MYC overrides HIF-1α to regulate proliferating primary cell metabolism in hypoxia

Courtney A. Copeland1,2, Benjamin A. Olenchock1,2, David R. Ziehr1,3,2, Sarah McGarrity1,2,4, Kevin Leahy1,2, Jamey D. Young5, Joseph Loscalzo1,2, and William M. Oldham1,2,‡

1 Department of Medicine, Brigham and Women’s Hospital, Boston, MA 02115, U.S.A.  
2 Department of Medicine, Harvard Medical School, Boston, MA 02115, U.S.A.  
3 Department of Medicine, Massachusetts General Hospital, Boston, MA, 02114 U.S.A.  
4 Center for Systems Biology, School of Health Sciences, University of Iceland, Reykjavik, Iceland  
5 Departments of Chemical & Biomolecular Engineering and Molecular Physiology & Biophysics, Vanderbilt University, Nashville, TN 37240, U.S.A.

‡ Correspondence: [William M. Oldham <woldham@bwh.harvard.edu>](mailto:woldham@bwh.harvard.edu)

# Abstract

Hypoxia requires metabolic adaptations to sustain energetically demanding cellular activities. While the metabolic consequences of hypoxia have been studied extensively in cancer cell models, comparatively little is known about the metabolic response of primary cells to hypoxia. We performed metabolic flux analyses of human lung fibroblasts and pulmonary artery smooth muscle cells proliferating in hypoxia. Unexpectedly, hypoxia decreased glycolysis despite activation of hypoxia-inducible factor 1α (HIF-1α) and increased glycolytic enzyme expression. While HIF-1α activation by prolyl hydroxylase (PHD) inhibition did increase glycolysis in normoxia, this effect was abrogated by hypoxia. Multi-omic profiling revealed distinct molecular responses to hypoxia and PHD inhibition, suggesting a critical role for MYC in modulating HIF-1α responses to hypoxia. Consistent with this hypothesis, MYC knockdown in hypoxia increased glycolysis and MYC overexpression in normoxia decreased glycolysis stimulated by PHD inhibition. These data suggest that MYC signaling in hypoxia uncouples an increase in HIF-dependent glycolytic gene transcription from glycolytic flux.

# Keywords

hypoxia / metabolic flux analysis / hypoxia-inducible factor / prolyl hydroxylase / MYC

# Introduction

Cellular responses to hypoxia propel many physiologic and pathologic processes from wound healing and angiogenesis to vascular remodeling and fibrosis (W. D. Lee et al., 2019; Semenza, 2012). 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 energy demands 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; Semenza, 2012). 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). 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 downstream 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 demand through down-regulation of Na+/K+-ATPase, slowing protein translation, and decreasing cell proliferation (Hubbi and Semenza, 2015; Wheaton and Chandel, 2011). In particular, HIF-1α decreases cell proliferation by activating cyclin-dependent kinase inhibitor expression, inhibiting cell-cycle checkpoint progression (Gardner et al., 2001), and antagonizing pro-proliferative MYC signaling (Koshiji et al., 2004). 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). How these cells meet the metabolic needs of sustained proliferation in hypoxia and how these adaptations are regulated are active areas of investigation (Jain et al., 2020; Lee et al., 2020; Oldham et al., 2015). 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; Jiang et al., 2016; J. W. Lee et al., 2019; Meléndez-Rodríguez et al., 2019; Metallo et al., 2011; Wise et al., 2011). For example, stable isotope tracing and metabolic flux analyses identified a critical role for the reductive carboxylation of glutamine-derived α-ketoglutarate for lipid biosynthesis in supporting tumor growth (Gameiro et al., 2013; Metallo et al., 2011; Scott et al., 2011; Wise et al., 2011), and metabolomic studies identified aspartate as a limiting metabolite for cancer cell proliferation under hypoxia (Garcia-Bermudez et al., 2018). 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 primary cells. A more complete understanding of primary cell metabolic adaptations 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, including pulmonary hypertension and pulmonary fibrosis. 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) did increase glycolysis and lactate efflux; however, hypoxia blocked this response. These findings suggested that important hypoxia-dependent regulatory mechanisms override the metabolic consequences of HIF-1α-dependent glycolytic gene expression. Transcriptomic profiling identified a critical role for the transcription factor MYC in the adaptive response to hypoxia. Using knockdown and overexpression approaches, we demonstrated that MYC attenuates HIF-driven glycolysis in hypoxia and following HIF stabilization in normoxia.

