PERSPECTIVES

Hypoxia, HIF1 and glucose metabolism in the solid tumour

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Abstract | It has been known for many years that cellular metabolism within the solid tumour is markedly different from that of the corresponding normal tissue. The transcription factor hypoxia-inducible factor 1 (HIF1) has been implicated in regulating many of the genes that are responsible for the metabolic difference. However, it remains unclear how this 'aerobic glycolysis', originally described by Otto Warburg, offers tumour cells a growth advantage. As discussed in this Perspective, new data suggests that this metabolic switch may provide a benefit to the tumour not by increasing glycolysis but by decreasing mitochondrial activity.

The metabolism within a solid tumour is significantly different from that of the surrounding normal tissue. The major metabolic changes within the tumour were identified over 70 years ago by Otto Warburg[1]. He found that normal tissue uses glycolysis to generate approximately 10% of the cell's ATP, with mitochondria accounting for 90%. In tumour sections, however, over 50% of the cellular energy is produced by glycolysis with the remainder being generated at the mitochondria. Interestingly, this shift occurs even when there is enough O_2 present to support mitochondrial function, and it is termed aerobic glycolysis. The reliance of tumour cells on glycolysis for energy production causes them to consume more glucose because of the low efficiency of glycolysis in generating ATP. The mechanisms responsible for this shift are being unravelled, and they are giving us new insight and hypotheses as to how this metabolic profile can provide tumours with a growth advantage.

One of the most recognized reasons for altered tumour metabolism is the unique physiological stresses that exist within the tumour. The tumour microenvironment suffers from hypoxia (low levels of O_2), acidosis and increased interstitial fluid pressure[2]. These microenvironmental stresses are largely the result of poorly formed tumour vasculature[3]. Significant regions of the tumour are at a great distance from the supporting blood vessels, and this distance causes a gradient of diffusion-limited hypoxia, low levels of nutrients and increased levels of waste products[4]. Hypoxia is perhaps the most pervasive of these stresses and variably exists when O_2 delivery does not meet the demand within the tumour tissue. Tumour cells respond to these conditions, and adapt their metabolism to adjust the O_2 demand to meet the limited supply[5,6]. Perhaps the most important aspect of how cells respond to this unique microenvironment is the activity of the hypoxia-inducible factor 1 (HIF1) transcription factor[7]. The net result of hypoxic HIF1 activation is to shift energy production by increasing glycolysis and decreasing mitochondrial function. HIF1 was initially identified because of its response to low O_2 concentrations, but it is now apparent that HIF1 can be regulated by other factors such as oncogene activation or loss of tumour suppressors. For example, HIF1 accumulates in tumour cells after activation of oncogenes such as Ras, *SRC* and phosphoinositide 3-kinase (PI3K), or loss of tumour suppressors such as *VHL* (von Hippel–Lindau) or *PTEN*, even under normoxic conditions[8]. Furthermore, increased levels of metabolites such as succinate and fumarate, or O_2 by-products such as free radicals, in a tumour can also stabilize HIF1α[9]. The net result of these genetic and physiological changes within the tumour is to induce HIF1 and actively shift energy production from mitochondrial to glycolytic sources in both hypoxic as well as oxygenated regions of the tumour.

Activation of HIF1 and the HIF1 transcriptional programme have two major effects on metabolism that serve to equilibrate O_2 consumption with O_2 supply. First, HIF1 stimulates glycolytic energy production by transactivating genes involved in extracellular glucose import (such as GLUT1, also known as *SLC2A1*) and enzymes responsible for the glycolytic breakdown of intracellular glucose (such as phosphofructokinase 1 (*PFK1*) and aldolase). Glycolysis is the biochemical process that breaks glucose down to pyruvate and generates two molecules of ATP[10]. This low-yield energy production is sufficient to supply ATP for cellular energetics if the supply of glucose is adequate. Pyruvate can be further broken down at the mitochondria through the process of oxidative phosphorylation, which uses O_2 and generates CO_2, H_2O and about 18 additional molecules of ATP. However, HIF1 also downregulates oxidative phosphorylation within the mitochondria by transactivating genes such as pyruvate dehydrogenase kinase 1 (*PDK1*)[11,12] and MAX interactor 1 (*MXI1*)[13]. These two effects reduce the O_2 demand of tumour cells within hypoxic tissue while still supplying sufficient energy to the cell. By accomplishing both these major effects, HIF1 alone can drive the major metabolic changes within the tumour that were identified by Otto Warburg[1]. The low-efficiency cancer cells must take up quantitatively much more glucose than the normal tissue. This discrepancy can be observed clinically: the glucose tracer 2-deoxyglucose can be labelled with the radioemitter ^{18}F, and its uptake by the tumour (and normal tissues) can be detected and quantified in a positron-emission tomography scanner.

