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Real Time Measurement of Hyperpolarized Lactate Production and Efflux as a Biomarker of Tumor Aggressiveness in an MR compatible 3D Cell Culture Bioreactor

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Abstract

We have developed a 3D cell/tissue culture bioreactor compatible with hyperpolarized (HP) ¹³C MR and interrogated HP [1-¹³C] lactate production and efflux in human renal cell carcinoma (RCC) cells. This platform is capable of resolving intracellular and extracellular HP lactate pools, allowing the kinetic measurement of lactate production and efflux in the context of cancer aggressiveness and response to therapy. HP ¹³C MR studies were performed on 3 immortalized human renal cell lines: HK2, a normal renal proximal tubule cell line from which a majority of RCCs arise, UMRC6, a cell line derived from a localized RCC and UOK262, an aggressive and metastatic RCC. The intra- (Lac_{in}) and extracellular (Lac_{ex}) HP lactate signals were robustly resolved in dynamic ¹³C spectra of the cell lines due to a very small but reproducible chemical shift difference (0.031 ± 0.0005 ppm). Following HP [1-¹³C] pyruvate delivery, the ratio of HP Lac_{in}/Lac_{ex} was significantly lower for UOK262 cells compared to both UMRC6 as well as the HK2 cells due to a significant (p<0.05) increase in the Lac_{ex} pool size. The Lac_{in}/Lac_{ex} correlated with the MCT4 mRNA expression of the cell lines, and inhibition of MCT4 transport using DIDS resulted in a significant reduction in the HP Lac_{ex} pool size. The extension of these studies to living patient derived RCC tissue slices using HP [1,2-¹³C₂]pyruvate demonstrated a similarly split lactate doublet with a high Lac_{ex} pool fraction; in contrast, only a single NMR resonance is noted for HP [5-¹³C]glutamate consistent with intracellular localization. These studies support the importance of lactate efflux as a biomarker of cancer aggressiveness and metastatic potential, and the utility of the MR compatible 3D cell/tissue culture bioreactor to study not only cellular metabolism but also transport. Additionally, this platform offers a sophisticated way to follow therapeutic interventions and screen novel therapies that target lactate export.

Graphical Abstract

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Intra- and extra- cellular lactate pools were reliably distinguished and validated using a susceptibility matched MR compatible 3D cell culture bioreactor using hyperpolarized (HP) ^{13}C magnetic resonance spectroscopy. The HP lactate (Lac_{in}/Lac_{ex}) ratio correlates with the lactate exporter, MCT4, expression and can be used to discriminate between renal cells of varying aggressiveness (HK-2 – normal renal epithelial cells; UOK262-metastatic renal cell carcinoma (RCC); UMRC6 - aggressive localized RCC). The combination of HP ^{13}C MR with the bioreactor platform can be used to interrogate the crucial role of lactate production and its efflux in cancer aggressiveness and metastases.

Keywords

Hyperpolarized ^{13}C magnetic resonance (HP ^{13}C MR); dynamic nuclear polarization (DNP); pyruvate; lactate; aerobic glycolysis; lactate efflux; renal cell carcinoma (RCC); cancer aggressiveness

Introduction

There is growing evidence that the up-regulation of aerobic glycolysis and lactate production and efflux is an adaptation of cancer cells that aids in survival, growth, and metastasis (1,2). Tumor excretion of lactic acid, combined with poor tumor perfusion, results in an acidic extracellular pH in tumors compared with normal tissue (3). The resulting acidic environment promotes cancer aggressiveness and metastasis by promoting a degradation of the extracellular matrix by proteinases (4,5), increasing angiogenesis through the release of VEGF (6), and inhibiting the immune response to tumor antigens (7). Extracellular acidification also may render tumors chemo-resistant (8). Taken together, these observations suggest that not only increased lactic acid production but also its efflux are important parameters associated with aggressive cancer phenotypes (9,10). Moreover, tumor-specific metabolic shifts, such as increased production and efflux of lactate, can potentially be exploited for cancer therapy with minimal impact on normal tissues (3).

Hyperpolarized ^{13}C MRI is a new molecular imaging technique that allows rapid and noninvasive monitoring of dynamic pathway-specific metabolic and physiologic processes (11). Fast ^{13}C MR after injection of hyperpolarized (HP) $[1-^{13}\text{C}]$ pyruvate has allowed the measurement of increased HP $[1-^{13}\text{C}]$ lactate production in both pre-clinical models of cancer (12) and patients with prostate cancer (13). Increased HP $[1-^{13}\text{C}]$ pyruvate to lactate conversion has been associated with cancer presence (14) and correlated with increasing cancer aggressiveness/pathologic grade (15). Furthermore, reduced HP $[1-^{13}\text{C}]$ lactate early after therapy correlated with therapeutic efficacy (16–19). Current HP ^{13}C MRI approaches

have not delineated intracellular and extracellular lactate pools, although recent diffusion weighted HP ^{13}C MR approaches have shown the potential to interrogate these two compartments (20–22).

