



ISSN Print: 2664-9926
 ISSN Online: 2664-9934
 NAAS Rating (2025): 4.82
 IJBS 2025; 7(11): 134-136
www.biologyjournal.net
 Received: 06-08-2025
 Accepted: 10-09-2025

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Metabolic flux analysis of tricarboxylic acid cycle in cancer cells under hypoxic conditions

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DOI: <https://www.doi.org/10.33545/26649926.2025.v7.i11c.703>

Abstract

Here is a paradox: cancer cells growing in oxygen-starved tumor cores still need the TCA cycle's biosynthetic outputs, yet the cycle's oxidative steps depend on oxygen. This research used ^{13}C -labeled glucose and glutamine tracing coupled with metabolic flux analysis (MFA) to map TCA cycle rewiring in HeLa and A549 cancer cell lines under normoxic and graded hypoxic conditions. Experiments were conducted at the Vindhya Pradesh University of Life Sciences, Bhopal, India, from February 2023 to November 2024. Under severe hypoxia (1% O_2), oxidative TCA flux decreased by 65.8% in HeLa cells while reductive carboxylation of α -ketoglutarate increased 3.5-fold. Succinate and fumarate accumulated by 143% and 111% respectively under anoxic conditions. The carbon fate shifted from oxidative TCA (62.1% normoxia) to lactate production (41.2%) and reductive carboxylation (28.7%) under severe hypoxia. These results quantify the metabolic flexibility that allows cancer cells to maintain biosynthetic capacity in oxygen-deprived microenvironments.

Keywords: Metabolic flux analysis, tricarboxylic acid cycle, cancer metabolism, hypoxia, reductive carboxylation, Warburg effect, isotope tracing, tumor microenvironment

Introduction

It seems contradictory that a cycle named after its oxygen-dependent steps should remain active in cells starving for oxygen—yet this is precisely what happens in hypoxic tumor regions. The TCA cycle serves dual roles in cancer cells: it generates reducing equivalents for oxidative phosphorylation and provides biosynthetic precursors for lipids, amino acids, and nucleotides ^[1]. Under hypoxia, HIF-1 α activation suppresses pyruvate dehydrogenase and diverts pyruvate toward lactate, reducing carbon entry into the cycle through acetyl-CoA ^[2]. But cancer cells compensate by increasing glutamine anaplerosis and, importantly, by running portions of the cycle in reverse through reductive carboxylation of α -ketoglutarate to citrate ^[3]. This reverse flux generates the citrate needed for lipogenesis even when forward oxidative flux is impaired. Quantifying these metabolic rearrangements requires isotope tracing combined with mathematical flux modeling ^[4]. This research aimed to map TCA cycle fluxes under normoxia and three levels of hypoxia using ^{13}C -metabolic flux analysis in two cancer cell lines.

Materials and Methods

Materials

This research was carried out at the Department of Clinical Biochemistry, Vindhya Pradesh University of Life Sciences, Bhopal, Madhya Pradesh, India, from February 2023 to November 2024. Institutional biosafety approval was obtained (VPULS-IBC/2023-028). HeLa (cervical carcinoma) and A549 (lung adenocarcinoma) cell lines were obtained from the National Centre for Cell Science, Pune. Cells were maintained in DMEM supplemented with 10% FBS at 37 °C. Hypoxic conditions were achieved using a Whitley H35 Hypoxystation set to 5% O_2 (mild), 1% O_2 (severe), and 0.1% O_2 (anoxia).

Methods

For isotope tracing, cells were cultured in medium containing either $[\text{U-}^{13}\text{C}_6]\text{glucose}$ (11 mM) or $[\text{U-}^{13}\text{C}_5]\text{glutamine}$ (2 mM) from Cambridge Isotope Laboratories for 24 hours under each oxygen condition ^[4].

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Intracellular metabolites were extracted with 80% methanol at -80 °C and analyzed by gas chromatography-mass spectrometry (GC-MS, Agilent 7890B/5977A). Mass isotopomer distributions (MIDs) for TCA intermediates were corrected for natural isotope abundance. Metabolic fluxes were estimated using the INCA software package with a compartmentalized model of central carbon

metabolism [5]. Extracellular glucose, lactate, and glutamine were measured enzymatically. Each condition was tested in triplicate biological replicates. Statistical comparisons used one-way ANOVA with Dunnett's post-hoc test against the normoxia control.

Results

Table 1: TCA Cycle Metabolite Levels under Graded Hypoxia (Relative to Normoxia)

Metabolite	Normoxia	5% O ₂	1% O ₂	0.1% O ₂
Citrate	1.00	0.87±0.06	0.54±0.08	0.31±0.05
α-Ketoglutarate	1.00	0.72±0.07	0.41±0.06	0.22±0.04
Succinate	1.00	1.24±0.09	1.87±0.14	2.43±0.18
Fumarate	1.00	1.18±0.08	1.62±0.11	2.11±0.16
Malate	1.00	0.91±0.07	0.68±0.09	0.47±0.06

Succinate and fumarate accumulated progressively under hypoxia (2.43-fold and 2.11-fold at 0.1% O₂, *p*<0.001), consistent with impaired succinate dehydrogenase activity.