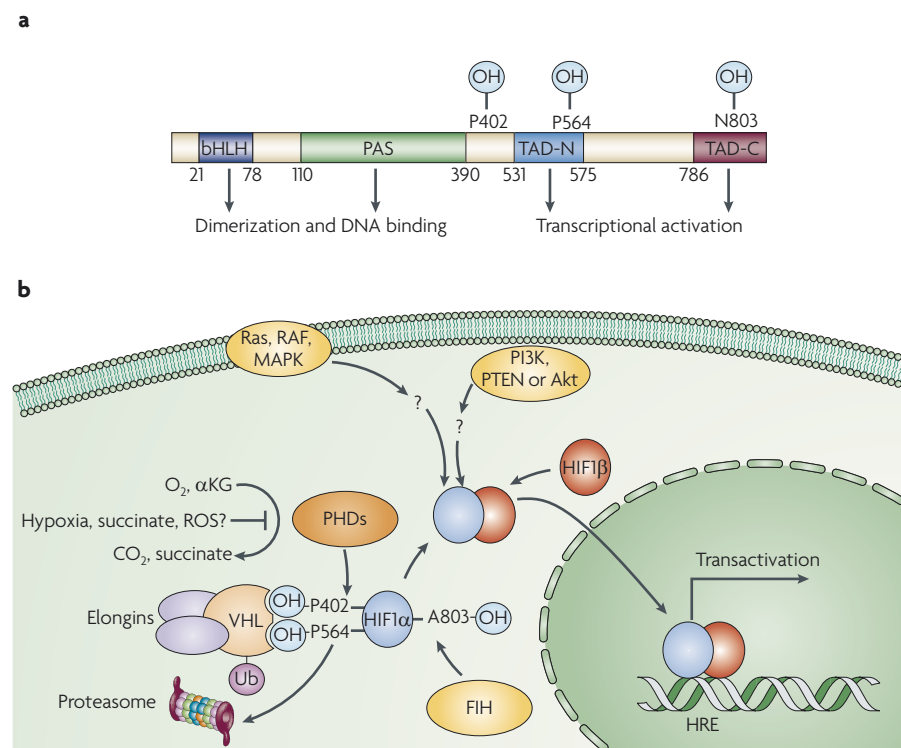This Opinion article discusses how HIF1 is capable of regulating the observed

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b

Figure 1 | **Mechanisms of hypoxia-inducible factor 1α (HIF1α) stabilization. a |** HIF1α domain structure. Sites of proline (P) hydroxylation are indicated in the O_2-dependant degradation domain of the human protein. Asparagine (N) hydroxylation in the carboxy-terminal transactivation domain by factor inhibiting HIF (FIH) regulates HIF1 activity but not stability. **b |** The classical O_2 sensing pathway is through O_2-dependent enzymatic hydroxylation at P402 and/or P564 on HIF1α. This modification is due to one of the three prolyl hydroxylase (PHD) enzymes, which mediate recognition of the VHL–elongins complex and ubiquitination (Ub) of HIF1α and hence targeting for proteasomal degradation. Oncogenic activation, associated with activation of the Ras–RAF–MAPK (mitogen-activated protein kinase), phosphoinositide 3-kinase (PI3K), PTEN or Akt pathways, can also cause HIF1α accumulation through unknown mediators. Tricarboxylic acid cycle intermediates such as succinate and fumarate, or perhaps mitochondrial reactive oxygen species (ROS), can inhibit the activity of PHDs, also stabilizing HIF1α. Stabilized HIF1α associates with HIF1β, which binds to cognate hypoxia-responsive elements (HREs) in target genes. Activation of HIF-responsive genes has a variety of effects: for example, vascular endothelial growth factor stimulates angiogenesis, lysyl oxidase stimulates metastasis and carbonic anhydrase IX regulates pH. αKG, α-ketoglutarate.

metabolic alterations within the tumour, in regions of both hypoxia and normoxia. These HIF1-dependent changes can then reconcile some of the physiological observations that encompass aerobic as well as hypoxic glycolysis. Finally, I present hypotheses as to why these metabolic changes can result in a growth advantage for tumours that are HIF1-positive and metabolically altered.

Regulation of HIF1

The HIF1 transcription factor is so named because of the cellular machinery that stabilizes HIF1α in hypoxia. This response to hypoxia is certainly the most well-understood aspect of HIF1's ability to regulate metabolism. Hypoxia is caused by a mismatch between O_2 delivery and consumption, so many of the gene expression changes are designed to bring this mismatch back in line. This can be achieved with decreases in consumption (through altered metabolism) and/or increases in delivery (through erythropoietin and vascular endothelial growth factor production). However, additional factors have been recognized that can cause HIF1 accumulation, and could also regulate metabolism in aerobic conditions. Aspects that are unique to the solid tumour, such as oncogenic mutations and the accumulation of intermediate metabolites, are also important because they can contribute to HIF1 regulation in better-oxygenated areas.

Hypoxia. HIF1 is a heterodimeric transcription factor with HIF1α or HIF2α (also known as EPAS1) as the O_2-responsive subunit, and HIF1β (also known as ARNT) as the constitutively expressed subunit[14]. HIF1, containing either HIF1α or HIF2α, recognizes similar hypoxia-responsive elements (HREs) in the promoters of target genes; however, it appears that slightly different groups of genes are activated by the two subunits[15]. Importantly, HIF1α seems to contain a unique transactivation domain that allows preferential activation of the hypoxia-responsive glycolytic genes[16]. HIF1α and HIF2α seem to have somewhat different effects regulating hypoxic responses in cell types such as renal cancers, but the understanding of this is limited[17].

HIF1α is unstable in well-oxygenated tissues owing to ubiquitin-mediated degradation, but rapidly becomes stable in hypoxic conditions (FIG. 1). Degradation is mediated through hydroxylation of prolines 402 and 564 of human HIF1α[18–21]. O_2 is a low-affinity substrate for the prolyl hydroxylases (PHDs, which confer sensitivity to O_2), so when O_2 tension (pO_2) drops HIF1α is not hydroxylated and thus becomes stabilized[22]. The hydroxylated prolines are recognized by VHL[23] complexed to elongins B and C. VHL acts with NEDD8 as an E3 ubiquitin ligase that modifies HIF1α and targets it for degradation[24]. When VHL expression is lost in renal cancers, HIF1α and HIF2α are constitutively stable (even in normoxia) resulting in constitutive transactivation of target genes and loss of VHL-mediated tumour suppression[25]. The activity of HIF1 can be inhibited by the asparagine hydroxylase factor inhibiting HIF (FIH, also known as HIF1AN), which hydroxylates asparagine 803 in the carboxy-terminal transactivation domain of human HIF1α. Hydroxylation-mediated regulation is thus the major mechanism for HIF1α regulation in normal tissue, and modifies HIF1 activity in tumour cells. However, there are several other changes that can also increase HIF1α protein levels, independent of hypoxia.