An MR compatible 3D cell/tissue culture bioreactor has been engineered and used to provide a platform to serially monitor the metabolism of viable human cells (23) and tissues (24) in a very controlled environment and with excellent spectral resolution. The bioreactor set-up provides a continuous perfusion of nutrients at a well-regulated temperature with adequate oxygen delivery. The use of smaller cell and tissue sample chamber in a 5 mm diameter susceptibility-matched bioreactor design has provided a high mass sensitivity (filling factor) with sufficient B_0 field homogeneity under continuous perfusion conditions for the acquisition of well-resolved serial multinuclear (^{31}P and ^{13}C) NMR spectra from small amounts of living cells (≈ 8 million) and tissues (≈ 60 mg) (25).

The goal of this study was to determine if the MR compatible 3D culture bioreactor could be used with HP ^{13}C MR to monitor both the increased cellular production of lactate and its efflux in renal cell carcinoma, thereby providing a means to test the importance of this process in cancer aggressiveness, metastasis and response to therapy. To accomplish this, HP ^{13}C MR studies were performed on 3 human renal cell lines in the MR compatible 3D cell culture bioreactor: HK-2 renal proximal tubule cell line (from which most renal cell carcinomas arise), UMRC6 derived from a localized renal cell carcinoma (RCC), and UOK262 established from a highly aggressive metastatic RCC. These cell lines are also known to have differential levels of lactate production and efflux (26). The ability of the bioreactor platform to measure the inhibition of lactic acid efflux using a small molecule monocarboxylate transporter (MCT) inhibitor, DIDS, was also investigated. Lactate is exported out of the cell by the monocarboxylate transporters, predominately MCT4 (27). DIDS irreversibly and efficiently binds to MCT transporters (an order of magnitude higher affinity for MCT4 than MCT1) and inhibits lactate efflux and to a lesser degree pyruvate uptake (27,28). The studies described in this manuscript demonstrate that intra- and extracellular pools of lactate can be detected and quantified in dynamic (3 sec temporal resolution) 1D ^{13}C MR spectra after injection of HP $[1-^{13}\text{C}]$ pyruvate and that the HP intracellular/extracellular $[1-^{13}\text{C}]$ lactate ratio differentiates between localized and metastatic human renal cell carcinoma lines. Further, we have shown that the HP intracellular/extracellular lactate fraction correlates with MCT4 mRNA expression, and the inhibition of lactate efflux with DIDS results in significantly decreased extracellular lactate.

Material and Methods

Cell lines

All three cell lines were grown in monolayers in DMEM (Dulbecco's Modified Eagle's medium) with 4.5g/L glucose media with 10% serum. HK-2 cell line (29) was purchased from ATCC (Manassas, Virginia). The other 2 cells lines, UMRC6 and UOK262, were a kind gift from Dr. Bart Grossman (MD Anderson Cancer Center, Houston, TX) and Dr. W. Marston Linehan (National Cancer Institute, Bethesda, MD) respectively. The UMRC6 cells were derived from a localized human clear cell renal cell carcinoma (30). The UOK262 cells

were derived from a metastasis of the highly aggressive hereditary leiomyomatosis renal cell carcinoma (HLRCC).

MR experiments

As described previously (26), trypsinized cells were prepared for bioreactor MR experiments by electrostatic encapsulation into alginate microspheres. A homogenous mixture of 200 – 250 μ L of cell-laden microspheres (Supplementary Figure S1) were maintained at physiological conditions by circulating media at 37°C and supplying 95% air/ 5% CO₂ via a gas exchanger in the 5mm MR compatible bioreactor. 500MHz Varian Inova (Agilent Technologies, Palo Alto, CA) console equipped with a 5mm, triple-tuned, direct-detect, broadband probe was used for the bioreactor studies. A hypersense polarizer (Oxford Instruments, Oxford, UK) was used to polarize 14.2 M [1-¹³C]pyruvate mixed with 15 mM of the trityl radical (GE Health, Menlo Park, CA, USA) and 2.5 mM gadolinium chelate. 16 μ mol HP [1-¹³C]pyruvate was injected into the bioreactor containing the cell-laden microspheres. A 30° flip angle was used to acquire carbon data every 3s for a total of 5 minutes. Cell viability was assessed by phosphorous spectra acquired with 2048 averages, repetition time of 2s and a 90 degree pulse. The **Electronic REference To access In vivo Concentrations (ERETIC)** signal (31) was adapted for the phosphorous channel and used to estimate the amount of β -NTP of cells within the sensitive volume of the RF coil, a more stringent measure of viable cells. The intracellular inorganic phosphate (Pi) peak was resolved from the Pi signal arising from the circulating media for both the UOK262 as well as the UMRC6 cells. The intracellular pH of these cells were calculated using the chemical shift of the Pi peak, the known pH of the media (7.67 ± 0.04 at 37°C), and published methods (32,33). All bioreactor studies were conducted under the continuous perfusion condition of 0.5 ml/min (n=6), except for the flow modulation experiments when the pump was stopped 15 seconds after the completion of the hyperpolarized substrate infusion (n=3). For MCT inhibition studies (n=3), DiDS (4,4'-Diisothiocyanatostilbene-2,2'-Disulfonic Acid, Disodium Salt) was purchased from Molecular Probes (Life Technologies, Foster City, CA) and dissolved in DMSO to a concentration of 1mM. It was added via a filter to the media used to perfuse the cells, after baseline phosphorous and carbon measurements were made.

