MYC overrides HIF to regulate proliferating primary cell metabolism in hypoxia

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# Abstract

# Keywords

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# Introduction

Cellular responses to hypoxia propel many physiologic and pathologic activities from wound healing and angiogenesis to vascular remodeling and fibrosis ([Lee et al., 2019a](#ref-lee2019); [Semenza, 2012](#ref-semenza2012b)). These activities require cells to continue energetically demanding tasks, such as macromolecular biosynthesis and proliferation, despite limited oxygen availability. Since respiration is the most efficient way for cells to produce energy, cell metabolism must adapt to meet energetic needs when oxygen supply is limiting. Understanding how these metabolic adaptations sustain critical cellular processes in hypoxia is fundamentally important to our understanding of human health and disease.

Cells typically respond to hypoxia by shifting energy production away from respiration and toward glycolysis. This response is mediated primarily by stabilization of the hypoxia-inducible transcription factor 1α (HIF-1α). HIF-1α activates the transcription of glucose transporters, glycolytic enzymes, lactate dehydrogenase, and pyruvate dehydrogenase kinase, while decreasing the expression of tricarboxylic acid (TCA) cycle and electron transport chain enzymes ([Lee et al., 2020](#ref-lee2020b); [Semenza, 2012](#ref-semenza2012b)). Although HIF-1α is constitutively expressed, it is hydroxylated by prolyl hydroxylase enzymes (PHDs) in normoxia and targeted for proteasomal degradation. PHDs are the principal oxygen sensors in metazoan cells ([Kaelin and Ratcliffe, 2008](#ref-kaelin2008a)). PHDs are α-ketoglutarate-dependent dioxygenase enzymes that require molecular oxygen for their enzymatic activity. When oxygen tension falls, PHD activity decreases, leading to HIF-1α stabilization and activation of its associated transcriptional program. Overall, this transcriptional program should increase glycolytic capacity and divert glucose-derived pyruvate from oxidative phosphorylation toward lactate fermentation to maintain glycolytic ATP production.

In addition to metabolic changes designed to maintain energy supply, hypoxic cells also reduce energy demands through down-regulation of Na+/K+-ATPase, slowing protein translation, and attenuating cell proliferation ([Hubbi and Semenza, 2015](#ref-hubbi2015); [Wheaton and Chandel, 2011](#ref-wheaton2011)). In particular, HIF-1α decreases cell proliferation by activating cyclin-dependent kinase inhibitor expression, inhibiting cell cycle checkpoint progression ([Gardner et al., 2001](#ref-gardner2001)), and antagonizing pro-proliferative MYC signaling ([Koshiji et al., 2004](#ref-koshiji2004)). Despite these canonical effects of HIF-1α activation, there are many examples where cells continue to proliferate despite hypoxic stress, including cancer cells, stem cells, and lung vascular cells ([Hubbi and Semenza, 2015](#ref-hubbi2015)). How these cells meet the metabolic needs of sustained proliferation in hypoxia and how these adaptations are regulated are an active area of investigation ([Jain et al., 2020](#ref-jain2020); [Lee et al., 2020](#ref-lee2020b); [Oldham et al., 2015](#ref-oldham2015)). Since hypoxia is a prominent feature of cancer biology as tumor growth outstrips blood supply, most detailed metabolic studies of hypoxic cell metabolism have used tumor cell models, yielding important insights into the metabolic pathobiology of cancer ([Garcia-Bermudez et al., 2018](#ref-garcia-bermudez2018); [Jiang et al., 2016](#ref-jiang2016); [Lee et al., 2019b](#ref-lee2019a); [Melendez-Rodriguez et al., 2019](#ref-melendez-rodriguez2019); [Metallo et al., 2011](#ref-metallo2011); [Wise et al., 2011](#ref-wise2011)). For example, stable isotope tracing and metabolic flux analyses identified a critical role for reductive carboxylation of glutamine-derived α-ketoglutarate for lipid biosynthesis in supporting tumor growth ([Gameiro et al., 2013a](#ref-gameiro2013a); [Metallo et al., 2011](#ref-metallo2011); [Scott et al., 2011](#ref-scott2011); [Wise et al., 2011](#ref-wise2011)), and metabolomic studies identified aspartate as a limiting metabolite for cancer cell proliferation under hypoxia ([Garcia-Bermudez et al., 2018](#ref-garcia-bermudez2018)). By contrast, comparatively little is known about metabolic adaptations of primary cells to hypoxia. Indeed, the importance of reductive carboxylation or aspartate biosynthesis remains to be elucidated in these cells. A more complete understanding of primary cell metabolic adaptation to hypoxia would provide an important context for understanding how metabolic reprogramming supports normal cellular responses to hypoxia, how these responses may be (mal)adaptive in a variety of disease contexts, and how the hypoxia metabolic program in primary cells differs from that observed in cancer cells.