Oncogenes. When it was determined that HIF1α protein stability was actively regulated, several genes that could regulate this process were identified. The expression of oncogenes such as HRAS-V12 leads to the accumulation of HIF1α in both normoxic and hypoxic conditions[26]. Because of this effect, tumour cells often exhibit increased HIF1 target gene activation *in vitro* and later analysis showed that activation of the Ras–MAPK (mitogen-activated protein kinase) pathway results in accumulation of

HIF1α in a number of model systems[27]. In addition, the expression of the oncogenes encoding SRC[28] and ERBB2 (REF. 29) can also increase HIF1α protein and target gene expression in normoxic conditions. The exact mechanism(s) responsible for the effects of these oncogenes is not yet understood, although HIF1α is hyperphosphorylated, indicating that activated kinases responding to oncogenic stress are candidates.

In addition, activation of the PI3K pathway can also result in HIF1α accumulation in normoxia[30]. Direct activation of PI3K itself or of the downstream kinase Akt, or loss of the inhibitory PTEN tumour suppressor, leads to increased HIF1α levels and activity. Although this effect might be cancer-type-specific, there appears to be a correlation with cancer types that undergo PTEN loss or Akt amplification during tumorigenesis (such as glioblastoma and prostate cancer cells)[31]. Interestingly, the mechanism does not appear to involve direct phosphorylation of HIF1α by Akt[30]. Hydroxylation and degradation of HIF1α appears intact in these cells[32], but HIF1α translation appears to be increased, which is thought to be due to Akt-dependent activation of mTOR (also known as FRAP1)[33]. Alternatively, secondary effects of the oncogenes on cell proliferation or metabolism could also contribute to the increased accumulation of HIF1α.

Secondary effects. In addition to HIF1 driving glucose metabolism, there are glucose metabolites that can regulate HIF1α through a positive feedback mechanism. The impact of this regulation was recently appreciated when it was found that the genes for succinate dehydrogenase (SDH) and fumarate hydratase (FH) were in fact tumour suppressors[9] contributing to transformation in malignant pheochromocytomas and leiomyomata, respectively. These enzymes of the tricarboxylic acid (TCA) cycle normally generate reducing equivalents for the electron transport chain in the mitochondria. However, when mutated, they form a block to the flow of metabolites and result in an accumulation of succinate and/or fumarate respectively. One effect of these increased levels is to generate a condition termed 'pseudohypoxia'. Because the HIF1α PHDs convert 2-oxoglutarate (α-ketoglutarate) and O_2 to succinate and CO_2, high levels of succinate can slow this enzyme, leading to HIF1α stabilization. Mutations in SDH or FH have therefore been shown to cause HIF1 to be stabilized in normoxic cells. A

number of other endogenous 2-oxoacids such as pyruvate and lactate have also been determined to stabilize HIF1α, providing a novel feed-forward mechanism[34]. Reversing this pathway of HIF1α stabilization with exogenous α-ketoglutarate derivatives may offer therapeutic approaches to treat these tumours[35].

It has also been proposed that the reactive oxygen species (ROS) generated as by-products of electron transport at the mitochondria can signal to stabilize HIF1α in hypoxia[36–40]. The interaction of mitochondrially generated superoxide with the HIF degradation machinery is not well-established, but it has been reported that hydrogen peroxide can oxidize the Fe^{2+} that is required as a cofactor for PHD activity. The resulting Fe^{3+} cannot function in hydroxylation and the loss of PHD activity in turn results in HIF1α stabilization[41]. However, this is controversial, with other groups reporting that HIF1α can be stabilized in hypoxia in cells lacking mitochondrial function[42,43].

HIF1 regulation of glucose metabolism
The largest functional group of genes consistently regulated by HIF1 in a number of cell types are associated with glucose metabolism[44–46] (TABLE 1). Glycolysis relies on linear processing of metabolites, so many of the enzymes in this pathway are induced together. HIF1 increases the expression of the transporters necessary for the entry of glucose into the cell, the genes involved in the enzymatic breakdown of glucose to pyruvate and the enzymes involved in the clearance of pyruvate. Pyruvate is not used by mitochondria in the hypoxic cell but instead is converted to lactate by lactate dehydrogenase (LDH)

to be released into the extracellular space. Recent data suggest that HIF1α can coordinate this process under aerobic as well as hypoxic conditions[47].

Mechanisms for HIF1-increased glycolysis. HIF1 can increase the rate of glucose uptake, primarily through the induction of the facultative glucose transporters GLUT1 and GLUT3 (SLC2A3) (REFS 48,49). These transporters belong to a superfamily comprising 13 facultative transporters as members[50]. However, GLUT1, GLUT3 and GLUT4 (SLC2A4) regulate the majority of glucose uptake owing to their tissue-wide expression pattern, number of molecules per cell and affinity for glucose[51]. Because these transporters move glucose down its concentration gradient from relatively high blood levels to relatively low intracellular levels, simply increasing the quantity of the protein increases the flux of glucose into the hypoxic cell. In tumour cells, a major physiological inducer of glucose transporters is HIF1 (REF. 52).