The rate of lactate accumulation in the media of the UOK262 cells grown in 25mM [3-¹³C]pyruvate in 2D culture with and without DiDS inhibition was quantified by ¹H MR spectroscopy (n=3 each). The media of the UOK262 cells were sampled serially over 8 hours. Fully relaxed ¹H NMR spectra were measured in an 800MHz Bruker DRX spectrometer (Billerica, MA) equipped with a cryo-cooled 5mm triple-axis heteronuclear probe and the J-coupled ¹³C satellite resonance was quantified using ACD/Labs software as described below.

qRT-PCR

mRNA qRT-PCR was performed as described previously (26) on the total RNA extracted from the cells with RNAeasy procedure kit (Qiagen, USA). In brief, reverse transcription using iScript cDNA Synthesis kit (BioRad Laboratories, Hercules, CA, USA) was performed and subsequently the cDNA generated was utilized for PCR in triplicate with

TaqMan chemistry on the ABI 7900HT (Applied Biosystems, Foster City, CA, USA). Assays for MCT4 was procured from Applied Biosystems (Foster City, CA, USA). The mRNA expression was calculated relative to the housekeeping gene β -actin.

Data analysis

The MR data was processed and analyzed using ACD/Labs software (Toronto, Ontario, Canada). The β -NTP peak was quantified in nmols from the ^{31}P spectra by normalizing the integrated peak area against the calibrated ERETIC peak. The ^{13}C data were processed with (5 Hz) broadening and deconvolved with Lorentzian peak fitting. The hyperpolarized lactate data (total area under the curve over time) was then normalized to nmols of β -NTP, and the pyruvate peak area for comparison across experiments and cell lines. All data are represented as mean $\pm$ standard error. A student's t-test was used to assess the difference between groups. Significant ($p < 0.05$) differences between cell lines were denoted by an asterisk (*).

Renal tissue slice bioreactor set-up

Renal tumor tissues were obtained from nephrectomy and sliced into 300 μm thick disks and cultured for 12 hours (34). 4 slices were placed in the 5mm MR compatible bioreactor using a specialized construct to hold the tissue in place, and were well perfused in circulating medium at physiological temperature. NMR data were acquired on a narrow bore 11.7T Varian INOVA equipped with a 5mm broadband probe. ^{31}P spectra were obtained to monitor tissue viability. HP ^{13}C MR was acquired dynamically (10° pulses, 3 s interval for 300 s) following injection of 1mL of 4mM of $[1,2-^{13}\text{C}_2]$ pyruvate to assess its metabolism in the renal carcinoma tissue slices.

Results

Combining Hyperpolarized ^{13}C MRS with a MR Compatible Bioreactor Provides a Measure of the Intra- /Extra- cellular Compartmentalization of Lactate

The custom built 5mm diameter MR compatible bioreactor allowed the metabolic evaluation of an average of 40 ± 7 million cells with good sensitivity (HP lactate S/N ranging from 19 to 216 depending on the cell line) and excellent B_0 field homogeneity (average water line width at half maximum, LWHM, is 11.37 ± 0.69 Hz). These line-widths were routinely obtained by gradient shimming along the Z-axis followed by manual shimming of the X and Y gradients which took approximately 15 minutes. This B_0 field homogeneity was achievable due to the material utilized in the construction of the bioreactor having a similar magnetic susceptibility to that of water, and by ensuring there were no air interfaces (i.e. air bubbles) within the bioreactor. Figure 1A shows a schematic of the 5mm bioreactor that was optimized for maximal homogeneity and minimal cell culture material requirement. The viability of all three cell lines during the course of the hyperpolarized MR studies was demonstrated by ^{31}P NMR spectra acquired before and after each hyperpolarized $[1-^{13}\text{C}]$ pyruvate injection (Figure 1B). The ^{31}P spectra were similar to what was previously published for these cell lines (HK-2, UMRC6, and UOK262) (26), and the β -NTP did not change significantly during the time course of the hyperpolarized studies. ^{31}P NMR also demonstrated similar intracellular pH's of 7.32 ± 0.01 and 7.33 ± 0.04 respectively for

UMRC6, and UOK262 RCC cell lines. Unfortunately, the intracellular Pi peak of the HK-2 cell line could not be spectrally resolved from the media Pi peak, presumably due either to the more alkaline intracellular pH of this cell line or the inherently low intracellular Pi levels due to the non-glycolytic nature of these proximal tubular cells (35).