# 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 used metabolic flux analysis to model how cell metabolism supports cell proliferation. Metabolic flux analysis fits cell proliferation rate, extracellular flux measurements, and 13C isotope labeling patterns to a computational model of cell metabolism (Antoniewicz, 2018). 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). These models assume that cells are at a metabolic pseudo-steady state over the experimental time course (Buescher et al., 2015). Exponential growth phase is thought to reflect metabolic pseudo-steady state as cells in culture steadily divide at their maximal condition-specific rate, provided nutrient supply does not become limiting (Ahn and Antoniewicz, 2011; Buescher et al., 2015). 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 (**Figure 1A**). We selected 0.5% oxygen for hypoxia as this level yielded the most reproducible phenotypic differences compared to 21% oxygen, while being physiologically relevant and above the KM of cytochrome *c* oxidase (electron transport chain complex IV) for oxygen (Lee et al., 2020; Wenger et al., 2015). From this starting point, we identified the optimal cell seeding density and time course to capture exponential cell growth (**Figure 1B**). Hypoxia decreased cell proliferation rates (**Figure 1C**), but slower growth was not associated with decreased cell viability (**Figure 1 - figure supplement 1A**). As anticipated, hypoxic cells demonstrated robust stabilization of HIF-1α associated with up-regulation of its downstream targets, glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA) (**Figure 1D-H**). These changes persisted for the duration of the experimental time course.

We next determined the extracellular fluxes of glucose (GLC), lactate (LAC), pyruvate (PYR), and amino acids (**Figure 1I-J**). Flux calculations incorporated cell growth rate, extracellular metabolite concentrations, metabolite degradation rates, and medium evaporation rate (Murphy and Young, 2013) (**Figure 1 - figure supplement 1B-C**). Interestingly, while we observed a modest increase in glucose uptake, we found that hypoxia actually decreased lactate efflux (**Figure 1I**). This finding was confirmed by measuring the rate of [U-13C3]-lactate produced from LFs cultured with [U-13C6]-glucose (**Figure 1 - figure supplement 2**). Hypoxia decreased lactate efflux despite activating HIF-1α and increasing glycolytic enzymes expression (**Figure 1D-H**).

To test if more severe hypoxia would augment glycolysis, we cultured cells in 0.2% ambient oxygen (**Figure 1 - figure supplement 3**). Under these conditions, we observed no change in glucose or lactate fluxes, similar to 0.5% oxygen culture. To test if this unexpected response was unique to LFs, we studied PASMCs under 0.5% oxygen conditions (**Figure 1 - figure supplement 4**). Similar to LFs, we observed no change in glucose uptake and reduced lactate efflux in PASMCs. Together, these data suggest that hypoxia uncouples HIF-1α target gene expression and glycolytic flux in proliferating primary cells.

Since hypoxia did not increase glycolysis in LFs, we wanted to determine how these cells responded to HIF-1α stabilization in normoxia. To activate HIF-1α, LFs were treated with the PHD inhibitor molidustat (BAY, 10 μM) using a similar time course as our hypoxia experiments (**Figure 2**). Like hypoxia, BAY decreased cell growth rate (**Figure 2B-C**) and activated the HIF-1α transcriptional program (**Figure 2D-H**). Unlike hypoxia, HIF-1α stabilization in normoxia markedly increased glucose uptake and lactate efflux (**Figure 2I**). Although hypoxia and BAY treatments increased in HIF-1α, GLUT1, and LDHA, to a similar degree, the glycolytic response differed markedly between these treatments.

## 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 (**Figure 1J, Figure 1 - figure supplement 3J, Figure 1 - figure supplement 4J, Figure 2J**). To our knowledge, this is the first comprehensive extracellular flux profiling of key metabolic substrates in primary cells. In LFs, overall, changes in extracellular fluxes were modest, with hypoxia generally decreasing the fluxes of all measured metabolites. These findings were similar with 0.2% oxygen exposure (**Figure 1 - figure supplement 3J**).

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., 2013; Metallo et al., 2011; Wise et al., 2011). 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). Unlike LFs, PASMCs did exhibit a trend toward increased glutamine uptake (**Figure 1 - figure supplement 4J**), 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 @ref(fig:m1)J**). Hypoxia increases collagen expression in these cells (Liu et al., 2013) 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).

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; Mann et al., 2021). Previous studies have shown that hypoxia up-regulates arginase expression in hypoxic PASMCs (Chen et al., 2009; Xue et al., 2017) to support polyamine and proline synthesis required for cell proliferation (Li et al., 2001). 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 @ref(fig:m2)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-1α activation in normoxia and hypoxia.

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# Author contributions

W.M.O. conceived and designed the analysis. C.A.C., B.A.O., D.R.Z., S.M., K.L., and W.M.O. collected the data. J.D.Y. and W.M.O. contributed data or analysis tools. W.M.O. performed the analysis. W.M.O. drafted the manuscript. All authors participated in interpreting the results and revising the manuscript. All authors approve the final submission.

# Conflict of interest

The authors declare that they have no conflicts of interest.

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# Figures

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| **Figure 1 -** |

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