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Separation of extra- and intracellular metabolites using hyperpolarized ^{13}C diffusion weighted MR ☆

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ABSTRACT

This work demonstrates the separation of extra- and intracellular components of glycolytic metabolites with diffusion weighted hyperpolarized ^{13}C magnetic resonance spectroscopy. Using *b*-values of up to 15,000 s mm^{-2}, a multi-exponential signal response was measured for hyperpolarized [1-^{13}C] pyruvate and lactate. By fitting the fast and slow asymptotes of these curves, their extra- and intracellular weighted diffusion coefficients were determined in cells perfused in a MR compatible bioreactor. In addition to measuring intracellular weighted diffusion, extra- and intracellular weighted hyperpolarized ^{13}C metabolites pools are assessed in real-time, including their modulation with inhibition of monocarboxylate transporters. These studies demonstrate the ability to simultaneously assess membrane transport in addition to enzymatic activity with the use of diffusion weighted hyperpolarized ^{13}C MR. This technique could be an indispensible tool to evaluate the impact of microenvironment on the presence, aggressiveness and metastatic potential of a variety of cancers.

1. Introduction

The use of hyperpolarized ^{13}C pyruvate has shown clinical potential in identifying and characterizing tumors by measuring its real-time conversion to hyperpolarized ^{13}C lactate [1]. These measurements are a direct consequence of increased glycolysis, a hallmark of cancer cells [2]. Studies in pre-clinical animal models of cancers have shown increased hyperpolarized ^{13}C lactate production with increasing cancer grade and aggressiveness [3]. Another common feature of aggressive and metastatic cancer cells is an acidic extracellular environment [4], a process that promotes tumor growth and invasion. This acidification is, in part, due to increased tumor lactate production and higher lactate efflux via the upregulated monocarboxylate transporter 4 (MCT4) [5,6], which co-transport lactate and protons out of the cell. Thus, measuring not only the overall production of hyperpolarized ^{13}C lactate, but also its transport may improve the ability to non-invasively identify aggressive tumors with high metastatic potential using hyperpolarized ^{13}C MR.

A prior publication has shown that hyperpolarized ^{13}C pyruvate can be used to differentiate human metastatic renal cell carcinoma (RCC) cells from indolent RCC cells using an *ex vivo* MR-compatible

Abbreviations: ADC, apparent diffusion coefficient; MR, magnetic resonance; MRS, magnetic resonance spectroscopy; DNP, dynamic nuclear polarization; RCC, renal cell carcinoma; DMEM, Dulbecco's Modified Eagle's medium; DIDS, 4,4′-Diiso thiocyanostilbene-2,2′-disulfonic acid; MCT, monocarboxylate transporter; LDH, lactate dehydrogenase; TCA, tricarboxylic acid; L_{intra}, intracellular lactate; L_{extra}, extracellular lactate; L_{total}, total lactate; P_{intra}, intracellular pyruvate; P_{extra}, extracellular pyruvate; P_{total}, total pyruvate; DMSO, dimethyl sulfoxide.

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bioreactor, a cell perfusion system [7]. In that *ex vivo* study, metastatic RCC UOK262 cells rapidly transported hyperpolarized ^{13}C lactate out of the cells during the course of the hyperpolarized ^{13}C MR experiment [7]. Additionally, it was recently shown that the extra- and intracellular hyperpolarized ^{13}C lactate signal can be directly measured in RCC cells in the same bioreactor system, and confirmed that the metastatic RCC cells have higher extracellular lactate pool [8]. By correlating the hyperpolarized lactate signals to the relative expression levels of MCT4 between the indolent and metastatic RCC cells, these studies demonstrated that the localization of hyperpolarized ^{13}C lactate between the extra- and intracellular environment could provide valuable information concerning tumor aggressiveness and metastatic potential. However, these previous *ex vivo* studies relied on the detection of small frequency shifts, which cannot be directly translated to *in vivo* imaging due to challenges in obtaining sufficient spectral resolution.

Diffusion weighted MR has been extensively used to assess the localization of various metabolites, both *in vitro* to study their extra- and intracellular distributions and *in vivo* to characterize tumor tissue microstructure based on water's apparent diffusion coefficient (ADC) [9]. Recently, there have been several publications that have used diffusion weighted MR to measure the diffusion coefficients of hyperpolarized ^{13}C metabolites, in solution [10], in cell suspensions [11] and *in vivo* [12–14]. These studies showed how changes in the ADCs could indicate the extra- and intracellular localization of the hyperpolarized ^{13}C metabolites. Yet, each of these studies used relatively small diffusion weighting gradients, or *b*-values, and thus measured only single diffusion coefficients from mono-exponential signal responses that did not provide extra- and intracellular separation. Proton diffusion studies have shown that using a wide range of *b*-values, with large values upwards of 3000 s mm^{-2}, results in a multi-exponential signal response that is indicative of the various diffusion environments, both *in vitro* in cells [15,16] and *in vivo* [17].

