (C). (L) Quantitative analysis of the effects of GLs (green) and Glc (blue) depletion on HIF-1α levels; bands are highlighted in corresponding colors in (B) and (C). (M) Quantitative analysis of the effects of GLs (green) and Glc (blue) depletion on HIF-1α levels; bands are highlighted in corresponding colors in (B) and (C). (N) Quantitative analysis of the effects of GLs (green) and Glc (blue) depletion on HIF-1α levels; bands are highlighted in corresponding colors in (B) and (C).
(Supplemental Fig 5A) and could not contribute significantly to the degradation of HIF-α.

Computing cell death of Pyr, we found a dramatic difference between WM containing Gc/Sln and Gc/Gln (Fig 6A, B). Despite a pronounced increase in p-Akt and deep hypoxia of hypoxic cells without Gc (Fig 3A), HIF-2α levels were lower than in cells supplied with Gc. Under anoxia, Gc/Sln significantly reduced the HIF-2α levels, and decreased p-Akt 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-AMPAα levels were observed in Gc + media under anoxia (Fig 6C, D), indicating that such a specific protein phosphorylation pattern may be required for (or coupled with) a down-regulation of HIF signaling in (GO) 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 anoxia for 3 h in WM with and without Gc (2 μM DM was applied 2 h prior to the onset of anoxia). No effects of DM on HIF-2α, p-AMPAα, or AMPK levels were observed (Fig 6E, F). However, in anoxia cells deprived of Gc, the levels of extracellular LDH increased upon treatment with DM, indicating a reduction of cell viability (Fig 6G, H). In cells supplied with Gc/Gln, this effect was significant (p < 0.01). To address such a difference in ATP and LDH release in these cells, we performed a time course ATP analysis and found that upon DM treatment cellular ATP levels decreased faster than in the control (Fig 6H). More consistent energy crisis in DM (+) cells led to an activation of apoptosis, as seen as a decrease in the level of PARP in 4 h anoxia followed by 2 h reperfusion (Fig 6G).

Overall, these data demonstrate that an increase in p-AMPAα levels in PC12 cells is always associated with reduced production of HIF-α upon OGD, whereas Akt/mTOR phosphorylation and Gc-dependent accumulation of HIF-α are tightly linked.

Effects of prolonged Gc and Gln deprivation on HIF-2α accumulation

PC12 cells actively produce ATP through oxidative and glycolytic pathways [35] and therefore have a high capacity for adaptation to stress conditions, such as deprivation of O2 and metabolic substrates. HIF-2α signaling in these cells usually dominates, although both HIF-1 and HIF-2 pathways become activated at lower O2 levels [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 eliminate contributions of possible differences in O2 and ATP in HIF-2α levels during prolonged incubation of cells in hypoxic conditions, we stabilized HIF-2α under normoxia by treating cells with a PHD inhibitor DMOG (1 mM for 4 h) [37]. Then, we grew cells for 24-28 h at 37°C in different WM (Fig 3A) and observed a decrease (~20%) in the total protein content in cells deprived of Gc and grown in WM with Gc/Pyr. Upon treatment of these cells with DMOG only a small increase in HIF-2α levels was seen, along with a notable reduction of Akt/Ser473 phosphorylation (Fig 5B, C). In cells deprived of Gc, treatment with DMOG caused only minor increase in HIF-2α, with a concurrent elevation of p-AMPAα and, intriguingly, a decrease in ATP levels (Fig 5D). We hypothesized that the capacity of Gc(−) cells to accumulate HIF-2α can be restored by inhibiting AMPK activity. To test it, we pre-incubated cells with ATP in 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-incubation of AMPK.

Relatively small differences in HIF-2α mRNA between samples (Fig 5E) led us to propose that prolonged Gc and Gln deprivation reduces HIF-2α production by novel protein synthesis at the translational level. This was confirmed in experiments with MG132, which failed to increase stabilization of HIF-2α by DMOG in cells grown for 24 h in WM with Gc/Pyr or Gc/Gln/Pyr (Fig 5F).

