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Evidence for transketolase-like TKTL1 flux in CHO cells based on parallel labeling experiments and ^{13}C-metabolic flux analysis

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ABSTRACT

The pentose phosphate pathway (PPP) is a fundamental component of cellular metabolism. It provides precursors for the biosynthesis of nucleotides and contributes to the production of reducing power in the form of NADPH. It has been hypothesized that mammalian cells may contain a hidden reaction in PPP catalyzed by transketolase-like protein 1 (TKTL1) that is closely related to the classical transketolase enzyme; however, until now there has been no direct experimental evidence for this reaction. In this work, we have applied state-of-the-art techniques in ^{13}C metabolic flux analysis (^{13}C-MFA) based on parallel labeling experiments and integrated flux fitting to estimate the TKTL1 flux in CHO cells. We identified a set of three parallel labeling experiments with [1-^{13}C]glucose + [4,5,6-^{13}C]glucose, [2-^{13}C]glucose + [4,5,6-^{13}C]glucose, and [3-^{13}C]glucose + [4,5,6-^{13}C]glucose and developed a new method to measure ^{13}C-labeling of fructose 6-phosphate by GC-MS that allows intuitive interpretation of mass isotopomer distributions to determine key fluxes in the model, including glycolysis, oxidative PPP, non-oxidative PPP, and the TKTL1 flux. Using these tracers we detected a significant TKTL1 flux in CHO cells at the stationary phase. The flux results suggest that the main function of oxidative PPP in CHO cells at the stationary phase is to fuel the TKTL1 reaction. Overall, this study demonstrates for the first time that carbon atoms can be lost in the PPP, by means other than the oxidative PPP, and that this loss of carbon atoms is consistent with the hypothesized TKTL1 reaction in mammalian cells.

1. Introduction

The pentose phosphate pathway (PPP) is a fundamental component of cellular metabolism. Together with glycolysis, it is believed to be one of the oldest metabolic pathways with a very ancient evolutionary origin (Keller et al., 2014). The main role of PPP is to generate precursors for synthesis of nucleotides (from ribose 5-phosphate, R5P), aromatic amino acids in microbial systems (from erythrose 4-phosphate, E4P), and reducing equivalents in the form of NADPH for reductive biosynthetic reactions (e.g. fatty acid and amino acid biosynthesis) and to combat oxidative stress. The PPP consists of two branches, an irreversible oxidative branch (oxPPP) and a reversible non-oxidative branch (noxPPP).

Recently, novel hidden reactions were uncovered in *Escherichia coli* in PPP using ^{13}C-tracer experiments (Nakahigashi et al., 2009). It has been hypothesized that mammalian cells may also contain a hidden reaction in PPP catalyzed by transketolase-like protein 1 (TKTL1) (Coy et al., 2005). Transketolase-like protein 1 is an enzyme encoded by the TKTL1 gene that is closely related to the classical transketolase (TKT) gene (Coy et al., 1996). Expression of TKTL1 has been linked to poor prognosis of various cancers (Coy et al., 2005; Grimm et al., 2014; Kayser et al., 2011; Krockenberger et al., 2007; Langbein et al., 2006; Song et al., 2015; Volker et al., 2007), and was found to correlate with high rates of cell proliferation (Hu et al., 2007; Xu et al., 2009). Additionally, gene silencing of TKTL1 by RNAi was shown to strongly inhibit cell growth of cancer cells (Shi et al., 2015; Xu et al., 2009), thus suggesting TKTL1 as a potential target for therapeutic interventions (Coy et al., 2005; Langbein et al., 2008; Volker et al., 2007). TKTL1 is believed to catalyze the reaction that cleaves xylulose 5-phosphate (X5P) to produce glyceraldehyde 3-phosphate (GAP) and acetyl-CoA (AcCoA); however, there is still very little experimental evidence for this reaction in mammalian systems (Meshalkina et al., 2013). In microbial systems, a similar reaction (i.e. phosphoketolase) was recently discovered in cyanobacteria (Xiong et al., 2016) and clostridia (Liu et al., 2012) when grown on xylose as the carbon source.