To address these questions, we have developed models of bioenergetic carbon flux in human lung fibroblasts (LFs) and pulmonary artery smooth muscle cells (PASMCs) cultured in 21% or 0.5% oxygen. These cells may be exposed to a wide range of oxygen concentrations *in vivo*, continue to proliferate despite hypoxic culture conditions *in vitro*, and play important roles in the pathology of non-cancerous diseases in which tissue hypoxia is a prominent feature. We found that hypoxia fails to increase glycolysis in these primary cells despite robust up-regulation of the HIF-1α transcriptional program. In normoxia, HIF-1α stabilization by the PHD inhibitor molidustat (BAY-85-3934, “BAY”) ([Flamme et al., 2014](#ref-flamme2014)) did increase glycolysis and lactate efflux; however, hypoxia blocked this response. These findings suggested the existence of important hypoxia-dependent regulatory mechanisms that override the metabolic consequences of HIF-1α-dependent up-regulation of glycolytic gene expression in human primary cells. Transcriptomic profiling suggested a critical role for the transcription factor MYC in the adaptive response to hypoxia. Using knockdown and overexpression approaches, we demonstrate that MYC attenuates HIF-driven glycolysis in hypoxia and following BAY treatment.

# Results

## Hypoxia uncouples HIF-dependent glycolytic gene expression from glycolytic metabolic flux

The goal of this study was to characterize hypoxia-induced metabolic changes in proliferating primary LFs and PASMCs. To accomplish this goal, we adopted a metabolic flux analysis (MFA) technique that enabled us to link intracellular metabolic fluxes to cell proliferation rates. MFA fits cell proliferation rate, extracellular flux measurements, and 13C intracellular isotope labeling patterns to a computational model of cell metabolism ([Antoniewicz, 2018](#ref-antoniewicz2018)). This analysis reconstructs comprehensive flux maps that depict the flow of carbon from extracellular substrates, through intracellular metabolic pathways, and into cell biomass and metabolic by-products ([Young, 2014](#ref-young2014)). These models assume that cells are at a metabolic pseudo-steady state over the experimental time course ([Buescher et al., 2015](#ref-buescher2015)). Exponential growth phase is thought to reflect metabolic pseudo-steady state as cells in the culture steadily divide at their maximal condition-specific rate, provided nutrient supply does not become limiting ([Ahn and Antoniewicz, 2011](#ref-ahn2011); [Buescher et al., 2015](#ref-buescher2015)). Thus, we first set out to define experimental conditions to capture exponential growth phase in normoxic and hypoxic cultures.

Cells were seeded and placed into hypoxia for 24 h prior to sample collection to provide adequate time for activation of the hypoxia-dependent transcriptional program (**Fig ??A**). We selected 0.5% oxygen for hypoxia as this level yielded the most reproducible phenotypic differences compared to 21% oxygen culture while being physiologically relevant and above the KM of cytochrome *c* oxidase (electron transport chain complex IV) for oxygen ([Lee et al., 2020](#ref-lee2020b); [Wenger et al., 2015](#ref-wenger2015)). From this starting point, we identified the optimal cell seeding density and time course to capture exponential cell growth (**Fig ??B**). LFs cultured in 0.5% oxygen grew more slowly than LFs cultured in 21% oxygen (**Fig ??C**), but slower growth was not associated with decreased cell viability (**Fig S1A**). As anticipated, hypoxic cells demonstrated robust stabilization of HIF-1α protein associated with up-regulation of downstream targets, such as glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA) (**Figs ??D-H**). These changes persisted for the duration of the experimental time course.

Having identified experimental conditions for exponential growth, we next determined extracellular fluxes of glucose (GLC), lactate (LAC), pyruvate (PYR), and amino acids (**Figs ??I-J**). Flux calculations incorporated changes in cell number, extracellular metabolite concentrations, metabolite degradation rates, and medium evaporation over time ([Murphy and Young, 2013](#ref-murphy2013a)) (**Figure S1**). Interestingly, while we observed a modest increase in glucose uptake, we found that hypoxia actually decreased lactate efflux (**Figs ??I**). This decrease in lactate efflux occurred despite activation of the HIF-1α transcriptional program as reflected by increased expression of GLUT1 and LDHA. To test if more severe hypoxia would augment glycolysis, we culture cells in 0.2% ambient oxygen (**Fig S2**). These results were similar to 0.5% oxygen culture. We observed no change in glucose or lactate fluxes. To test if this unexpected response was unique to LFs, we next studied PASMCs under 0.5% oxygen conditions (**Fig S3**). PASMCs grew faster than LFs, and so samples were collected every 12 h for 48 h for these cells. Again, similar to LFs at 0.5% and 0.2% oxygen, we observed no change in glucose uptake and reduced lactate efflux in PASMCs regardless of HIF-1α stabilization. Together, these data suggest that hypoxia uncouples HIF target gene expression and glycolytic flux in proliferating primary cells.

