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Article

Oxidation of Alpha-Ketoglutarate Is Required for Reductive Carboxylation in Cancer Cells with Mitochondrial Defects

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SUMMARY

Mammalian cells generate citrate by decarboxylating pyruvate in the mitochondria to supply the tricarboxylic acid (TCA) cycle. In contrast, hypoxia and other impairments of mitochondrial function induce an alternative pathway that produces citrate by reductively carboxylating α-ketoglutarate (AKG) via NADPH-dependent isocitrate dehydrogenase (IDH). It is unknown how cells generate reducing equivalents necessary to supply reductive carboxylation in the setting of mitochondrial impairment. Here, we identified shared metabolic features in cells using reductive carboxylation. Paradoxically, reductive carboxylation was accompanied by concomitant AKG oxidation in the TCA cycle. Inhibiting AKG oxidation decreased reducing equivalent availability and suppressed reductive carboxylation. Interrupting transfer of reducing equivalents from NADH to NADPH by nicotinamide nucleotide transhydrogenase increased NADH abundance and decreased NADPH abundance while suppressing reductive carboxylation. The data demonstrate that reductive carboxylation requires bidirectional AKG metabolism along oxidative and reductive pathways, with the oxidative pathway producing reducing equivalents used to operate IDH in reverse.

INTRODUCTION

Proliferating cells support their growth by converting abundant extracellular nutrients like glucose and glutamine into precursors for macromolecular biosynthesis. A continuous supply of metabolic intermediates from the tricarboxylic acid (TCA) cycle is essential for cell growth, because many of these intermediates feed biosynthetic pathways to produce lipids, proteins, and nucleic acids (Deberardinis et al., 2008). This underscores the dual roles of the TCA cycle for cell growth: it generates reducing equivalents for oxidative phosphorylation by the electron transport chain (ETC) while also serving as a hub for precursor production. During rapid growth, the TCA cycle is characterized by large influxes of carbon at positions other than acetyl-coenzyme A (acetyl-CoA), enabling the cycle to remain full even as intermediates are withdrawn for biosynthesis. Cultured cancer cells usually display persistence of TCA cycle activity despite robust aerobic glycolysis and often require mitochondrial catabolism of glutamine to the TCA cycle intermediate α-ketoglutarate (AKG) to maintain rapid rates of proliferation (Icard et al., 2012; Hiller and Metallo, 2013).

Some cancer cells contain severe, fixed defects in oxidative metabolism caused by mutations in the TCA cycle or the ETC. These include mutations in fumarate hydratase (FH) in renal cell carcinoma and components of the succinate dehydrogenase (SDH) complex in pheochromocytoma, paraganglioma, and gastrointestinal stromal tumors (Tomlinson et al., 2002; Astuti et al., 2001; Baysal et al., 2000; Killian et al., 2013; Niemann and Müller, 2000). All of these mutations alter oxidative metabolism of glutamine in the TCA cycle. Recently, analysis of cells containing mutations in FH or ETC complexes I or III or exposed to the ETC inhibitors metformin and rotenone or the ATP synthase inhibitor oligomycin revealed that turnover of TCA cycle intermediates was maintained in all cases (Mullen et al., 2012). However, the cycle operated in an unusual fashion characterized by conversion of glutamine-derived AKG to isocitrate through a reductive carboxylation reaction catalyzed by $NADP^+$/NADPH-dependent isoforms of isocitrate dehydrogenase (IDH). As a result, a large fraction of the citrate pool carried

five glutamine-derived carbons. Citrate could be cleaved to produce acetyl-CoA to supply fatty acid biosynthesis and oxaloacetate (OAA) to supply pools of other TCA cycle intermediates. Thus, reductive carboxylation enables biosynthesis by enabling cells with impaired mitochondrial metabolism to maintain pools of biosynthetic precursors that would normally be supplied by oxidative metabolism. Reductive carboxylation is also induced by hypoxia and by pseudohypoxic states caused by mutations in the *von Hippel-Lindau* (*VHL*) tumor-suppressor gene (Metallo et al., 2012; Wise et al., 2011).

Interest in reductive carboxylation stems in part from the possibility that inhibiting the pathway might induce selective growth suppression in tumor cells subjected to hypoxia or containing mutations that prevent them from engaging in maximal oxidative metabolism. Hence, several recent studies have sought to understand the mechanisms by which this pathway operates. In vitro studies of IDH1 indicate that a high ratio of NADPH/$NADP^+$ and low citrate concentration activate the reductive carboxylation reaction (Leonardi et al., 2012). This is supported by data demonstrating that reductive carboxylation in *VHL*-deficient renal carcinoma cells is associated with a low concentration of citrate and a reduced ratio of citrate:AKG, suggesting that mass action can be a driving force to determine IDH directionality (Gameiro et al., 2013b). Moreover, interrupting the supply of mitochondrial NADPH by silencing nicotinamide nucleotide transhydrogenase (NNT) suppresses reductive carboxylation (Gameiro et al., 2013a). This mitochondrial transmembrane protein catalyzes the transfer of a hydride ion from NADH to $NADP^+$ to generate NAD^+ and NADPH. Together, these observations suggest that reductive carboxylation is modulated in part through the mitochondrial redox state and the balance of substrate/products.