Chronic hypoxia modulate the effects of substrates on HIF stabilization

Continuous exposure to low O2 markedly affects metabolism, bioenergetics and HIF-signaling in PC12 cells [38]. To study the effects of metabolic substrates on HIF-2α accumulation in chronically hypoxic cells, we continuously grew (15 days) and differentiated them (5 days) at a monolayer at 25 O2 (Fig 6A). The hypoxic cells exhibited elevated HIF-2α levels and a shift towards glycolytic phenotype with increased ECAT rate (Supplemental Fig 5G). However, a capacity of the cells to accumulate HIF-2α upon treatment with DMOG was strongly decreased (Fig 6B).

Measuring using Nanocube probe, O2 levels in chronically hypoxic cells varied in the range of 5-25 μM, depending on the substrate composition (Fig 5G). Interestingly, when Gc was available, HIF-2α was elevated regardless of the presence of Gln, a cell supplied with Gln and Pyr but deprived of Gc, despite the lowest levels of Gc/5-10 μM, HIF-2α was barely detectable and did not increase upon treatment with DMOG or MG132 (Fig 6D). Remarkably, in these cells both active (Akt and Edk) and negative (AMPA) regulators of HIF-2α production were activated (Fig 5D, E). Cellular ATP was similar in all samples, therefore lower levels in a factor preventing HIF-2α accumulation with Gc was ruled out (Fig 6G). Inhibition of GLUT with 2-DG did not affect HIF-2α level upon treatment with DMOG (Supplemental Fig 5B).

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

Discussion

The involvement of HIF pathways in cancer cell metabolism and transcription factor 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 [39-41]. Although O2 level is an extremely important parameter for hypoxia research, it is rarely measured, and the published experimental protocols erroneously report on the atmosphere, O2-free 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 glycolysis and glutaminolysis play differential roles in cancer cell hypoxia and metabolism.
Fig 4 – Dichotomy in AMPKα and Akt phosphorylation and effect of AMPK inhibition on cell viability upon OGD. (A) Western blotting analysis of HEF-2α and phosphorylation of HEF-regulating proteins in Glu/Gln or Glu/Gln media at different atmospheric O2. (B) Quantitative analysis of (A) normalized to corresponding values in mononuclear cells supplied with Glu/Gln in WM containing Glu/Gln at 4% O2. Akt/Erk phosphorylation is increased; however HEF-2α levels are significantly lower, than in WM containing Glu/Gln. Under anoxia, an increase in HEF-2α levels is abolished in cells supplied with Glu/Gln. 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. (E) Upon 3 h OGD, treatment with an AMPK inhibitor (DM 2 mM for 5 h) does not affect HEF-2α, p-Akt or p-AMPKα levels, but causes a slight decrease in Erk1/2 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 Glu/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 Glu/Gln, the level of PAPP (16 kDa band) was reduced upon AMPK inhibition. Data are normalized to the values in WM containing Glc (1.0). Asterisks indicate significant difference; in (B) - between corresponding values in WMs containing Glu/Gln and Glu/Gln. In (C) and (D) - from anoxic Glc (-) sample, in (F) - from Glc (+) sample, in (G) - between DM (+) and DM (-) cells. In (A), (B) and (E), n = 3; in (C, n = 4. Error bars represent SD.
Fig. 5 - Changes in HIF-2α levels upon prolonged Glc or Glc deprivation. (A) Layout of the experiment. (B) Western blotting analysis of HIF-2α and phosphorylation of Akt-regulating proteins in cells supplied with Glc/Gln/Pyr, Glc/Gln/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, Erk and AMPKα phosphorylation. Data are normalised to the values in WM containing Glc/Gln/Pyr (1 ng/mL). (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 proteasome inhibitor MG132 (10 μM) on HIF-2α protein level, shown as fold change relative to mock-treated cells. Asterisks indicate significant difference, in (B) and (F) from corresponding values in Glc/Gln/Pyr medium; in (C) from corresponding values in two other media; in (D) from corresponding value in mock control. 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 slowly warms glycolysis and yields no ATP, causes a compensatory increase in Ophos flux and cell-wide desammoniation, while Glc deprivation decreases Ophos flux, increases ATP and activates glycolysis [40].