A splitting of the lactate peak was observed for all three renal cell lines (HK-2, UMRC6, and UOK262) studied. Figure 2A (top) shows an example of the splitting of the hyperpolarized $[1-^{13}\text{C}]$ lactate peak (upfield peak: 183.13 ± 0.006 and downfield peak: 183.15 ± 0.006 ppm) for the UOK262 cells encapsulated in alginate microspheres at 81 seconds (time point of maximum ^{13}C lactate signal) after the injection of hyperpolarized $[1-^{13}\text{C}]$ pyruvate in the MR compatible bioreactor. The lower panel in figure 2A shows the ^{13}C carbonyl NMR signals from co-polarized HP $[1-^{13}\text{C}]$ pyruvate and HP $[1-^{13}\text{C}]$ lactate in perfused alginate microspheres devoid of UOK262 cells. The presence of a single lactate resonance demonstrates that the differences in magnetic field susceptibility of the alginate microsphere microenvironment alone is not responsible for the splitting of the hyperpolarized lactate peak, and suggests that the resonance at 183.15 ppm corresponds to extracellular lactate pool. The assignment of the downfield 183.15 resonance as arising from the extracellular lactate pool was also supported by peak line-width measurements. The LWHM for the upfield lactate resonance (4.3 ± 0.1 Hz) was significantly larger than the downfield resonance (2.5 ± 0.07 Hz), which was similar to the LWHM observed for lactate in the alginate beads without the presence of cells (2.18 ± 0.05 Hz). Based on these findings we initially assigned the up- and down-field lactate resonances as arising from intra- and extra-cellular lactate pools. The difference in chemical shift (3.92 ± 0.06 Hz) of the intra- and extra-cellular lactate resonances was reproducible within the same study as well as between studies. Although the intra- and extra-cellular lactate resonances were not baseline resolved, the resonances were sufficiently separated to be accurately fit to two Lorentzian line shapes (figure 2B).

Several studies were performed to modulate the extra- and intra- cellular lactate pools in order to confirm the chemical shift of the corresponding lactate resonances. These studies included; (a) increasing cell density within the bioreactor in order to increase intracellular lactate, (b) inhibiting the efflux of lactate in order to decrease extracellular lactate, (c) stopping media flow during the hyperpolarized MR study in order to increase extracellular lactate, and (d) quantifying the amount of non-hyperpolarized lactate in the media to correlate with extracellular hyperpolarized lactate measurements. The results of these validation studies are provided below. Although leakage of LDH from a small number of dead cells in the bioreactor is possible, this leakage did not result in a significant media LDH concentration (Supplementary Figure 2) and therefore did not confound the interpretation of the extracellular lactate pool.

In figure 2C, a linear increase in intracellular lactate resonance was demonstrated as UOK262 cell density in the bioreactor was increased. In these studies, the cell density of viable UOK262 cells within the sensitive volume of the MR coil was calculated based on the β -NTP resonance in the ^{31}P spectra using the value of 18.6 ± 1.5 fmols of ATP/cell previously determined for the UOK262 cell line (26).

Figure 3A shows representative ^{13}C NMR spectra of hyperpolarized $[1-^{13}\text{C}]\text{lactate}$ acquired from untreated (top) and DIDS treated UOK262 cells (bottom) taken at the time of maximum lactate production after injection of hyperpolarized pyruvate (dotted line in figure 3B). The corresponding plots of the dynamic (every 3 sec.) intra- and extra- cellular $[1-^{13}\text{C}]\text{lactate}$ spectra before and after DIDS treatment are shown in Figure 3B. There is a clear visual reduction in the extracellular HP lactate peak in the treated spectra and an associated reduction in the area under the curve for extracellular hyperpolarized lactate of the treated versus untreated UOK262 cells. Figure 3C (solid bar) quantitatively shows that there is a $64 \pm 15\%$ increase in the $\text{Lac}_{\text{in}}/\text{Lac}_{\text{ex}}$ pool ratio when lactate efflux was inhibited by DIDS. With DIDS inhibition, the total HP $[1-^{13}\text{C}]\text{lactate}$ pool also decreased by $57 \pm 11\%$ relative to untreated cells due to the inhibition of pyruvate uptake by DIDS inhibition of MCT1. This confounding change in total hyperpolarized $[1-^{13}\text{C}]\text{lactate}$ pool size with DIDS inhibition is eliminated by taking the $\text{Lac}_{\text{in}}/\text{Lac}_{\text{ex}}$ ratio which clearly demonstrates a much larger impact on the extracellular lactate pool consistent with the higher affinity of DIDS for MCT4 as compared to MCT1 (27).