In this study, we have applied state-of-the-art methods in

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^{13}C-metabolic flux analysis (^{13}C-MFA) based on parallel labeling experiments (Antoniewicz, 2015b; Crown and Antoniewicz, 2013) to provide evidence for the existence of TKTL1 flux in CHO cells. First, we determined that isotopic tracers that are traditionally used for quantifying metabolic fluxes in PPP, including [1,2-^{13}C]glucose and [1-^{13}C]glucose+[U-^{13}C]glucose, are not well suited to estimate the TKTL1 flux. We then applied recently developed techniques for optimal design of labeling experiment (Antoniewicz, 2013; Crown et al., 2012; Crown and Antoniewicz, 2012) to identify novel isotopic tracers that were optimal for quantifying TKTL1 flux with high precision. Moreover, we developed a new method for measuring ^{13}C-labeling of fructose 6-phosphate by GC–MS to improve flux resolution. Using these new tracers and new labeling measurements we detected a significant TKTL1 flux in CHO cells at the stationary phase. This study thus demonstrates for the first time that carbon atoms can be lost in the PPP, by means other than the oxPPP, and that this loss of carbon atoms is consistent with the hypothesized TKTL1 reaction.

2. Materials and methods

2.1. Materials

Culture materials were purchased from Cellgro (Mediatech, Manassas, VA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). [1-^{13}C]Glucose (99.5 atom% ^{13}C), [2-^{13}C]glucose (99.5%), [3-^{13}C]glucose (99.5%), and [4,5,6-^{13}C]glucose (99.8%) were purchased from Cambridge Isotope Laboratories (Andover, MA). Glucose stock solutions were prepared at 250 g/L in phosphate buffer saline (PBS). For experiments involving tracer mixtures, new glucose stock solutions were prepared by mixing the appropriate stock solutions at the desired ratio. The growth medium was Dulbecco's modified Eagle medium (DMEM, Cat. No. 10-013-CV) supplemented with 10% fetal bovine serum (FBS, Cat. No. 35-011-CV) and 1% penicillin-streptomycin solution (PS, Cat No. 30-004-CI).

2.2. Cell culture and parallel labeling experiments

CHO-K1 cells (ATCC Cat. No. CCL-61) were grown as monolayer culture in T-25 flasks (Corning, NY, Cat. No. 430639) in a humidified incubator at 37 °C with 5% CO_2 until stationary phase (day 5 of culture) as described previously (Ahn and Antoniewicz, 2013). On day 5, a bolus of glucose was added to the cultures containing one of the following tracer combinations: a 1:1 mixture of [1-^{13}C]glucose and [4,5,6-^{13}C]glucose; a 1:1 mixture of [2-^{13}C]glucose and [4,5,6-^{13}C]glucose; or a 1:1 mixture of [3-^{13}C]glucose and [4,5,6-^{13}C]glucose. After 9 h incubation with the tracers, cells were harvested and intracellular metabolites were extracted for GC–MS analysis as described previously (Ahn and Antoniewicz, 2011). Previously, we validated that intracellular metabolites in upper metabolism reach isotopic steady state within 3 h after the addition of ^{13}C-glucose tracers under these conditions (Ahn and Antoniewicz, 2011; Ahn and Antoniewicz, 2013).

2.3. Gas chromatography-mass spectrometry analysis

GC–MS analysis was performed using an Agilent 7890A GC equipped with a DB-5ms (30 m × 0.25 mm i.d. × 0.25 μm; Agilent J&W Scientific) capillary column, interfaced with a Waters Quattro Micro GC–MS/MS (Milford, MA) operating under ionization by electron impact at 70 eV and 200 °C ion source temperature. The injection port and interface temperatures were both 250 °C. Helium flow was maintained at 1 mL/min. Mass spectra were recorded in selected ion recording (SIR) mode with 30 ms dwell time. Mass isotopomer distributions were obtained by integration of ion chromatograms (Antoniewicz et al., 2007a), and corrected for natural isotope abundances (Fernandez et al., 1996) using the Metran software (Yoo et al., 2008).

