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Ratiometric analysis in hyperpolarized NMR (I): Test of the two-site exchange model and the quantification of reaction rate constants

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Abstract

Conventional methods for analyzing the *in vivo* hyperpolarized ¹³C-NMR (HP-MR) data from the lactate dehydrogenase (LDH) reaction usually make assumptions on the stability of rate constants and/or the validity of two-site exchange model. In this study, we developed a framework to test the validity of the assumption of stable reaction rate constants and the two-site exchange model *in vivo* via ratiometric fitting of the time courses of signal ratio $L(t)/P(t)$. Our analysis provided evidence that the LDH enzymatic kinetics observed by HP-MR is in near-equilibrium and satisfies the two-site exchange model for only a specific time window. Additionally we quantified both the forward and reverse exchange rate constants of the LDH reaction for the transgenic and mouse xenograft models of breast cancer using the ratio fit (RF) method we developed that includes only two modeling parameters and is less sensitive to the influence of instrument settings/protocols such as flip angles, degree of polarization, and tracer dosage. We further compared the RF method with a conventional two-site exchange modeling method, i.e., the differential equations fitting (DEF) method using both the experimental and simulated HP-MR data. The RF method appeared to fit better than the DEF method for the reverse rate constant on the mouse tumor data with less relative errors on average, whereas the DEF method also resulted in a negative reverse rate constant for one tumor. The simulation results indicated that the accuracy of both methods depends on the width of transport function (TF), noise level, and rate constant ratio; one method may be more accurate than the other one based on experimental/biological conditions aforementioned. We were able to categorize our tumor models into specific conditions of the computer simulation and estimate the errors of rate quantification. We also discussed possible approaches to developing more accurate rate-quantification methods for HP-MR.

Keywords

rate constant; two-site exchange; net flux model; ratio fit; ratiometric analysis; pyruvate; lactate; lactate dehydrogenase

Introduction

In recent years, hyperpolarized NMR (HP-MR) has been used to noninvasively probe tissue functions and metabolisms, yielding useful *in vivo* physiological information (1–4). One of the key interests of the HP-MR experiments is to determine the reaction rate constants for a specific enzymatic reaction *in vivo*. The rate constants are important indicators of the enzyme activities and are useful for understanding the biology, biochemistry, and pathology in healthy and diseased tissues. It has been shown that the apparent reaction rate constants can be quantified *in vivo* with the modeling of the HP-MR data (5–7).

To extract reaction rate constants, various methods including the major ones, i.e., the two-site chemical exchange model and the net-flux model have been used to fit the time courses of the hyperpolarized ^{13}C -NMR signals from a specific enzymatic reaction, e.g. the LDH reaction in tissue (5,6,8). Both models assume unchanged or stable reaction rate constants during the time period of interest, which may not be true in general in a complex biological system with feed-back/feed-forward and adaptive mechanisms. The net-flux model parameterizes the tracer injection rate, the mean arrival time for tracers to travel from blood to tissue, and the forward reaction rate converting pyruvate to lactate; however, it neglects the reverse reaction rate from lactate to pyruvate. The two-site exchange model includes both forward and backward reaction rate constants, but focuses more on the time period after the pyruvate signal reaches a maximum in tissue and neglects the influence of blood transport and reperfusion of the tracers. The variable blood transport properties may lead to difficulty in objectively determining the proper time range for the validity of two-site exchange modeling. Furthermore, both methods model the time courses of NMR signals and acknowledge that these rate constants are apparent and only partially reflect the true rate constants due to the aforementioned instrument/protocol/biological factors.

In this study, we developed the ratiometric analysis of hyperpolarized ^{13}C -pyruvate NMR data obtained from breast cancer mouse models to bi-directionally quantify the reaction rate constants. We first tested whether the two-site exchange model and the reaction-rate-stability assumption were valid *in vivo*. Our analysis results consistently showed that both assumptions were valid only in a specific time window. By fitting the lactate/pyruvate ratios in this time window with the RF method, we could quantify both the forward and backward rate constants with only two fitting variables in all the tumor models, whereas the differential equation fit (DEF) of the two-site exchange model failed in one data set and exhibited relatively larger relative errors in the determination of the reverse rate constant. We further compared the RF and DEF methods by simulating the HP-MR data with various noise levels, transport functions (TF) widths, and rate constant ratios, and then solving for the reaction rate constants. We also classified the tumor data into specific conditions of simulation. These analyses provide new insights on how to develop more accurate approaches for HP-MR data quantification based on the specific experimental/biological condition in the future.