Given that hypoxia exposure did not increase glycolysis in LFs, we next wanted to determine how these cells responded to HIF-1α stabilization in normoxia. To accomplish this, LFs were treated with the PHD inhibitor molidustat (BAY, 10 μM) using a similar time course as our hypoxia experiments. Cells were treated with BAY for 24 h to activate the HIF transcriptional program prior to sample collection (**Fig ??A**). As with hypoxia, BAY decreased cell growth rate (**Figs ??B-C**) and activated the HIF-1α transcriptional program (**Figs ??D-H**). Compared to hypoxia, BAY treatment resulted in a similar activation of HIF-1α target gene transcription and protein expression. Unlike hypoxia, however, HIF-1α stabilization in normoxia markedly increased glucose uptake and lactate efflux (**Fig ??I**), as expected based on the pattern of glycolytic gene expression that we observed. Interestingly, although hypoxia and BAY treatments resulted in similar increases in HIF-1α, GLUT1, and LDHA, the glycolytic response was markedly different.

## Extracellular fluxes are treatment and cell-type dependent

In addition to glucose and lactate, we also determined the extracellular fluxes of pyruvate and amino acids (**Figs ??J, ??J**). To our knowledge, this is the first comprehensive extracellular flux profiling of key metabolic substrates in these primary cells. In LFs, overall, changes in these fluxes were modest, with hypoxia generally decreasing the fluxes of all measured metabolites. These findings were similar with 0.2% oxygen exposure (**Fig S2J**).

Notably, we observed a significant decrease in glutamine consumption in hypoxic LFs. This finding contrasts with previous studies of cancer cell metabolism demonstrating increased glutamine uptake as a key feature of the metabolic response to hypoxia ([Gameiro et al., 2013b](#ref-gameiro2013); [Metallo et al., 2011](#ref-metallo2011); [Wise et al., 2011](#ref-wise2011)). In these systems, glutamine-derived α-ketoglutarate was reductively carboxylated by isocitrate dehydrogenase enzymes to generate citrate for lipogenesis. In addition, glutamine has been shown to support TCA cycling in hypoxia in a Burkitt lymphoma model ([Le et al., 2012](#ref-le2012)). Unlike LFs, PASMCs did exhibit a trend toward increased glutamine uptake (**Figure S3J**), suggesting a greater reliance on these metabolic pathways in their adaptive response to hypoxia.

In LFs, among all of the measured amino acid fluxes, proline consumption uniquely increased (**Fig ??J**). Hypoxia increases collagen expression in these cells ([Liu et al., 2013](#ref-liu2013a)) and proline constitutes ~ 10% of the total amino acid content of collagens. Together, these data suggest an important contribution of extracellular proline to collagen production in hypoxic LFs as has been observed in other fibroblast cell lineages ([Szoka et al., 2017](#ref-szoka2017)).

In PASMCs, we observed increased consumption of the branched-chain amino acids (BCAAs) leucine and valine as well as arginine (**Figure S3J**), which was not observed in LFs. BCAAs are transaminated by branch chain amino transferase enzymes to branched chain α-keto acids (BCKAs). BCKAs are further metabolized to yield acyl-CoA derivatives for lipogenesis or oxidation ([Crown et al., 2015](#ref-crown2015); [Mann et al., 2021](#ref-mann2021)). Previous studies have shown that hypoxia up-regulates arginase expression in hypoxic PASMCs ([Chen et al., 2009](#ref-chen2009); [Xue et al., 2017](#ref-xue2017)) to support polyamine and proline synthesis required for cell proliferation ([Li et al., 2001](#ref-li2001)). Interestingly, activation of these metabolic pathways in hypoxia was not observed in LFs and suggests distinct metabolic vulnerabilities of these different cell types.

Compared to hypoxia treatment, BAY demonstrated more modest effects on amino acid fluxes generally (**Figure ??J**). In particular, glutamate efflux was not affected by BAY treatment, while it was reduced by hypoxia. Alanine efflux was increased by BAY treatment, but decreased by hypoxia. In addition to the glucose and lactate fluxes noted above, these findings further highlight fundamental differences in the metabolic consequences of HIF activation in normoxia and hypoxia.