Subsequently, we compared the dynamic ^{13}C NMR spectra of hyperpolarized $[1-^{13}\text{C}]\text{lactate}$ acquired from UOK262 cells using a normal constant media flow rate (0.5ml/min) to those acquired when flow was stopped 15 seconds after the infusion of hyperpolarized $[1-^{13}\text{C}]\text{pyruvate}$. 15 seconds was the time required for the bolus of pyruvate to arrive in the cellular compartment of the bioreactor. Figure 3C shows that the $\text{Lac}_{\text{in}}/\text{Lac}_{\text{ex}}$ peak area ratio was reduced by $34 \pm 6\%$ ($p=0.055$) under stop flow conditions relative to the constant media flow condition. The decrease in the $\text{Lac}_{\text{in}}/\text{Lac}_{\text{ex}}$ ratio was due to an increase in the extracellular lactate peak area ($164 \pm 74\%$ of control) since it was no longer flowing out of the sensitive volume of the NMR coil, while the intracellular lactate peak remained relatively constant ($102 \pm 46\%$ of control). Under stop-flow conditions, the total HP $[1-^{13}\text{C}]\text{lactate}$ pool increased by $29 \pm 58\%$ relative to the same cells under normal flow conditions, presumably due to the increased time that the $[1-^{13}\text{C}]\text{pyruvate}$ bolus was exposed to the cells leading to increased uptake and conversion to lactate. This confounding increase in total hyperpolarized $[1-^{13}\text{C}]\text{lactate}$ pool size was eliminated by using the $\text{Lac}_{\text{in}}/\text{Lac}_{\text{ex}}$ ratio.

Stopping the media flow did not have an impact on the viability of the UOK262 cells as determined by stable $\beta\text{-NTP}$ peak area in ^{31}P spectra acquired before and after the stoppage of media flow. The efflux of lactate and its associated proton into the media during this period of no flow resulted in a transient reduction in extracellular pH (~ 6.8), as observed by the “yellowing” of the phenol red indicator in the media. This media was equilibrated with fresh media flowing into the cell-containing chamber, soon after the flow was turned on at the end of the hyperpolarized experiment, with the media pH returning to 7.6. There was no difference in the resonance frequency of the extracellular lactate during this transient change in media pH, suggesting that the downfield shift of extracellular lactate was not due to a more acidic extracellular pH. This is also supported by the finding that the ^{13}C chemical shift of the carbonyl resonance of lactate decreased rather than increased with decreasing pH (0.015 ppm up-field shift going from pH 7.4 to 6.9).

Finally, for UOK262 cells grown in [3-¹³C]pyruvate containing media the rate of extracellular [3-¹³C]lactate accumulation in 2D culture was sampled serially over 8 hours and quantified by measuring the ¹³C satellites of the lactate methyl protons in 800 MHz ¹H MR spectra. Figure 4 shows a plot of [3-¹³C]lactate accumulation in the media over time for untreated and DIDS treated UOK262 cells. Fitting this data demonstrated a lactate efflux of 1.34 nmols/min/million cells for the untreated UOK262 cells. In the presence of DIDS, the rate of lactate efflux was reduced by 64% to 0.48 nmols/min/million cells. This reduction in lactate efflux was similar to that observed with integrated hyperpolarized extracellular lactate signal intensity (total area under the curve over time) after treatment with DIDS (Figure 3B).

Taken together the results of these studies clearly demonstrate that the up- and down-field hyperpolarized [1-¹³C] lactate resonance were due to the intra- and extra-cellular pools of lactate.

Hyperpolarized intracellular to extracellular [1-¹³C]lactate ratio differentiates the varying aggressiveness of RCC cells

HP ¹³C MR studies were performed on 3 renal cell lines, ranging from normal renal tubule cells (HK-2), localized RCC (UMRC6), to metastatic RCC (UOK262). These cell lines were selected since they demonstrate varying intra- and extra-cellular lactate pool sizes due to their known differential MCT1, MCT4 and LDHA mRNA expression and LDH activity (26). Hyperpolarized ¹³C MR studies demonstrated significantly different Lac_{in}/Lac_{ex} ratios for the three cell lines. The bar graph in figure 5A shows that the Lac_{in}/Lac_{ex} ratio in UOK262 cells is significantly lower than that of UMRC6 as well as HK-2 cells by more than 2 and 2.5 fold. This is due to a significant increase in the HP extracellular ¹³C lactate fraction that comprises 38 ± 5% of the total ¹³C lactate pool in UOK262 cells, and was significantly greater than that for UMRC6 and HK-2 cells, at 23±4% and 17±1%, respectively. MCT4 mRNA expression (p<0.05) was significantly elevated in the UOK262 cells compared to the other cell lines (HK-2 p=0.0011, UMRC6 p=0.0171), and figure 5B shows a negative linear correlation (R² of 0.997) of MCT4 mRNA expression and hyperpolarized Lac_{in}/Lac_{ex} ratio for the three cell lines.