Labeling of intracellular metabolites was determined by GC–MS analysis of *tert*-butyldimethylsilyl (TBDMS) derivatives as described previously (Ahn and Antoniewicz, 2011). Specifically, mass isotopomer distributions of 3-phosphoglycerate (3PG fragment *m/z* 585), phosphoenolpyruvate (PEP fragment *m/z* 453), and dihydroxyacetone phosphate (DHAP fragment *m/z* 484) were quantified. Labeling of glucose in the medium was determined by GC–MS analysis of the di-o-isopropylidene propionate derivative of glucose (Antoniewicz et al., 2011). Specifically, the mass isotopomer distribution of the fragment at *m/z* 301 was quantified, which contains all six carbon atoms glucose (Antoniewicz et al., 2011).

2.4. GC–MS analysis of intracellular fructose 6-phosphate

In this work, we have also developed a new method to measure ^{13}C-labeling of intracellular fructose 6-phosphate (F6P) by GC–MS, after dephosphorylation of F6P (and FBP) with alkaline phosphatase to fructose, followed by methoxylamine (MOX)-TMS derivatization. The protocol for the enzymatic dephosphorylation was a modified version of the method described in (White, 2004): 100 μL of water and 50 μL of glycine buffer (0.1 M of glycine in pH 10.4, 1 mM of Zn acetate, and 1 mM of $MgCl_2$) were added to dried intracellular metabolites. Next, 5 μL (ca. 0.3 IU) of alkaline phosphatase from *E. coli* (Sigma, Cat. No. P4377-100UN) was added and the samples were briefly vortexed. After incubation at 37 °C for 1 h, the samples were dried under nitrogen gas at 37 °C. The dephosphorylated metabolites were then dissolved in 33 μL of 2 wt% methoxylamine hydrochloride in pyridine and incubated at 37 °C for 90 min Next, 67 μL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)+1% chlorotrimethylsilane (TMCS) was added (Thermo Scientific, Cat. No. TS-48915) and the samples were incubated at 60 °C for 30 min After an overnight incubation at room temperature, the derivatized samples were centrifuged and the clear liquid was transferred into GC vials for GC–MS analysis. For GC–MS analysis, 1 μL was injected in splitless mode. The oven temperature was held at 80 °C for 2 min, increased to 280 °C at 7 °C/min and held for 4.43 min The total run

Table 1
Metabolic network model for ^{13}C metabolic flux analysis. For each metabolite carbon atoms are identified using letters to represent successive carbon atoms.

Glycolysis			
v1	Gluc. ext (abcdef)	→	G6P (abcdef)
v2	G6P (abcdef)	↔	F6P (abcdef)
v3	F6P (abcdef)	↔	FBP (abcdef)
v4	FBP (abcdef)	↔	DHAP (cba)+GAP (def)
v5	DHAP (abc)	↔	GAP (abc)
v6	GAP (abc)	↔	3PG (abc)
v7	3PG (abc)	↔	PEP (abc)
v8	PEP (abc)	→	Pyr (abc)
Pentose phosphate pathway			
v9	G6P (abcdef)	→	Ru5P (bcdef)+CO_2 (a)
v10	Ru5P (abcde)	↔	X5P (abcde)
v11	Ru5P (abcde)	↔	R5P (abcde)
v12	X5P (abcde)	↔	EC2 (ab)+GAP (cde)
v13	F6P (abcdef)	↔	EC2 (ab)+E4P (cdef)
v14	S7P (abcdefg)	↔	EC2 (ab)+R5P (cdefg)
v15	F6P (abcdef)	↔	EC3 (abc)+GAP (def)
v16	S7P (abcdefg)	↔	EC3 (abc)+E4P (defg)
TKTL1 reaction			
v17	X5P (abcde)	→	AcCoA (ab)+GAP (cde)