Glucose has several possible metabolic fates once it is taken up by the cell. Intracellular glucose is quickly phosphorylated by hexokinase (HK) to glucose-6-phosphate. Both HK1 and HK2 can be induced by HIF1, but it appears that HK2 is more important for modifying glucose in hypoxia[53]. The charged glucose molecule cannot easily pass back through the plasma membrane and is trapped within the cell. Glucose-6-phosphate is then used in one of several pathways: as a structural component in the synthesis of glycoproteins, metabolized in the pentose shunt to generate ribose, used to synthesize glycogen or, predominantly, broken down to pyruvate in glycolysis (FIG. 2). HIF1 can channel glucose
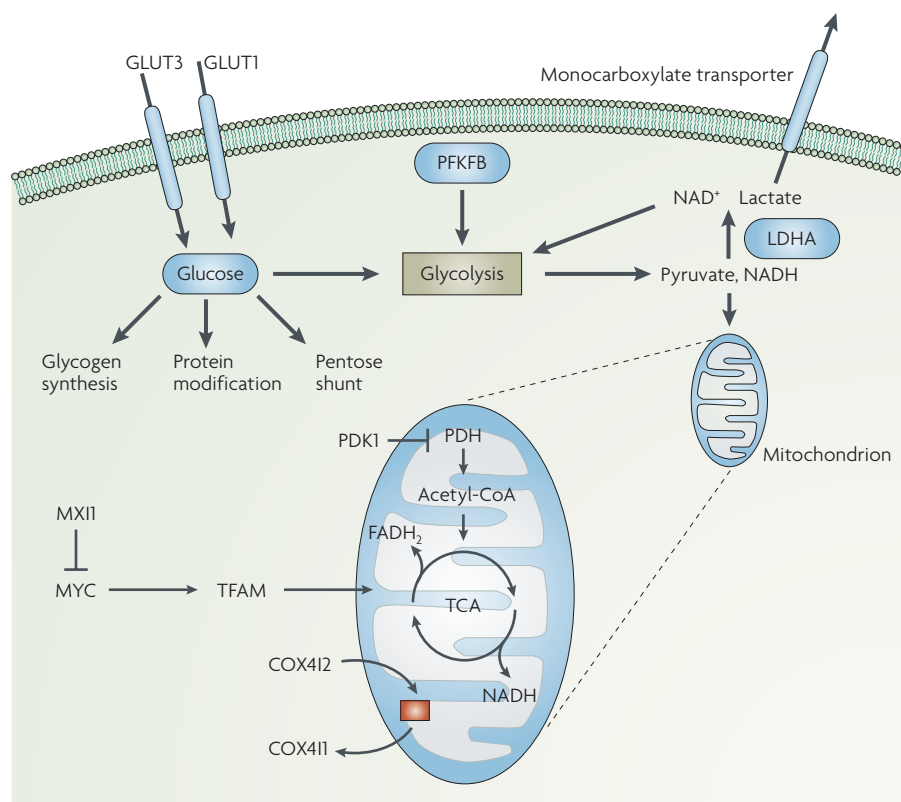
Table 1 | HIF1 targets that regulate glucose metabolism

Product(s) of HIF1 target gene(s)	Metabolic function	Refs
Glucose transporters GLUT1 and GLUT3	Glucose entry into the cell	49,119
HK2	Phosphorylation of glucose	53
PGI, PFK1, aldolase, TPI, GAPDH, PGK, PGM, enolase, PK, PFKFB1–4	Glycolysis	7,120–122
LDHA	Conversion of pyruvate to lactate	58
MCT4	Removal of lactate from the cell	59
PDK1, MXI1	Decreased mitochondrial activity	11–13
COX4I2, LON protease	Increased O_2 consumption in hypoxia	66

COX4I2, cytochrome oxidase isoform 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; HIF, hypoxia-inducible factor; HK, hexokinase; LDHA, lactate dehydrogenase A; MCT, monocarboxylate trasporter; MXI, max interactor; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PFKFB, 6-phospho-2-kinase/fructose 2,6 bisphosphatase; PGK, phosphoglycerate kinase; PGI, phosphoglucose isomerase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; TPI, triosephosphate isomerase.

Figure 2 | **Overview of hypoxia-inducible factor 1α (HIF1α)-mediated regulation of tumour cell metabolism.** HIF1 activation leads to an increase in several metabolic pathways (such as glycolysis) and a decrease in others (such as oxidative phosphorylation). First, there is increased uptake of glucose into the cell through the upregulation in expression of the glucose transporters GLUT1 and GLUT3. The intracellular glucose is then metabolized by the increased levels of the glycolytic enzymes. Increased glycolysis generates increased pyruvate, which is largely converted to lactate by HIF-inducible lactate dehydrogenase A (LDHA) and removed from the cell by the monocarboxylate transporter. In the mitochondria, decreased pyruvate flow into the tricarboxylic acid (TCA) cycle owing to HIF1-dependent pyruvate dehydrogenase kinase 1 (PDK1) induction, decreased mitochondrial biogenesis through MAX interactor 1 (MXI1) induction (which antagonizes MYC activity) and switched cytochrome oxidase subunit 4 isoform 1 (COX4l1) to the high-efficiency COX4l2 subunit. PDH, pyruvate dehydrogenase; PFKFB, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase.

into glycolysis by increasing the amounts of the enzymes involved in this process. All 12 enzymes necessary for glycolysis (shown in TABLE 1) are directly regulated — at least in part — by HIF1 (REF. 7) such that the entire process is coordinately stimulated by HIF1 (REF. 54). The rate of glycolysis is regulated primarily by substrate and product availability owing to the reversibility of each step, so one means of increasing glucose breakdown is increasing the expression of enzymes within the cell. However, one exception is the enzyme PFK1, which catalyses an irreversible reaction and is regulated allosterically through the abundance of fructose 2,6-bisphosphate. This metabolite is generated by PFKFB2, a member of the family of dual-functioning enzymes 6-phosphofructo-2-kinase/fructose

2,6-bisphosphatase (PFKFB1 to PFKFB4). PFKFB3 is also induced by HIF1 (REF. 55), and can be inhibited by TIGAR (C12orf5), a target of the tumour suppressor p53 (REF. 56). As a general rule, however, the increased expression of glycolytic enzymes favours the use of glucose in glycolysis. Glucose, two ADP and 2 NAD^+ are thus converted by glycolysis to 2 pyruvate, 2 ATP and 2 NADH.

