Journal of Magnetic Resonance 284 (2017) 86–93

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

The rate of lactate production from glucose in hearts is not altered by per-deuteration of glucose

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ARTICLE INFO

Article history:
Received 3 August 2017
Revised 13 September 2017
Accepted 14 September 2017
Available online 18 September 2017

Keywords:
Kinetic isotope effect
Heart
Perfusion
Glucose metabolism
NMR
Deuterium

ABSTRACT

This study was designed to determine whether perdeuterated glucose experiences a kinetic isotope effect (KIE) as glucose passes through glycolysis and is further oxidized in the tricarboxylic acid (TCA) cycle. Metabolism of deuterated glucose was investigated in two groups of perfused rat hearts. The control group was supplied with a 1:1 mixture of [U-$^{13}C_6$]glucose and [1,6-$^{13}C_2$]glucose, while the experimental group received [U-$^{13}C_6$,U-$^{2}H_7$]glucose and [1,6-$^{13}C_2$]glucose. Tissue extracts were analyzed by ^{1}H, ^{2}H and proton-decoupled ^{13}C NMR spectroscopy. Extensive ^{2}H-^{13}C scalar coupling plus chemical shift isotope effects were observed in the proton-decoupled ^{13}C NMR spectra of lactate, alanine and glutamate. A small but measureable (~8%) difference in the rate of conversion of [U-$^{13}C_6$]glucose vs. [1,6-$^{13}C_2$]glucose to lactate, likely reflecting rates of C—C bond breakage in the aldolase reaction, but conversion of [U-$^{13}C_6$]glucose versus [U-$^{13}C_6$,U-$^{2}H_7$]glucose to lactate did not differ. This shows that the presence of deuterium in glucose does not alter glycolytic flux. However, there were two distinct effects of deuteration on metabolism of glucose to alanine and oxidation of glucose in the TCA. First, alanine undergoes extensive exchange of methyl deuterons with solvent protons in the alanine amino transferase reaction. Second, there is a substantial kinetic isotope effect in metabolism of [U-$^{13}C_6$,U-$^{2}H_7$]glucose to alanine and glutamate. In the presence of [U-$^{13}C_6$,U-$^{2}H_7$]glucose, alanine and lactate are not in rapid exchange with the same pool of pyruvate. These studies indicate that the appearance of hyperpolarized ^{13}C-lactate from hyperpolarized [U-$^{13}C_6$,U-$^{2}H_7$]glucose is not substantially influenced by a deuterium kinetic isotope effect.

1. Introduction

Stable isotopes are widely used to investigate metabolism. ^{13}C labeling of substrates and analysis of the products by NMR spectroscopy is valuable because of the information encoded in the chemical shift and ^{13}C-^{13}C scalar coupling [1]. Since many applications are restricted by the poor sensitivity of ^{13}C and the low concentration of metabolic products, there is strong interest in hyperpolarization methods which temporarily increase ^{13}C polarization by 10,000-fold or more [2]. Since T_1 decay of ^{13}C polarization limits the duration of data acquisition, incorporation of ^{2}H prolongs the T_1 of ^{13}C in some molecules, enabling a longer period of observation [2]. Metabolism of hyperpolarized [U-$^{2}H_7$, U-$^{13}C_6$]glucose to hyperpolarized [U-$^{13}C_3$]lactate has been described in *Escherichia coli* [3], yeast [4], breast cancer cells [5] and lymphoma [6]. These findings are important because imaging of hyperpolarized lactate derived from glucose could provide a direct measure of glycolysis and thereby offer a fundamentally new method for investigating carbohydrate metabolism in vivo [6]. Conclusions about flux through the glycolytic pathway derived from these experiments are only valid if flux through the pathway is not influenced by ^{2}H in glucose. Some enzymes are known to be sensitive to isotopic ^{1}H to ^{2}H replacement in substrates and display a kinetic isotope effect (KIE). A primary isotope effect refers to an altered rate of reaction when a heavy isotope is in a chemical bond that is broken [3,7], while secondary isotope effects occur when no

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https://doi.org/10.1016/j.jmr.2017.09.007
1090-7807/

chemical bonds are broken but an altered rate is still observed [8–10]. The typical result is to slow the reaction kinetics, although exceptions have been reported [11]. The KIE in the individual reactions of glycolysis have been studied in isolated systems with the conclusion that a KIE during pyruvate enolization and in the triose phosphate isomerase reaction are substantial [12–15]. Little is known, however, about the effects of deuteration on overall flux of glucose through the entire pathway. Some models of complex metabolic systems assume that the KIE at each step are additive [16] implying that even small effects of ^{2}H at each reaction in glycolysis would jeopardize the assumption that propagation of hyperpolarized [U-$^{2}H_7$, U-$^{13}C_6$]glucose through the system is proportional to glycolysis.