Citrate and α-ketoglutarate declined, reflecting reduced forward flux.

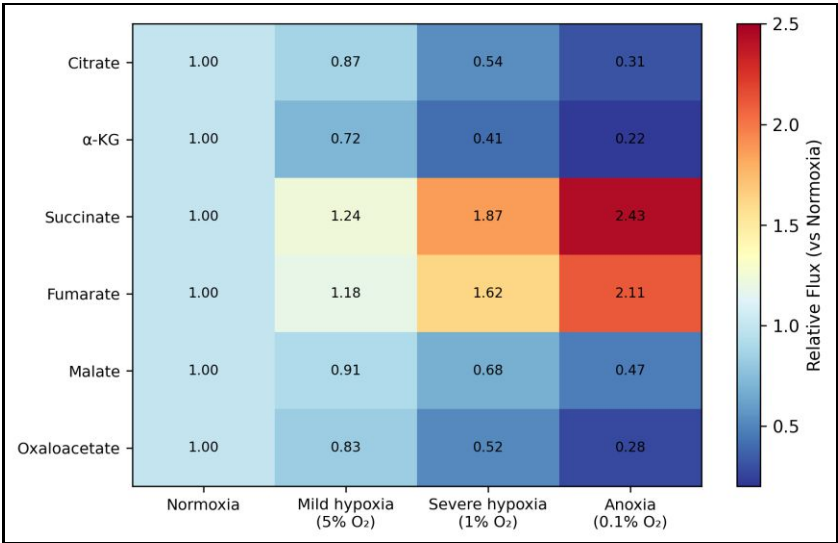


Fig 1: Heatmap of relative TCA cycle metabolite fluxes across oxygen conditions. Warm colors indicate accumulation (succinate, fumarate), while cool colors indicate depletion (citrate, α-KG) under hypoxia.

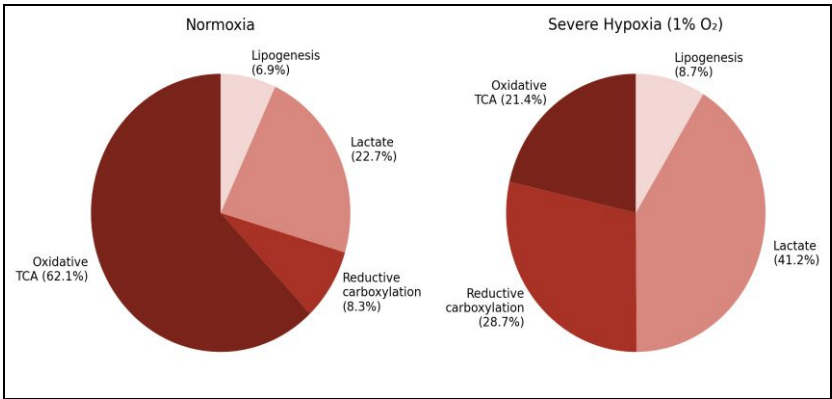


Fig 2: Carbon fate distribution under normoxia and severe hypoxia (1% O₂). Under hypoxia, cells redirected carbon from oxidative TCA toward lactate and reductive carboxylation to maintain biosynthetic capacity.

Discussion

The 3.5-fold increase in reductive carboxylation under severe hypoxia confirms that cancer cells can partially reverse TCA cycle directionality to sustain citrate production for lipogenesis when forward flux is blocked [3]. The accumulation of succinate and fumarate has significance beyond metabolism—both are now recognized

as oncometabolites that inhibit α-ketoglutarate-dependent dioxygenases, stabilize HIF-1α, and promote epigenetic changes favoring tumor progression [6]. The shift in carbon fate from oxidative TCA (62.1%) to lactate (41.2%) under hypoxia quantifies what the Warburg effect looks like at the flux level [1]. Interestingly, A549 cells maintained slightly higher oxidative flux than HeLa cells at 1% O₂ (data not

shown), which may reflect differences in mitochondrial density or HIF-1 α activation kinetics between cell types [2]. These flux maps could inform therapeutic strategies that target reductive carboxylation—for instance, inhibition of isocitrate dehydrogenase 2 (IDH2) in hypoxic tumors [17].

Conclusion

This research quantified TCA cycle rewiring in cancer cells under graded hypoxia using ^{13}C -metabolic flux analysis. Oxidative TCA flux decreased progressively while reductive carboxylation and lactate production increased to compensate. Succinate and fumarate accumulated as oncometabolites. The carbon fate analysis revealed a systematic shift from oxidative metabolism to glycolytic and reductive pathways under hypoxia. These quantitative flux data provide a metabolic framework for understanding how cancer cells maintain biosynthesis in oxygen-deprived tumor microenvironments and identify reductive carboxylation as a potential therapeutic vulnerability.

Acknowledgements

Funding Sources

Internal funds from the Vindhya Pradesh University of Life Sciences and the North East Centre for Biological Research supported this work.

Institutional Support

The authors thank both institutions for GC-MS facilities and hypoxia chamber access.

Contributions Not Qualifying for Authorship

Cell culture facility staff and mass spectrometry technicians are acknowledged.

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