Recently, Glc was shown to be sufficient to drive the Krebs cycle in Glc-independent manner in hypoxic cells overexpressing MCK oncogene [57]. We show here that availability of Glc is one of the main factors necessary for HIF-α accumulation at moderate hypoxia in actively growing PC12 cells, as Glc determines kinetics and level of cell desammoniation (Figs. 1 and 2). HIF-2α levels are known to increase exponentially at O2 below ~40 mmHg (or 30 μM) [41]. In all WM, Glc was added to rapidly rise when cells were transferred from 15% to 4% atmospheric O2 (Fig. 1). However, cells deprived of Glc were not sufficiently desammoniated for significant HIF-2α stabilization (Figs. 1 and 3). Incubation in the presence of Glc δ0 dropped below 10 μM and reached 5 μM (i.e. the optimal O2 level for HIF stabilization [58]). In WM containing Glc/Gln/Pyr, cells stayed below 10 μM for more than 50 min during the first 2 h of hypoxia, effectively accumulating HIF-α. On the other hand, desammoniation of cells supplied with Glc/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 strongly affect HIF levels under hypoxia.

Indeed, substrate availability appears to determine the rate of HIF-α stabilization, most probably by regulating cellular ATP levels and pathways involved in HIF-1α stabilization (Fig. 1). Unlimited Glc availability is essential for cancer cells even at high O2 levels [59], and becomes particularly important under deep hypoxia and anaemia, when Ophos flux decreases. Here we demonstrate that in PC12 cells deprived of Glc or Glc supply is compromised for HIF-α accumulation, which does not occur upon OGD (Figs. 1, 3 and 4). In accordance with this, HIF-2α levels strongly correlate with cellular ATP levels in anaemia, when energy is produced exclusively via glycolysis (Table 1 and Supplemental Fig. S1). These findings support the idea that ischemic-like HIF signaling might be inhibited or even turned off at a translational level [58,59], while cell metabolism becomes regulated by other players, e.g. via AMPK phosphorylation.

In agreement with [50,68], we found AMPKα phosphorylation elevated in all OGD samples (Figs. 3D and 4A–C). An increase in AMPK/ATP ratio followed by activation phosphorylation of AMPKα...
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 NG108-15 (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 nuclear and cytosolic stabilized HIF-2α levels in cells grown continuously in high versus low O2 (5%) 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 Nanolite probe in 30–100% 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 NG108 increases HIF-2α levels in Glc/Glu/Pyr medium. (E) Quantitative analysis of Erk, 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 WM with Glc/Glu/Pyr. Asterisks indicate significant difference. n=3, error bars represent SD (n=3). In (C) and (F) results are shown as a range of 10%, and ATP values (Mean±SD).

at Tyr172 is known to trigger a cascade of events directed towards a global metabolic reprogramming and cell survival. This includes energy preservation (via decreased catalytic processes, translation and cell proliferation), increased autophagy and mitochondrial biogenesis, coordination of oxidative and glycolytic metabolism and regulation of redox state [63]. Recently, AMPK has been shown to down-regulate aerobic glycolysis in cancer cells by reducing HIF-1α levels and TORC1 activity, and to suppress tumour growth in vivo [64], although the major role of AMPK in AMPK1 regulation under hypoxia and reperfusion is argued [65]. Our results demonstrate that PC12 cells can easily overcome short-term OGD (2 h) and restore their capacity to accumulate HIF-2α upon reinitiation of O2 and Glc supply (Supplemental Fig S2). Activation of AMPK can be essential to reduce ATP expenditure (including HIF-α translation) and increase cell survival during OGD [66]. In agreement to this, an inhibition of AMPK with DM caused a decrease in viability of cells supplied with Glc/Glu which experienced energy stress under anoxyia (Fig. 6E).

A decrease in HIF-α production upon OGD may also be related to a reduction of Akt (Ser473) phosphorylation. Normally, activated Akt triggers translocation of the GLUT1 to the plasma membrane and maintains hexokinase function [67], thus stimulating ‘aerobic glycolysis’ in the actively proliferating cancer cell....
[68] Akt phosphorylation, required for activation of TORC1 [17]
was elevated under hypoxia in all WtRbc tested (Figs. 1 and 4), in agreement with [48,59]. In anoxic cells (at least at 2 h time point), Akt phosphorylation is strongly linked to Gli availability. Interestingly, HIF-2 can also activate TORC1 and increase cell proliferation under hypoxia by up-regulating expression of the anionic acid carrier SLC7A9 [69].