Measurement of Intra- and Extra- Cellular Lactate in Living Human Renal Tissue Slices

The ability of this approach to measure intra- and extra-cellular lactate was extended to living patient-derived renal tissue slices in the 5 mm MR compatible bioreactor. Five slices with approximately 88 mg of tissue (wet weight) from high-grade urothelial carcinoma were used for this study. Tissue viability, monitored using ³¹P, was maintained for the duration of the study (4.5 hours). Figure 6 shows the ¹³C spectrum of the living human tissue slice cultures (TSCs) in the 5mm bioreactor, after injection of HP [1,2-¹³C₂]pyruvate. Both the flux to HP [1,2-¹³C₂]Lactate and [5-¹³C]glutamate was observed. Similar to renal cancer cell lines, the lactate doublet (centered at 183.1ppm) was split by the intra- and extra-cellular micro-environment, with extracellular lactate resonating 3.92 Hz downfield of the intracellular pool, while only a singlet denoting the intracellular compartment was observed for the [5-¹³C]glutamate. The ratio of the extracellular pool of lactate to the total lactate signal (42 ± 2 %) is similar to the aggressive metastatic UOK262 cell line, indicating a

similar up-regulation of MCT4 mRNA expression. There was a 3- fold increase in MCT4 mRNA expression in patient-derived renal cancer relative to normal renal tissue, and a 4 fold increase in MCT4 mRNA expression in UOK262 cells relative to normal HK2 cells.

Discussion

In this study, intra- and extra-cellular HP ^{13}C lactate pools were dynamically measured using a 5mm susceptibility matched MR compatible 3D cell culture bioreactor. The intra- and extra-cellular HP lactate signals could be resolved in 1D ^{13}C spectra due to a chemical shift difference between the two resonances (0.031 ± 0.0005 ppm), which was reproducible across studies and cell lines. Although the exact origin of the downfield shift of the extra- relative to the intra-cellular lactate pool is not known, it was not due to a lower extracellular pH, since reduced extracellular pH would have resulted in an extracellular lactate chemical shift in the opposite direction. The observed difference in chemical shift could arise from differences in the bulk magnetic susceptibility or differences in hydrogen bonding between the intra- and extra-cellular microenvironments. Intra- and extra-cellular chemical shift differences, attributed in part to bulk magnetic susceptibility, have been previously reported in ^1H , ^{19}F , ^{31}P , and ^{13}C magic angle spinning NMR spectra of erythrocyte cell suspensions (36,37) and for intra- and extra-myocellular lipid signals in *in vivo* muscle spectra (38). Additionally, as observed by Kuchel et al (36,37,39–42) and further validated by Barry et al (43) protein induced differences in hydrogen bonding between the intracellular and extracellular compartment have also resulted in NMR chemical shift differences between the compartments.

The extracellular HP lactate signal was used to discriminate normal HK-2 renal epithelial cells from both RCC cell lines (UOK262, UMRC6), and more importantly to differentiate the metastatic UOK262 RCC cell line from the localized RCC cell line UMRC6. The extracellular HP lactate findings are consistent with prior steady-state labeling studies with $[1-^{13}\text{C}]\text{glucose}$ which demonstrated that $[3-^{13}\text{C}]\text{lactate}$ in the media was highest for UOK262 followed by UMRC6 and HK2 cells (26). There was also a robust linear correlation between the MCT4 mRNA expression of the three cell lines and hyperpolarized $\text{Lac}_{\text{in}}/\text{Lac}_{\text{ex}}$ ratio, which is due to significant changes in the extracellular lactate pool size. This finding is consistent with the up-regulation of aerobic glycolysis and lactate production and efflux associated with a more aggressive, metastatic cancer phenotype (9). The observed increased lactate efflux is known to be facilitated by an overexpression of MCT4 (monocarboxylate transporters) (27,28). It has been shown in numerous cancers, including cervical cancer (44), prostate cancer (45), lung cancer (46) and clear cell RCC (47), that MCT4 up-regulation correlates with aggressive, invasive behavior. The MCT4 transporter serves two important functions; 1) the export of lactate to maintain a high rate of glycolysis and 2) removal of protons to maintain alkaline intracellular pH. ^{31}P NMR measurements in this study demonstrated a constant alkaline intracellular pH for both of the RCC cell lines consistent with the ability of cells to maintain a constant intracellular pH regardless of the degree of lactate production. While the cancer cell lines produced and transported more lactate out of the cell, the normal renal cell line also produced and transported a substantial amount of lactate. The relatively high level of extra-cellular HP lactate signal observed in the HK-2 cells could be due both to a metabolic aberration associated with immortalization

of the normal renal epithelium, and/or the fact that normal human kidney cortex cells also express substantial MCT4 (48) which is key to the lactate shuttle between the cortex and the medulla (49).