The purpose of this study was to compare the relative rates of conversion of [U-$^{13}C_6$]glucose and [U-$^{13}C_6$,U-$^{2}H_7$]glucose to alanine, lactate and acetyl-CoA in a highly oxidative tissue, the isolated rat heart. Metabolism of [1,6-$^{13}C_2$]glucose was used as an internal reference. The design takes advantage of the known effects of ^{2}H on the ^{13}C NMR spectrum [17]. Upon substitution of ^{1}H with ^{2}H, nearby ^{13}C signals are generally shifted upfield in proportion to bond distances and the number of substituted ^{2}H nuclei. Scalar coupling effects were also informative. Upon coupling of a ^{13}C nucleus to ^{2}H (I = 1), a 1:1:1 triplet is observed in the proton-decoupled ^{13}C NMR spectrum. Two ^{2}H nuclei bonded to a single ^{13}C produce a 1:2:3:2:1 quintet, and three covalently bonded ^{2}H nuclei generate a 1:3:6:7:6:3:1 septet in the ^{13}C NMR spectrum. Deconvolution of the ^{2}H-^{13}C couplings and chemical shift effects allowed measurement of complex mixtures of ^{2}H and ^{13}C isotopomers. To assist with the characterization of the resulting mixtures, a reference compound with different degrees of deuteration in lactate and pyruvate was prepared. In spite of the prior evidence for a significant KIE in some reactions of glycolysis and the suggestion that these effects are additive in vivo, deuteration of glucose had no measureable impact on the rate of production of lactate in the functioning heart. These results suggest that rate of production of hyperpolarized [U-$^{13}C_3$]lactate from hyperpolarized [U-$^{2}H_7$, U-$^{13}C_6$]glucose is not influenced by deuteration. In addition to information about metabolism of glucose to lactate, the initial goal of the study, this experiment provided an unexpectedly rich source of information about metabolism of glucose to alanine and glutamate. Production of alanine and glutamate was quite sensitive to the presence of deuterium in upstream glucose. We also observed proton exchange in the methyl group of lactate and alanine, previously described in isolated enzyme preparations [18] and now demonstrated in mammalian tissue.

2. Materials & methods

[1,6-$^{13}C_2$]glucose, [U-$^{13}C_6$]glucose, [3-$^{13}C_1$,3-3-3-$^{2}H_3$]sodium pyruvate and [U-$^{2}H_7$,U-$^{13}C_6$]glucose were purchased from Sigma-Aldrich Isotec (Miamisburg, OH) or Cambridge Isotope Laboratories (Tewksbury, MA) and used without further purification. The [3-$^{13}C_1$,3-3-3-$^{2}H_3$]sodium pyruvate was obtained as mixture of all ^{2}H isotopomers: [3-$^{13}C_1$,3-3-3-$^{2}H_3$],[3-$^{13}C_1$,3-3-$^{2}H_2$], [3-$^{13}C_1$,3-$^{2}H_1$] and [3-$^{13}C_1$]sodium pyruvate.