Pyruvate is a product of glycolysis and, as glycolysis increases, more pyruvate is produced that needs to be removed. Although it is not clear why, tumour cell growth can be inhibited when the means of pyruvate removal is genetically blocked by short hairpin RNA directed against *LDHA*[57]. It may be that accumulation of the end product of glycolysis slows further

activity of the pathway, or that LDHA is required for recycling the cytosolic NAD^+ necessary for further glycolysis. Under normoxic conditions pyruvate from the cytoplasm enters the mitochondria and the reducing equivalents in NADH are shuttled into the mitochondria (through the malate–aspartate or 3-phosphoglycerate shuttles) to be the substrates for oxidative phosphorylation. Pyruvate is oxidized to CO_2, generating additional mitochondrial NADH and $FADH_2$. These mitochondrial reducing equivalents donate electrons to the electron transport chain and combine with O_2 to produce water and a proton gradient that is used to generate ATP. However, as discussed below, the mitochondria within the hypoxic cell do not appear to function at maximal levels, and cytoplasmic pyruvate and NADH accumulate. An alternative pathway to dispose of these compounds is also activated by HIF1. *LDHA* is induced by HIF1 and this enzyme catalyses the conversion of pyruvate and NADH to lactate and NAD^+ (REF. 58). The lactate can then be removed from the cell through the action of the HIF-inducible plasma membrane monocarboxylate transporter 4 (MCT4, SLC16A4)[59]. This solves two problems simultaneously: removal of excess pyruvate and regeneration of NAD^+, which is required by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for additional cycles of glycolysis.

HIF1 modulates mitochondrial function
Papandreou and colleagues were the first to show HIF1 is both necessary (using gene knockout and knockdown cells) and sufficient (using VHL-negative renal cancer cells) for hypoxic downregulation of mitochondrial function[12]. These experiments also showed that HIF-negative cells had increased mitochondrial function, which under certain growth conditions such as high cell density leads to increased intracellular hypoxia. This consumption-driven hypoxia was most apparent in intermediate levels of hypoxia, such as 1–2% O_2. Recent studies have identified several mechanisms by which HIF1-regulated genes lead to the modulation of mitochondrial function, ultimately leading to decreased O_2 consumption and decreased intracellular hypoxia.

Mechanisms for HIF-dependent downregulation of mitochondrial function. Pyruvate, amino acids and fatty acids can all be used as fuel in the mitochondria to generate energy. Glutamine can contribute significantly to total energy production in

tumour cells, but these processes have not been shown to be induced by hypoxia[60]. Therefore, in most hypoxic cells the major carbon source is pyruvate, which is the end product of glycolysis. In hypoxic tumour cells with increased glycolysis and increased levels of pyruvate, there needs to be an active mechanism by which this increased pyruvate is excluded from the mitochondria. It was recently determined by several groups that *PDK1* is a direct transcriptional target of HIF1 (REFS 11,12). This gene is induced in a number of tumour cell types in response to hypoxia and HIF1 stabilization. PDK1 is a protein kinase that can phosphorylate the E1 subunit of the mitochondrial enzyme pyruvate dehydrogenase (PDH) and inactivate it[61]. PDH catalyses the committed, irreversible step in the mitochondria by which pyruvate is broken down into acetyl-CoA and CO_2, while generating NADH. The acetyl-CoA is consumed and directly donates two carbons into the TCA cycle at citrate synthase (CS). The reducing equivalents produced in this process from one pyruvate molecule (in the form of NADH and $FADH_2$) are used directly in the electron transport chain to eventually generate up to an additional 18 ATP. By inactivating PDH, PDK isoforms block the flow of pyruvate into the mitochondria[62]. Pyruvate does not enter the TCA cycle, and there is decreased generation of reducing equivalents to power the electron transport chain. This reduction in fuel leads to a reduction in oxidative phosphorylation, a reduction in total cellular O_2 consumption[12] and ROS generation[11].

A second mechanism has been reported by which HIF1 reduces the demand for O_2 by regulating mitochondrial biogenesis[13]. This mechanism requires a chronic state of HIF1 activation, and appears to be more evident in cells that have lost VHL and have constitutive HIF1 activation, such as in renal cell carcinoma. This mechanism involves HIF1-mediated reduction of the number of mitochondria per cell[13] due to the induction of *MXI1* (REF. 63). The basis for this effect rests on the role that the oncogenic transcription factor MYC has in driving mitochondrial biogenesis. MYC can dimerize with its activating partner MAX and directly transactivates the gene encoding transcription factor A (TFAM)[64]. TFAM contributes to the expression of the mitochondrial genome and the genes needed for mitochondrial DNA replication. HIF can directly activate another member of the Myc family, the negative regulator MXI1, which can displace

MAX and inactivate MYC. As a result TFAM expression, mitochondrial mass and mitochondrial O_2 consumption are reduced in renal cancer cells that have lost VHL[13]. However, MYC and HIF have been reported to cooperate in driving gene expression[65], so it is not clear why MYC is responsible for stimulating mitochondrial biogenesis when HIF and hypoxia reduce mitochondrial function. Furthermore, oncogenic transformation usually contributes to the glycolytic phenotype of cancer cells, so the fact that a potent oncogene like MYC contributes to mitochondrial function is surprising.