## Isotope tracing reveals altered substrate utilization in hypoxia

To investigate intracellular metabolic reprogramming in hypoxic cells, we performed 13C stable isotope tracing with [U-13C6]-glucose, [1,2-13C2]-glucose, and [U-13C5]-glutamine. Isotopic enrichment of downstream metabolites in glycolysis and the TCA cycle were determined by LC-MS (**Figs S3, S4**). Overall, relatively small changes in the patterns of isotope incorporation were observed following hypoxia or BAY treatment. The most substantial differences were observed in pyruvate (PYR), the terminal product of glycolysis, and citrate (CIT), a central metabolic node in TCA and fatty acid metabolism (**Figs ??A-C**). Both hypoxia and BAY treatments decreased incorporation of glucose-derived carbon into pyruvate (**Fig ??A**) (*i.e.,* the unlabeled, or M0, fraction was greater). This suggests a greater contribution from an unlabeled carbon source, such as extracellular pyruvate, lactate, or alanine, than from glucose to the intracellular pyruvate pool following PHD inhibition.

Total citrate labeling from [U-13C6]-glucose was unchanged across the treatment conditions (**Fig ??B**). As expected, we observed decreased M2 and M4 citrate isotopes, consistent with decreased pyruvate dehydrogenase activity in hypoxia. Interestingly, we observed increased M3 and M5 citrate isotopes. Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate after which all three pyruvate carbons are incorporated into citrate by citrate synthase. Thus, this labeling pattern suggests a more prominent contribution of pyruvate carboxylase to sustain TCA cycle anaplerosis despite pyruvate dehydrogenase inhibition following HIF-1α activation. By contrast to glucose labeling, much less citrate was labeled by glutamine with hypoxia or BAY with a more pronounced effect of BAY treatment (**Fig ??C**), suggesting a less important contribution of glutamine to TCA anaplerosis under these conditions. In addition, the overall fraction of M5 citrate resulting from reductive carboxylation of glutamine-derived α-ketoglutarate was low (< 7%) (**Fig ??D**). Although a hypoxia-mediated increase in M5 citrate was observed, the overall fraction was much less than the 10-20% levels previously reported in cancer cells ([Metallo et al., 2011](#ref-metallo2011); [Wise et al., 2011](#ref-wise2011)).

The stable isotope labeling patterns in PASMCs were generally similar to LFs (**Figure S5**). The most notable differences between LF and PASMC labeling were observed in citrate. Compared with LFs, a much lower fraction of total citrate was labeled by glucose in PASMCs. Less activity of pyruvate carboxylase in these cells was suggested by decreased M3 and M5 citrate isotopes after glucose labeling. Interestingly, the M5 citrate fraction in PASMCs was more consistent with previous reports from the cancer literature (**Fig ??D**), consistent with a more important role for glutamine metabolism for biomass synthesis in these cells.

## Glycolytic flux in hypoxia is closely coupled to cell growth rate

The mass isotopomer distribution for a given metabolite is determined by the complex relationship among the rate of isotope incorporation into the metabolic network, the contributions of unlabeled substrates, and fluxes through related pathways. To clarify how these labeling patterns reflect changes in intracellular metabolite fluxes, we next generated metabolic flux models incorporating the extracellular flux measurements and stable isotope tracing data described above. Preliminary labeling time courses indicated that, even after 72 h of labeling, intracellular metabolites did not reach isotopic steady state (**Fig S6**). Thus, we performed isotopically non-stationary metabolic flux analysis as implemented by Isotopomer Network Compartment Analysis (INCA) ([Jazmin and Young, 2013](#ref-jazmin2013); [Murphy and Young, 2013](#ref-murphy2013a); [Young et al., 2014](#ref-young2014a)) (**Figs ??E-F, S7, Tables S1-S3**).

Overall, LF metabolic fluxes were dominated by high rates of glucose uptake and glycolysis ()

proliferating LFs had high rates of glucose uptake and glycolysis. Approximately 10% of cytoplasmic pyruvate enters the TCA cycle with the balance converted to lactate (**Figure S7A**). Consistent with extracellular flux measurements and isotope labeling patterns described above, significant reductions in glycolysis, the TCA cycle, and amino acid metabolism were observed in the metabolic flux models of LFs cultured in hypoxia. A significant increase in pentose phosphate pathway flux was also observed, although the absolute flux through this pathway is low (**Figure ??A**). By contrast, HIF-1 activation by BAY in 21% oxygen increased glycolysis and lactate fermentation by ~50% (**Figure ??B**), but had a similar effect as hypoxia in decreasing serine and glutamine incorporation. Metabolite fluxes in DMSO-treated cells were similar to 21% oxygen controls.

# Discussion

# Materials and Methods

## Data Availability

# Acknowledgements

# Author Contributions

# Disclosures and competing interests

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# Tables

# Figure Legends

# Figures