2.1. Heart perfusions

The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Sprague Dawley rats, 250–300 g, were purchased from Charles River (Cambridge, MA). All animals were fed ad libitum. Isofluorance (1.5–2%) used for general anesthesia. Depth of anesthesia was assessed by observation of the respiratory rate, paw pinch reflex, and palpebral reflex of the animal. Under general anesthesia, hearts were rapidly excised and arrested in ice-cold perfusion medium. Hearts were perfused in the Langendorff mode using a non-recirculating medium at a constant perfusion pressure of 100 cm H_2O. The medium contained 25 mM $NaHCO_3$, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 1.25 mM $CaCl_2$ and was bubbled continuously with a 95/5 mixture of O_2/CO_2 gas to maintain a pH of 7.4. The perfusion medium was maintained at 37 °C using a water bath. Two sets of perfusion conditions were used. Hearts in group 1 were supplied with 5 mM [1,6-$^{13}C_2$] glucose plus 5 mM [U-$^{13}C_6$]glucose (n = 4) while hearts in group 2 were supplied with 5 mM [1,6-$^{13}C_2$]glucose plus 5 mM [U-$^{13}C_6$, U-$^{2}H_7$]glucose (n = 5). In both groups, the perfusate was treated with insulin (1 microunit/ml). Metabolism of [1,6-$^{13}C_2$]glucose to [3-$^{13}C_1$]lactate was assumed constant between the two groups. Hearts were freeze-clamped after 30 min of perfusion. Frozen tissue was extracted using perchloric acid [19,20], then dissolved in 5% D_2O, 0.5 mM DSS-d_3, 1 mM EDTA in H_2O at pH 7 and analyzed by ^{1}H, ^{2}H and ^{13}C NMR spectroscopy.

2.2. ^{1}H, ^{2}H and ^{13}C NMR spectroscopy

The principles and terminology for analysis of the ^{13}C NMR spectra have been described previously [1,20–22]. All high resolution NMR spectra were obtained using an Agilent 600 MHz VNMRS Direct Drive console using a 3 mm broadband probe. We assumed that once ^{2}H was released into intracellular water, it was no longer involved in metabolism. ^{13}C NMR spectra of the extracts were acquired as described previously [18,22–25]. The multiplets in the ^{13}C NMR spectrum were fit using a mixed Gaussian/Lorentzian lineshape in the TOPSPIN 3.5 Software (Bruker, Germany). Relative peak areas of the resonances adjacent to ^{12}C versus the ^{13}C satellites were measured in the ^{1}H NMR spectrum to obtain ^{13}C enrichments in the methyl carbon. The ^{13}C NMR spectra of glutamate were analyzed as described previously [21]. Significant differences were evaluated using GraphPad Prism (La Jolla, CA) using multiple t-tests with a significance level of $P < 0.05$.

2.3. Synthesis and NMR analysis of [3-^{13}C,3,3,3-$^{2}H_3$] lactate

A mixture of [3-$^{13}C_1$,3,3,3-$^{2}H_3$]-, [3-$^{13}C_1$,3,3-$^{2}H_2$]-, [3-$^{13}C_1$,3-$^{2}H_1$]-pyruvate and [3-$^{13}C_1$] pyruvate obtained as a mixture from Sigma Aldrich Isotec (Miamisburg, OH) was partially reduced by dissolving it in H_2O and palladium on carbon. The reaction vessel was attached to a Parr hydrogenator for 4 h to produce a mixture of approximately 50% lactate and pyruvate. The mixture was filtered and freeze-dried. The resulting mixture of was analyzed by ^{1}H, ^{13}C {^{1}H} and ^{2}H NMR spectroscopy. In all cases, the shorthand notation ^{13}C NMR indicates $^{13}C\{^{1}H\}$ NMR spectroscopy.

2.4. ^{2}H and ^{13}C NMR isotopomer analysis of pyruvate and downstream metabolites

These experimental conditions generate five different isotopomers of pyruvate (Fig. 1). Unlabeled pyruvate originates from glycogen (Fig. 1, [A]). Isotopomer [B] arises from the internal standard [1,6-$^{13}C_2$]glucose, and [C] is derived from the [U-$^{13}C_6$]glucose in the control hearts or from metabolism of [U-$^{13}C_6$, U-$^{2}H_7$]glucose to pyruvate followed by replacement of the ^{2}H by ^{1}H. During glycolysis, the deuterated glucose is converted to fructose-1,6-bisphosphate, which is then split into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G-3-P). G-3-P is derived from the 4, 5, 6 carbons of glucose and, therefore, contains two ^{2}H nuclei at the C3 position, while DHAP is derived from the 1, 2, 3 carbons of glucose where only one ^{2}H is retained in the C3 position. DHAP rapidly isomerizes to G-3-P creating a 1:1 mixture between two different deuterated species in [U-$^{13}C_3$]pyruvate with one ^{2}H,