The third reported mechanism by which HIF1 controls mitochondrial function in hypoxia is by altering the activity of cytochrome *c* oxidase (COX) so that it functions efficiently in conditions of reduced substrate availability[66]. As the final complex in the electron transport chain, cytochrome *c* oxidase uses O_2 as the terminal electron acceptor. The model calls for an isoform switch in one of the regulatory subunits of the cytochrome *c* oxidase complex. Human kidney cells express COX4I1 under normoxic conditions, and COX4I2 and the mitochondrial LON protease (LONP1) when HIF1 is induced[66]. The LON protease causes the turnover in the COX4I1 protein subunit *in situ*. The two isoforms differ biochemically and presumably provide an altered affinity for O_2 and an altered turnover rate of the catalytic COX1 and COX2 subunits[67]. This model appears to parallel the way that the yeast *Saccharomyces cerevisiae* reacts to anoxic conditions, which also relies on an isoform switch at the cytochrome *c* oxidase complex. In the better-studied *S. cerevisiae*, the COX V subunit (closely related to the mammalian COX4 protein) has a COXVa form expressed during normoxia and a COXVb form expressed during anoxia[68]. In kidney epithelial cells, when COX4I2 is ectopically overexpressed, there is an increase in O_2 consumption in cells grown in hypoxia[66]. Conversely, loss of function of COX4I2 in hypoxic cells resulted in reduced O_2 consumption[66]. This regulated O_2 consumption (as well as other findings) was interpreted to indicate more efficient use of the remaining O_2 by COX4I2 in hypoxia.

A metabolic tumour growth advantage?

Warburg made his seminal observations that tumour cells had higher glycolysis and lower oxidative phosphorylation than normal cells, but he was not able to explain how this metabolic shift translated into a growth

advantage for tumour cells *in vivo*[69]. There has been speculation that the increased glycolysis drives the phenotypical changes seen in the tumour cells. However, increased glycolysis is necessary in cells with reduced mitochondrial function because these are the only mechanisms for energy production in the cell. Perhaps, therefore, it is the reduced mitochondrial function that is responsible for the glycolytic increase and not *vice versa*. The reduced mitochondrial function could be responsible for the tumour cell growth advantage. Below I discuss two of the more established hypotheses that have been proposed to explain how downregulating mitochondrial function could be a benefit to tumour cells, and offer a third hypothesis. These three models are not mutually exclusive and so they might all contribute to the aggressive tumour phenotype that is a consequence of the metabolic shift.

Decreased mitochondrial function reduces ROS production. Metabolically produced ROS can come from the mitochondria[70] as a by-product of electron transport. Large amounts of ROS can be toxic to cells through their damaging effects on cellular macromolecules[71]. A small fraction of single electrons that are passing through complex 3 of the electron transport chain can 'leak' out from ubisemiquinone cofactors during the Q cycle. These free electrons are captured by molecular oxygen at high affinity to form the superoxide radical O_2^-. Superoxide and its breakdown products have pleiotropic effects on signalling processes within normal and tumour cells. However, the role for ROS in hypoxia-induced death is controversial[72,73]. In support of this toxic ROS concept, groups have shown increased survival in cells exposed to hypoxia that are competent in the enzymatic detoxification of ROS systems (such as superoxide dismutase)[73]. One must take into account production of ROS from non-mitochondrial sources[74] and after re-oxygenation[75]. There is also significant controversy as to the effect of hypoxia on ROS production. Some reports suggest that ROS production can be stimulated by hypoxia[37,39], whereas others have shown no increase[76].

A reduction in mitochondrial function should also reduce the amount of ROS generated as a by-product of respiration[77]. The mechanisms by which HIF1 reduces mitochondrial function have been reported to result in reduced ROS production[11,13,66]. In these studies, the decreased ROS production has been correlated with increased
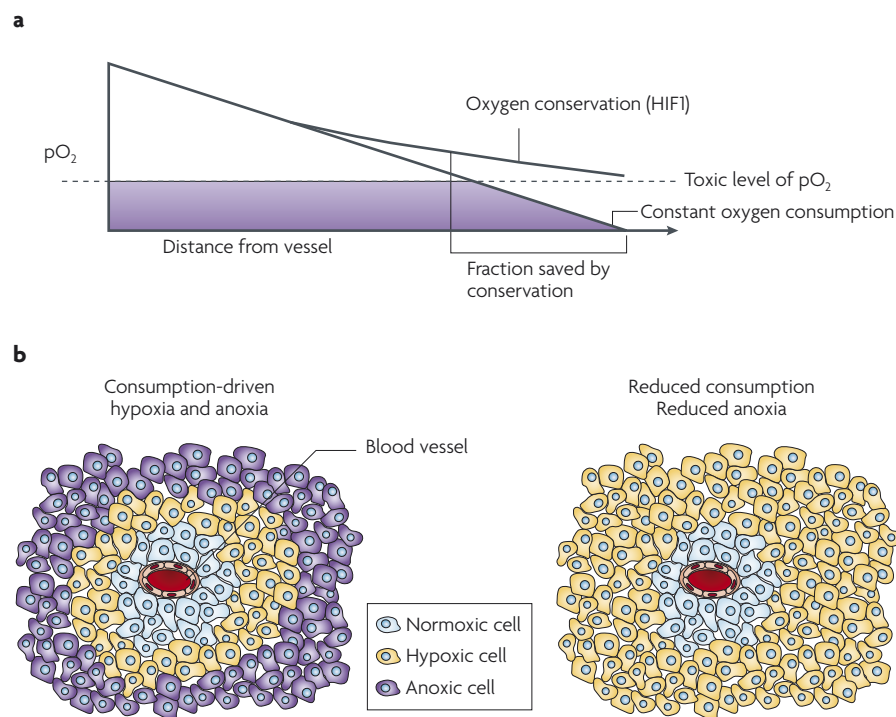
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Figure 3 | **A hypothesis of how the Warburg effect gives tumour cells a growth advantage through reduced mitochondrial function. a** | A graphical representation showing that by slowing the consumption of O_2 in the hypoxic cells, O_2 diffuses further and fewer cells reach anoxic levels that are toxic. Mild hypoxia can support cellular growth. **b** | A cartoon showing the same concept. Because the area of cells in the tumour cord increases as the square of the radius from the blood vessel, there are a great number of cells at risk of O_2-induced arrest or death in the periphery that are saved by O_2 conservation. HIF1, hypoxia-inducible factor 1; pO_2, O_2 tension.

survival *in vitro*. Some groups[11,13,66] but not others[72,78,79] have observed increased survival for HIF1-competent cells compared with HIF1-deficient cells exposed to hypoxia *in vitro*. Part of the difficulty in comparing these studies is the complexity of measuring ROS: there are many different short-lived species that are typically measured indirectly through their actions on reporter molecules such as fluorescein derivatives.