In our experiments, levels of p-VEGFR (Ser2346) do not correlate with Akt, Erk, or AMPK phosphorylation (Figs. 1, 3, 6). The rather poor match of phosphorylation patterns may arise from the differences in the kinetics of (de)phosphorylation events, which are separated by time for these proteins. This difference can also explain why Akt is hyperphosphorylated in HRE−/− cells at 2 h but not in HIF-2α KO cells at 24 h, and why Akt levels reach their maximum after 2 h and then noticeably decrease, while HIF levels increase progressively (Figs. 3, 4). In HRE−/− cells, which are using Gli as their main metabolic, a decay of Akt and Erk phosphorylation is more gradual, than in the presence of Gli.

Gli, the most abundant free amino acid, is taken up by cells through a number of transporters and is metabolised mainly through glutaminolysis. It is also a precursor of N-acetyl-l-glutamine, therefore Glu deprivation or inhibition of CSE1 with BPTES cellular or KG levels decrease and activity of PHD proteins may decrease accordingly [65]. This could potentially lead to PHD-dependent stabilization of HIF-α subunits in VM without Glu, which was not the case as demonstrated in the experiments with VM containing Glc/Gal/Pyruvate (Fig. 7). On the other hand, i.e., low-glutamine cells deprived of Glu, KG, levels can be sufficient for PHDs to hydroxylate HIF-α even at reduced availability of KG. Interestingly, an increase in K levels has been shown to contribute to HIF-1 activation by interacting with a NCB end of PHD6 [57], such an inhibition of PHDs by K pyruvate via glycolysis (RHIF-2α levels are much higher than in the presence of Gal/Glu (Fig. 4).

In a simplified manner, proposed effects of short-term (several hours) substrate deprivation on HIF-2 levels are summarized in Fig. 7A. The possibility that HIF-2 accumulation is regulated via a negative feedback mechanism in the PHD2 transcriptional level was ruled out, as no difference in HIF-inducible PHD2 and PHD3 protein after a short-term hypoxia/anoxia was seen (Supplemental Fig. 5A). It is worth to note that upon continuous substrate deprivation the impact of Glu, Glu and Pot to HIF signaling may become strongly affected by many other factors.

In our next knowledge shown how for the first time, an effect of short-term (~24 h) HIF-2 levels and pattern of protein phosphorylation in cells treated with DMOG or a proteasome inhibitor MG132 (Fig. 5F) led us to propose a scenario for down-regulation of HIF-2 response (Fig. 7B). With the assumption that all effects may be cell specific, we believe that HIF-2α translation is inhibited in both cases, but a Glu deficiency causes an inactivation of WT and ERK pathways, while withdrawal of Glu leads to an activation of AMPK pathway. A decrease in pro-proliferative Akt/Erk signaling makes perfect sense for the cells deprived of Glu, which is the major contributor to the cellular biomass. In turn, in actively requiring cells without Glu, p-AMPK levels can increase in an attempt to activate mitochondrial biogenesis through the p53-1α [72] and in response to an elevation of ROS production by Gal, demonstrated in mouse brain and human ipecysts [73,74]. It is attractive to propose that inhibition of AMPK can be used for the amelioration of pro-proliferative HIF-2 signaling and energy consumption, thus leading to exacerbated energy stress and death of cancer cells. Indeed, the capacity to accumulate HIF-2α was partially restored in cells treated with 1M in mid-term substrate deprivation experiments. However, we were unable to substantially increase HIF-2α levels by pharmacological inhibition of AMPK (Fig. 7C).

Similarly, almost no changes in HIF-2α were seen in cells treated with 1M upon short-term Glu and pot deprivation (Fig. 7F). To this end it is worthy to note that DMOG, although broadly used, is rather non-specific inhibitor of AMPK pathway, shown to cause an AMPK-independent inhibition of AMPK signaling [75], activation of apoptosis [76] and increase in p-Erk/2 (see text [64]). The latter was actually seen in cells supplied with Glc/Glu/Pyruvate (Fig. 7A, F).

These observations suggest that more specific strategies for AMPK inhibition may be necessary to re-activate HIF-2 signaling and target cancer cells limited in Glu and Pyruvate supply. A decrease in cellular ATP levels upon treatment with DMOG observed in cells deprived of Glu for 24 h is agree with an activation of AMPK (Fig. 5B). This result is intriguing and requires further analysis of the cell energetics buffer in these conditions, both under normoxia and hypoxia.