In the studies presented here, large diffusion gradients were used to investigate the extra- and intracellular weighted distribution of hyperpolarized [1-^{13}C] pyruvate and its metabolites in RCC cells perfused in a MR compatible 3D cell culture bioreactor. Using *b*-values up to 15,000 s mm^{-2}, a multi-exponential signal response was measured for the various hyperpolarized ^{13}C metabolites and by fitting the fast and slow asymptotes of these curves, their extra- and intracellular weighted diffusion coefficients were determined. Additionally, the dynamics of these extra- and intracellular weighted hyperpolarized ^{13}C metabolite pools were assessed in real-time, including a demonstration of the effect of inhibiting MCT4 mediated efflux of hyperpolarized ^{13}C lactate. These studies demonstrate the importance of membrane transport, in addition to enzymatic activity, in understanding the metabolic flux of hyperpolarized ^{13}C metabolites. As opposed to previous diffusion weighted studies of hyperpolarized ^{13}C metabolites, both the high *b*-values used in this study and the large difference between the intra- and extracellular diffusion environments, which allow for a more thorough separation of the two signal pools, provide a measurement of the intracellular weighted diffusion coefficients that may be closer to the true intracellular diffusion coefficient. This improved intracellular weighted lactate ADC measured in this work may aid the design and interpretation of future *in vivo* diffusion weighted imaging clinical studies.

2. Methods

2.1. RCC cell line experiments in an NMR compatible bioreactor

Two different renal cell carcinoma (RCC) cell lines were used. UMRC6 cells are representative of localized human clear cell RCC [18], and were a gift from Dr. Bart Grossman (MD Anderson Cancer Center, Houston, TX; obtained January, 2010; authenticated using STR profiling, October 2012). UOK262 cells are derived from a metastasis of the highly aggressive hereditary leiomyomatosis RCC (HLRCC), which is characterized by mutation of the TCA cycle enzyme fumarate hydratase [19]. UOK262 cells were a gift from Dr. W. Marston Linehan (National Cancer Institute, Bethesda, MD; obtained May, 2010; authenticated using STR profiling, October 2012). All cells were grown in Dulbeco's Modified Eagle's Medium (DMEM) with 4.5 g L^{-1} glucose. The cells were passaged serially and were used for assays and magnetic resonance experiments between passages 2 to 10 and at 60–80% confluency.

For bioreactor experiments, cells were electrostatically encapsulated into 3.5% w/v alginate microspheres, as previously described [20,21], and then loaded into an NMR spectrometer compatible bioreactor [7,20]. Cell-free alginate microspheres were created by the same process. Approximately 800 μL of either cell-free microspheres or microspheres with cells were loaded into the bioreactor. Only for the ^{1}H diffusion weighted imaging experiment (see below) the microspheres with and without cells were layered into the bioreactor (Fig. 2b). The bioreactor was perfused with DMEM H-21 media at a flow rate of 2 mL min^{-1} and kept at 37 °C with water-jacketed perfusion lines and 95% air/5% CO_2 with a gas exchanger.

All MR studies were performed on a 14.1 T Varian INOVA spectrometer (600 MHz ^{1}H/150 MHz ^{13}C) microimaging system (Agilent Technologies), equipped with a 10 mm broadband probe and 100 G cm^{-1} gradients. Probe temperature was controlled at 37 °C. ^{31}P spectra were acquired before and after each hyperpolarized study to assess cell viability and measure the number of cells within the bioreactor, as previously described [7]; TR = 3 s, 512 or 1024 averages, 90° flip-angle.

2.2. Diffusion weighted studies of ^{1}H water

A ^{1}H pulsed gradient single spin echo sequence with rectangular 90° and 180° RF pulses was used to measure the extra- and intracellular diffusion coefficients of water: TR = 2.5 s, TE = 26 ms, gradient pulse duration δ = 9 ms, gradient pulse separation Δ = 16 ms and without averaging. The diffusion weighting or *b*-value was arrayed by changing the gradient amplitudes *G* from 0 to 46 G cm^{-1}, resulting in *b*-values 0–15,944 s mm^{-2}. Diffusion gradients were applied in the transverse direction, i.e., G_x or G_y. The flow was stopped for the duration of these scans to eliminate the effects of flow.

The diffusion coefficients of extra- and intracellular weighted water was determined by fitting the first 7 points (i.e., fast decaying asymptote) or last 7 points (i.e., slowly decaying asymptote) of the multi-exponential signal response to the equation [16,22]

$$\ln(S_i/S_0) = -b \cdot D \tag{1}$$

where S_i is the signal at a specific *b*, or diffusion weighting, S_0 is the signal without diffusion weighting and *D* is the diffusion coefficient. The *b*-value is defined as:

$$b = n\gamma^2 G^2 \delta^2 (\Delta - \delta/3) \tag{2}$$

in which γ is the gyromagnetic ratio and *n* is 1 for single spin echo experiments and 2 for double spin echo experiments, used here for ^{1}H and ^{13}C experiments, respectively. Since the system's maximum slew rate was used, the effect of the gradient ramps were negligible on total *b*-value and hence were neglected from these calculations. For the cell-free alginate microspheres, only the fast decaying asymptote was calculated.

For these experiments, a three-way analysis of variants (ANOVA) was used to identify statistical differences between extracellular weighted diffusion coefficients, followed by a Tukey's