Reduced mitochondrial function yields increased anabolic substrates. One requirement of rapidly dividing tumour cells is that they must duplicate all their constituents every cell division. This biosynthetic process requires the cell to manufacture large amounts of precursors for proteins, nucleic acids and lipids[80]. Tumour cells have been shown to have increased metabolic turnover in normoxia owing to activation of oncogenes such as Akt[81] and *HRAS*[82]. The increased metabolic activity generates several of these precursors as products of the glycolytic pathway (such as ribose for nucleic acid synthesis) or the TCA cycle (such as citrate for lipid synthesis). Increased glucose uptake in the tumour cell and decreased consumption of metabolites for energy in the mitochondria allows for more of the substrates to be used for these alternative purposes.

The HIF-dependent reduction of mitochondrial function should regulate the quantity of TCA cycle intermediates available for lipid biosynthesis. By regulating the flow of pyruvate into the mitochondria (through PDK1 expression) and the turnover of carbons in the TCA cycle (through COX4 subunit determination) HIF1 can regulate the availability of TCA cycle intermediates for lipid synthesis. In *de novo* lipid synthesis citrate is shuttled out of the mitochondria to the cytoplasm where the enzyme ATP citrate lyase generates acetyl-CoA and oxaloacetate[83]. The acetyl-CoA is used for lipid synthesis by acetyl-CoA carboxylase. The proliferative advantage of cells with high ATP citrate lyase activity can be appreciated because inhibition of the enzyme can block tumour cell growth *in vitro*[83].

However, in growth factor-dependent haematopoietic cells HIF1 appears to be able to regulate macromolecular synthesis and proliferation, even in normoxia[47].

By increasing glycolysis and producing lactate instead of citrate for lipid synthesis, HIF1 actually slows the growth of these cells, promoting survival[47]. In glioma cells, however, there is an increased flux of carbons into the TCA cycle through the incorporation of glutamine, which should not be sensitive to HIF1 regulation[84]. In hypoxic tumour cells, PDK1 has reduced the flow of pyruvate into the TCA cycle. However, alteration of the COX4 isoforms has also reduced the flow of carbons used to power the electron transport chain. The net effect of reducing the inflow and outflow could result in excess carbons available for lipid synthesis. This way, even with reduced mitochondrial function, acetyl-CoA can still be generated in hypoxic tumour cells to support proliferation.

Reduced mitochondrial function conserves O_2 for alternative use. It was originally thought that the glycolytic genes were induced in hypoxia because there was not enough O_2 to support oxidative phosphorylation in the mitochondria[85]. However, this hypothesis now appears to be incorrect, and two observations support this contention. First, HIF1α, HIF1 target genes and the corresponding metabolic changes are readily induced in most cells tested at mild hypoxia of 2–3% pO_2, low enough to induce HIF1 activity[14,78] but high enough to support robust oxidative phosphorylation. O_2 has been shown to be limiting as a substrate for oxidative phosphorylation at concentrations below 0.5%[86]. Second, as described above, HIF1 uses several mechanisms that actively downregulate mitochondrial function through PDK1 induction, MXI1 induction and COX4I2 expression. Therefore, HIF1 appears to conserve O_2 as the environmental availability decreases but before it is limiting.

The idea that O_2 availability can be limiting in tumour growth was first recognized over 50 years ago by Thomlinson and Gray[87]. They reported that in human tumours cells were limited in their growth to within about 10 cells of a blood vessel. This distance was defined as the 'diffusion limit' for O_2 to reach the outermost cell. The limit is a function of the O_2 content within the blood vessel and the rate of consumption within the tumour cells as O_2 diffuses out from the vessel (FIG. 3). Decreasing consumption is theoretically the most efficient way to extend this diffusion limit *in vivo*[88]. The cells that are exposed to this O_2 gradient are able to proliferate[89] and are not subjected to anoxia-induced cell death[78,79]. Furthermore, the conserved

O_2 is available for non-energy-producing cellular functions that require O_2. For example, several types of enzymes use O_2 as a substrate either directly, or indirectly. Some of these enzymes are oxidases[90], hydroxylases[91], protein disulphide isomerases[92] and histone demethylases[93]. This non-mitochondrial O_2 consumption is essential for sterol synthesis and oxidative protein folding, and can account for up to 10–30% of total cellular O_2 consumption[94,95].

Therapeutic implications of the model

Whether it is through HIF1 activation or alternative oncogenic changes, or a combination of reasons, tumour cells have increased glycolysis and reduced oxidative phosphorylation. The tumour cells are thought to be 'addicted' to the energy produced by glycolysis whereas the normal cells still get significant energy from the mitochondria. This metabolic shift represents a difference between normal and cancerous tissue, and as such a possible target for anti-tumour therapy. Several groups are devising schemes to metabolically target glycolysis, hypoxia and HIF1 in the solid tumour.