Although most of the in vitro studies with cell lines, including PCT2 cells, are routinely conducted in 18-21% atmosphere oxygen, these O2 levels must be defined as hypoxia, because normal physiological oxygenation of mammalian tissues varies between 14% and 20% (% similarly to partial pressure of 72–85 mmHg or dissolved concentration of 10–17 μM) [77]. Therefore to obtain data relevant in physiological normoxia we analysed the effects of substrate deprivation on HIF-2a signaling using PCT2 cells continuously maintained at 3% atmospheric oxygen. We recently showed that such chronically hypoxic conditions become equivalent to OGD conditions and induce the capacity to accumulate HIF-2α in response to OGD deprivation [39]. Here we demonstrate that in chronically hypoxic cells Glu no longer plays a critical role in HIF-2α production, which becomes distinctly Glu-dependent (Fig. 5B). Shown here for the first time, this phenomenon is most probably associated with a general pre-glycolytic rearrangement of metabolism in PC22 cell under hypoxia (Supplemental Fig. 3). Here, therefore, Glu, as an indispensable source of nitrogen [78], remains essential for proliferation of hypoxic cells, which depend upon Glu availability (data not shown).

Interestingly, in chronically hypoxic cells without Glu, despite of their deeper deoxygenation and higher p-HIF2α and p-Akt levels, HIF-2α was noticeably lower than in Glu (+)-cells (Fig. 6C). Similar aberration were observed in transgenic hypoxic cells supplied with Gal/Glu/Pyruvate treated with MG132 as well as in cells supplied with Gal/Glu/Pyruvate (Figs. 1A, 2A, 3A, and 4A). Since AMPK phosphorylation pattern was different in transiently and chronically hypoxic cells without Glu, we hypothesized that in certain metabolic environments HIF-2α accumulation can be inhibited on post-transcriptional level in a manner independent on Akt, Gli and AMPK pathways. Transcriptional regulation can be ruled out based on the results of qRT-PCR analysis (Fig. 5) [18]. This observation does not clarify the mechanism involved, although makes further investigation of them attractive.

Conclusively, we believe that the pattern of HIF-2α accumulation in tissues can be strongly affected by Glu, Gli and Pyruvate availability and utilization, which differentially regulate O2 and ATP levels, activity of Akt/Gli/AMPK pathways and other factors driving HIF-2α expression.
Fig 7 - Summarized 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 AMPK-Pepk dependent pathway and AMPK flux via AMPK activation is also shown to contribute to these factors. Without Glc several conditions required for HIF elevation are not met: (1) HIF levels are sufficient for HIF protein to initiate HIF-α degradation; (2) the levels of p-Akt and p-p38 do not increase; (3) mitochondrial ATP flux decrease, the latter also occurs in the presence of Glc, when cells limited to O2, actively consume it and become anoxic. In such anoxic cells as well as in O2 deprived cells, Glc becomes the major factor maintaining ATP levels and capacity of cells to produce-stable HIF. Cells deprived of Glc not only rapidly lose ATP, required for translation, but also shut down major 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 blue, 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 moderate metabolic deprivation. In differentiated PC12 cells grown for 24-28 h without Glc or Glc, a decrease in HIF-α is coupled to and may be regulated via inactivation of AMPK or activation of AMPK pathways, respectively. Inhibition of AMPK pathway can therefore lead to an increase in concentration of HIF-α levels in cells without Glc. To test this hypothesis, cells were grouped in different WIs as indicated (C) and pre-incubated with an AMPK inhibitor DM (2 μM) for 4 and 24 h and then treated with DM1 (4 μM) in the presence or absence of DM. Western blotting analysis demonstrated that HIF-α levels did not change significantly upon AMPK inhibition, however, a peculiar trend was observed in cells supplied with Glc but deprived of Glc and particularly CaCl2/Pyr. In front, 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) hypoglycemia in patients with diabetes mellitus [82] and liver carcinoma [83]; (iii) reduced glutamine synthesis activity in astrocytes [84]. Our data add to the understanding of key metabolic substrates as major contributors to HIF signaling and promising targets for prevention and therapy of cancer and ischemia.

Conflict of interests

DP is an employee of tCell Sciences 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.2015.06.005.

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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