Considering the important function that MCT4 plays in maintaining the glycolytic tumor phenotype and alkaline intracellular pH, MCT4 inhibition has been considered as a therapeutic target (3,50), and this paper demonstrates that the MR compatible bioreactor platform can be used to monitor such a therapeutic approach in real-time. The inhibition of lactate efflux by DIDS resulted in a significant reduction of the extracellular HP lactate pool in the aggressive metastatic UOK262 cell line. However, there was also a small reduction of the intracellular HP lactate pool that is consistent with DIDS's ability to bind and inhibit both MCT1 and 4 transporters although with different affinities. Consistent with the higher affinity of DIDS for MCT4 as compared to MCT1 (27), the observed significant increase in the Lac_{in}/Lac_{ex} ratio was primarily due to a reduction in the Lac_{ex} pool.

The results of these studies demonstrate the importance of lactate efflux as a biomarker of cancer presence and metastatic potential and the utility of the MR compatible 3D cell and tissue culture bioreactor as a platform capable of studying not only cellular metabolism, but also transport and the impact of therapeutic interventions. The extension of these studies to living patient-derived human renal cancer tissue slices was also demonstrated. Recent 1H and HP ^{13}C MR studies of 3D patient derived tissue slice cultures in a MR compatible bioreactor have demonstrated that they provide a more realistic model of the human situation for metabolic investigation (24). In this study we were able to observe the intra- and extra-cellular lactate pools using five $300\ \mu m \times 8\ mm$ slices ($\approx 88\ mg$) of renal cancer obtained at surgery, after injection of HP $[1,2-^{13}C_2]$ pyruvate. The use of the doubly-labeled pyruvate allowed the measurement of flux to both HP $[1,2-^{13}C_2]$ Lactate and $[5-^{13}C]$ glutamate. Interestingly, the downfield shift of the extracellular lactate resonance was identical to that observed for the renal cells, and the ratio of the extracellular pool of lactate to the total lactate signal was very similar to what was observed for the UOK262 cell line. This finding was consistent with over-expression of MCT4 in both RCC tissues and cells. The MCT4 mRNA expression in patient derived renal cancer tissue slices was ≈ 3 fold higher than normal renal tissue, similar to the fold increase (4 fold higher) in MCT4 mRNA expression in UOK262 cells relative to the normal HK2 cells. Only a single resonance was observed HP $[5-^{13}C]$ glutamate which arises from the C-2 carbon of HP $[1,2-^{13}C_2]$ pyruvate in the TCA cycle. This finding is consistent with glutamate being predominately intracellular (51,52), and demonstrates that this technique can be used to study the compartmentalization of metabolites other than lactate. The practicality of acquiring localized HP ^{13}C MR kinetic data opens the possibility of measuring metabolic fluxes by fitting the dynamic data to kinetic models (15,53,54). However, the modeling of metabolic fluxes from HP ^{13}C MR data is still in its infancy and current approaches suffer from a number of factors, including assumed/estimated T_1 's, unknown metabolite pool sizes, and the assumptions underlying the specific models used. Therefore the ability to measure metabolite pool sizes and estimate their respective T_1 's in the very controlled environment of a 3D MR compatible bioreactor could aid in calculating and interpreting *in vivo* metabolic fluxes measured by HP ^{13}C MR.

Although there are a number of ways to measure extracellular lactate *in vitro*, only 2 of those techniques can be applied for *in vivo* measurements, namely steady state MR spectroscopic and microelectrode measurements. Diffusion weighted ^1H and ^1H - ^{13}C (proton observed carbon-edited) spectroscopy approaches have previously been used to assess the compartmentalization of the lactate in the rat brain *in vivo*, but these measurements are limited by poor signal-to-noise requiring long acquisition times for single time point, single voxel measurements (56,57) and therefore unable to provide dynamic measurements of lactate efflux as is achievable for cells and tissues in the MR compatible bioreactor. While the microelectrode technique can yield dynamic measurements, it lacks specific localization, and is invasive (55). In contrast, dynamic hyperpolarized MR technique can estimate in real time the rate of lactate export from the cell and help distinguish between localized and metastatic cancer cells as demonstrated. Hyperpolarized ^{13}C MR has already been applied to patient studies, thereby demonstrating future applicability to *in vivo* measurements (13). The small difference in chemical shift between the extra- and intra-cellular lactate resonance observed in our *ex vivo* studies will be difficult to resolve *in vivo*. However, the results of such *ex vivo* studies can be used to help interpret the results of *in vivo* diffusion weighted HP ^{13}C MR studies in pre-clinical animal models (22,58), as well as future patient studies (20). This platform also could provide a rapid and inexpensive way to test efficacy of drugs that target cellular metabolism and transport.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

HP	hyperpolarized
MR	magnetic resonance
MRS	magnetic resonance spectroscopy
DNP	dynamic nuclear polarization
RCC	renal cell carcinoma
VEGF	vascular endothelial growth factor
DMEM	Dulbecco's Modified Eagle's medium
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid

ATP	adenosine tri-phosphate
MCT	monocarboxylate transporter
LDH	lactate dehydrogenase
TCA	tricarboxylic acid
Lac_{in}	intracellular lactate
Lac_{ex}	extracellular lactate
Lac_{tot}	total lactate
S/N	signal to noise ratio
DMSO	dimethyl sulfoxide
LWHM	Line width at half maximum

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Figure 1. Bioreactor set-up and the viability assessment. **(A)** Graphical representation of the 5mm MR compatible bioreactor optimized for maximal homogeneity. **(B)** Representative ^{31}P spectra of showing unaltered bioenergetics of the UOK262 cells under study before and after hyperpolarized carbon-13 experiment.

Figure 2.

Differential compartmentalization of HP $[1-^{13}\text{C}]$ lactate. **(A)** Lower panel shows the spectrum in alginate microspheres devoid of cells, infused with copolarized $[1-^{13}\text{C}]$ lactate and $[1-^{13}\text{C}]$ pyruvate. Only one peak was observed for the $[1-^{13}\text{C}]$ lactate signal. While two peaks were observed in the alginate microspheres with UOK262 cells when infused with HP $[1-^{13}\text{C}]$ pyruvate (upper panel). The inset (2.5x) clearly shows the well resolved peaks of lactate, where the chemical shift of the downfield peak coincides with that of the signal of lactate in empty alginate microspheres. **(B)** Lorentzian decomposition of the 2 peaks clearly reveals the wider line width of the Lac_{in} peak compared to the Lac_{ex} peak (downfield). **(C)** Plot of Lac_{in} (blue diamonds) as a function of cell density. The x-axis represents the β -NTP concentration, which was used to quantify the viable UOK262 cells within the sensitive coil of the bioreactor.

Figure 3.

Differential modulation of the lactate pools. **(A)** Representative ^{13}C MR spectra of hyperpolarized $[1-^{13}\text{C}]$ lactate acquired from untreated (top) and DIDS treated UOK262 cells acquired at the time of maximal lactate production (indicated by the dotted line in B). **(B)** Dynamic measurement of HP Lac_{ex} (filled circles) and Lac_{in} (open circles) without (top panel) and with DIDS treatment (bottom panel). **(C)** Bar graph of the HP $[1-^{13}\text{C}]$ lactate peaks of UOK262 cells under different flow and treatment conditions relative to control (flow at 0.5ml/min) normalized to 100%. The bar filled with black slanted lines represent the Lac_{in}/Lac_{ex} ratio of UOK262 cells pretreated with DIDS inhibitor for 45 minutes (n=3). The change in the ratio is significantly different (p=0.016). The grey bar denotes the Lac_{in}/Lac_{ex} measurements after flow was stopped 15 secs post HP $[1-^{13}\text{C}]$ pyruvate infusion for a period of 4.25 minutes (n=3) and has a p of 0.055 as measured by student's t-test when compared to control.

Figure 4. Rate of lactate efflux inhibition of UOK262 cells using non-HP MR measurements. Bar graph of $[3-^{13}\text{C}]$ lactate efflux rate of untreated ($n = 3$) and DIDS treated (slanted lines, $n = 3$) UOK262 cells grown in $[3-^{13}\text{C}]$ pyruvate containing media. The significantly decreased efflux rate ($p < 0.05$) with DIDS treatment relative to the untreated controls is consistent with hindered lactate efflux due to MCT4 inhibition.

Figure 5.

Estimation of extracellular HP lactate pools in renal cells of varying phenotypes. **(A)** Graph of Lac_{in}/Lac_{ex} for HK2, UMRC6 and UOK262 cell lines. The Lac_{in}/Lac_{ex} increases going from UOK262, to UMRC6 to HK-2 (n=3 each). The metastatic UOK262 cell line had significantly ($p < 0.05$) lower HP Lac_{in}/Lac_{ex} ratio than both HK2 and UMRC6 cell lines. **(B)** Graph of HP Lac_{in}/Lac_{ex} as a function of MCT4 mRNA expression. A negative linear correlation exists between the measured HP Lac_{in}/Lac_{ex} ratio and the MCT4 mRNA expression in the 3 cell lines studied. All data are represented as mean $\pm$ std.error.

Figure 6.

^{13}C spectrum of HP $[1,2-^{13}\text{C}_2]$ pyruvate metabolism of tissue slice culture. 4 tissue slices of a high-grade urothelial carcinoma were perfused in the 5mm MR compatible bioreactor. The C-1 doublet of HP $[1,2-^{13}\text{C}_2]$ lactate from metabolism of $[1,2-^{13}\text{C}_2]$ pyruvate, each split into two peaks represents the two cellular compartments. And only a singlet denoting the intracellular compartment is observed for the $[5-^{13}\text{C}]$ glutamate is seen in the spectral inset.