Direct inhibition of glycolysis. To date, most anticancer strategies targeting metabolism have tried to exploit the increased glycolytic activity of the tumour[96–98], rather than the reduced mitochondrial function. Two agents that have advanced to clinical trials are the non-metabolizable competitive inhibitor of glucose 2-deoxyglucose (2-DG) and the putative hexokinase inhibitor lonidamine[99]. *In vitro* studies have shown efficacy of these agents in their ability to kill tumour cells[100,101], especially when the cells were hypoxic[102]. However, single-agent anti-tumour activity of 2-DG in cells *in vivo* is seen at doses several-fold higher than can be tolerated in humans[103]. Unfortunately, even though the tumour has increased rates of glycolysis, there are still tissues that rely on glycolysis for normal function, such as the brain, heart and retina. The dose-limiting toxicity of 2-DG has been reported to be headache in patients[103].

Recent *in vitro* studies suggest that glycolytic inhibitors at sublethal doses may act to sensitize tumour cells to conventional chemotherapy or radiotherapy. Increased glucose metabolism is hypothesized to lead to resistance to conventional DNA-damaging agents. Combining 2-DG with doxorubicin or paclitaxel in xenograft models[104] or with radiotherapy *in vitro*[105,106] yielded additive toxicity. Mechanistic studies in several

cancer cell lines suggest that high levels of glycolysis may protect against apoptotic stimuli[96,107]. Perhaps this effect is not because of the increased glycolysis in these cells, but because of the decreased mitochondrial function that alters the apoptotic signalling through the mitochondria. If this is one mechanism by which altered metabolism is protective against chemotherapeutic death, then pharmacological inhibition of glycolysis may have limited utility in sensitizing tumour cells to chemotherapy or radiation-induced apoptosis. However, it is important to note that — given all the uses for glucose within the cell — the anti-tumour effects of 2-DG might be mediated by non-glycolytic effects[108,109].

HIF1 inhibitors with metabolic effects

Investigators are also attempting to inhibit the HIF1 transcription factor in an effort to block the metabolic effects as well as other physiological pathways that are regulated by HIF1 in the solid tumour (such as angiogenesis)[107,110,111]. HIF1 is an attractive target for anticancer therapy because it should have low activity in normal, well-oxygenated tissues, so side effects caused by inhibition should be minimal. Several agents and natural products have been reported to inhibit HIF1 activity through a number of mechanisms[112], although their efficacy remains to be demonstrated clinically. In addition, the effective inhibition of HIF1 may not completely inhibit glycolysis owing to the basal level of expression of many of the glucose-metabolizing enzymes. Direct glycolytic inhibitors or competitors such as 2-DG should be more effective at inhibiting glycolysis.

Recognizing these caveats concerning the ability to block glycolysis in tumours, our laboratory has focused on exploiting the HIF1 pathway that regulates O_2 consumption in the mitochondria. We showed that the small molecule HIF1 inhibitor echinomycin or the small molecule PDK1 inhibitor dichloroacetate (DCA) can increase O_2 consumption within the xenografted tumour[113]. In tumours with wild-type HIF1, treatment with either echinomycin or DCA was able to increase mitochondrial activity and increase O_2 consumption. Because the supply of O_2 to the tumour is limited, this increase in demand therefore makes the tumour more hypoxic. However, these drugs do not increase hypoxia in tumours grown from cells lacking HIF1, demonstrating a targeted specificity for hypoxia and HIF1. Increasing hypoxia makes cells resistant to radiotherapy or

chemotherapy but sensitizes cells to hypoxic cytotoxins such as tirapazamine. These results are consistent with recent reports that showed hypoxic tumours were more sensitive to tirapazamine-containing therapies in xenografted mouse tumours[114] and in clinical trials[115]. Although it appears that transcription factors like HIF1 can be targeted such that metabolic effects have anticancer application, it is important to note that targeting the downstream genes themselves may be a more controlled and/or specific approach.

Conclusions and future perspectives

Both of Warburg's original observations — increased aerobic glycolysis and decreased mitochondrial function — can therefore be attributed to activation of the HIF1 transcription factor in the tumour. However, this does not exclude a possible role for other oncogenic changes in altering the metabolic profile in the tumour. Perhaps oncogenic changes could contribute to the decreased mitochondrial function, just as they contribute to increased glycolysis. The p53 tumour suppressor can either reduce glycolysis when activated[56] or reduce O_2 consumption when mutated[116].

The glycolytic tumour phenotype has been exploited diagnostically with the fluorodeoxyglucose-uptake positron-emission tomography scan. This functional imaging yields more information than anatomical imaging alone. However, the metabolic changes in the tumour do not seem to be the Achilles heel that they were originally hoped to be. The use of glycolytic inhibitors may not be specific enough to kill the tumour cells without causing side effects in normal tissues that also rely on glycolysis. The first characteristic of a next-generation glucose-directed 'metabolic drug' would be decreased penetration of the blood–brain barrier, as that organ appears to be the most sensitive[103]. Second, a well-defined modulation of metabolic processes must be established for such drugs so that specific combinations could be rationally chosen. Third, inhibition of glycolysis may not be enough to directly kill the tumour cell, so adding combinations of inhibitors directed against other metabolic processes may be more effective. For example, a glycolytic inhibitor would potentiate the activity of a fatty acid synthase inhibitor by decreasing the acetyl-CoA available for lipid synthesis. Alternatively, a non-metabolizable glucose analogue could potentiate the activity of an inhibitor of protein glycosylation[109].

PERSPECTIVES

Comparatively little work has gone into the development of drugs that target mitochondrial function in the tumour. One existing drug that targets this activity is the PDK inhibitor DCA. Genetic knockdown of PDK1 has profound effects on xenografted tumour growth[117], as does pharmacological inhibition[118]. Although the mechanism of its toxicity is not clear, it could be that DCA causes enough of an increase in O_2 consumption that an acute drop in oxygenation results in anoxic cell death. Alternatively, secondary effects, such as increased hypoxia and targeting with hypoxic cytotoxins, could be exploited. Finally, combination strategies with HIF1 inhibitors may be able to achieve the benefits of inhibiting metabolic changes as well as simultaneously inhibiting other important tumour processes such as angiogenesis.

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