Here, we used metabolomics and stable isotope tracing to better understand overall metabolic states associated with reductive carboxylation in cells with defective mitochondrial metabolism and to identify sources of mitochondrial reducing equivalents necessary to induce the reaction. We identified high levels of succinate in some cells using reductive carboxylation and determined that most of this succinate was formed through persistent oxidative metabolism of AKG. Silencing this oxidative flux by depleting the mitochondrial enzyme AKG dehydrogenase substantially altered the cellular redox state and suppressed reductive carboxylation. The data demonstrate that bidirectional/branched AKG metabolism occurs during reductive carboxylation in cells with mitochondrial defects, with oxidative metabolism producing reducing equivalents to supply reductive metabolism.

RESULTS

Shared Metabolomic Features among Cell Lines with *cytb* or *FH* Mutations

To identify conserved metabolic features associated with reductive carboxylation in cells harboring defective mitochondrial metabolism, we analyzed metabolite abundance in isogenic pairs of cell lines in which one member displayed substantial reductive carboxylation and the other did not. We used a pair of previously described cybrids derived from 143B osteosarcoma cells in which one cell line contained wild-type mitochondrial DNA (143B*wt*) and the other contained a mutation in the *cytb* gene (143B*cytb*), severely reducing complex III function (Rana et al., 2000; Weinberg et al., 2010). The 143B*wt* cells primarily use oxidative metabolism to supply the citrate pool, while the 143B*cytb* cells use reductive carboxylation (Mullen et al., 2012). The other pair, derived from FH-deficient UOK262 renal carcinoma cells, contained either an empty vector control (UOK262EV) or a stably re-expressed wild-type *FH* allele (UOK262FH). Metabolites were extracted from all four cell lines and analyzed by triple-quadrupole mass spectrometry. We first performed a quantitative analysis to determine the abundance of AKG and citrate in the four cell lines. Both 143B*cytb* and UOK262EV cells had less citrate, more AKG, and lower citrate:AKG ratios than their oxidative partners (Figures S1A–S1C), consistent with findings from *VHL*-deficient renal carcinoma cells (Gameiro et al., 2013b).

Next, to identify other perturbations, we profiled the relative abundance of more than 90 metabolites from glycolysis, the pentose phosphate pathway, one-carbon/nucleotide metabolism, the TCA cycle, amino acid degradation, and other pathways (Tables S1 and S2). Each metabolite was normalized to protein content, and relative abundance was determined between cell lines from each pair. Hierarchical clustering (Figure 1A) and principal component analysis (Figure 1B) revealed far greater metabolomic similarities between the members of each pair than between the two cell lines using reductive carboxylation. Only three metabolites displayed highly significant ($p < 0.005$) differences in abundance between the two members of both pairs, and in all three cases, the direction of the difference (i.e., higher or lower) was shared in the two cell lines using reductive carboxylation. Proline, a nonessential amino acid derived from glutamine in an NADPH-dependent biosynthetic pathway, was depleted in 143B*cytb* and UOK262EV cells (Figure 1C). 2-hydroxyglutarate (2HG), the reduced form of AKG, was elevated in 143B*cytb* and UOK262EV cells (Figure 1D), and further analysis revealed that while both the L- and D- enantiomers of this metabolite were increased, L-2HG was quantitatively the predominant enantiomer (Figure S1D). It is likely that 2HG accumulation was related to the reduced redox ratio associated with *cytb* and *FH* mutations. Although the sources of 2HG are still under investigation, promiscuous activity of the TCA cycle enzyme malate dehydrogenase produces L-2HG in an NADH-dependent manner (Rzem et al., 2007). Both enantiomers are oxidized to AKG by dehydrogenases (L-2HG dehydrogenase and D-2HG dehydrogenase). It is therefore likely that elevated 2-HG is a consequence of a reduced NAD^+/NADH ratio. Consistent with this model, inborn errors of the ETC result in 2-HG accumulation (Reinecke et al., 2012). Exposure to hypoxia (<1% O_2) has also been demonstrated to reduce the cellular NAD^+/NADH ratio (Santidrian et al., 2013) and to favor modest 2HG accumulation in cultured cells (Wise et al., 2011), although these levels were below those noted in gliomas expressing 2HG-producing mutant alleles of isocitrate dehydrogenase 1 or 2 (Dang et al., 2009).

Finally, the TCA cycle intermediate succinate was markedly elevated in both cell lines (Figure 1E). We tested additional