Theory

In the HP-MR experiments, pyruvic acid with the carbon-13 label at the carboxylic carbon (C1) was used to probe the lactate dehydrogenase reaction,

Using the same symbol convention as ref. 5, $L(t)$ and $P(t)$ are the NMR signal intensities of lactate and pyruvate respectively; k_p is the first order reaction rate constant for the conversion of pyruvate to lactate, k_l is the reverse rate constant from lactate to pyruvate, ρ_l and ρ_p are the $1-^{13}\text{C}$ T_1 relaxation rates for lactate and pyruvate, respectively. In addition, we first assume that $\rho_l = \rho_p = \rho$ (This is rigorously valid in the limit of fast intracellular pool exchange; we will also analyze the conditions in which $\rho_l \neq \rho_p$ afterwards). From the differential equations of the two-site exchange model neglecting the initial blood transport process,

$$dP/dt = -\rho P - k_p P + k_l L, \quad (2a)$$

$$dL/dt = -\rho L + k_p P - k_l L, \quad (2b)$$

we can derive the following equation (see Appendix A)

$$q = -k_l R_{lp} + k_p, \quad (3)$$

where $R_{lp}(t) = L(t)/P(t)$ and $q = (dR_{lp}/dt)/(1 + R_{lp})$. If k_p and k_l are stable during the time course, $q(t)$ versus $R_{lp}(t)$ is linear with a negative slope and a positive intercept. We can therefore extract k_p and k_l from the slope and intercept.

From the general solutions of Eq. 2 (see Appendix A), we can also derive

$$R_{lp}(t) = \frac{r(1 + R_{lp}(t_0)) + [R_{lp}(t_0) - r] e^{-s(t-t_0)}}{1 + R_{lp}(t_0) + [r - R_{lp}(t_0)] e^{-s(t-t_0)}}, \quad (4)$$

where at time t_0 , $R_{lp}(t_0) = L(t_0)/P(t_0)$, $r = k_p/k_l$, and $s = k_p + k_l$. We can model $R_{lp}(t)$ according to Eq. 4 with only two variables r and s in order to obtain k_p and k_l . We called it the two-parameter ratio modeling method. To investigate whether $\delta\rho = 0$ is a good working assumption we also used the three-parameter model ($\delta\rho$ as the third parameter) (Eq. A12 in Appendix) to fit the time course of the lactate/pyruvate ratio.

Materials and Methods

Fig. 1 is a schematic outlining the approaches that were used in this study. We first evaluated the linearity of $q \sim R$ curve to identify a specific time window in which the two-site exchange model and the stable rate constant assumption were valid. We then applied the RF methods to the experimental HP-MR data from breast cancer mouse models, i.e., the $q \sim R$ fit using Eq. 3 and the two-parameter ratio fit using Eq. 4 to quantify the reaction rate constants. We identified the two-parameter ratio fit as the more accurate approach and compared it with the DEF method both in animal model data and computer simulations in order to gain insight on better approaches for HP-NMR reaction rate quantification.

All animal experiments were performed in accordance with protocols approved by the University of Pennsylvania institutional animal care and use committee.

Transgenic mouse models—We used MMTV-rtTa; TetO-NeuNT doxycycline-inducible bitransgenic mice that express the reverse tetracycline-inducible transactivator (rtTA) in the mammary gland epithelium under the control of the mouse mammary tumor virus (MMTV) promoter (9). Upon the administration of doxycycline, rtTA binds to the TetO regulator and induces the expression of the Neu oncogene, leading to the growth of mammary tumors spontaneously. The mammary tumors (#1, 2, 3) examined in this study ranged in size between 1 cm × 1 cm and 1.5 cm × 1.5 cm.

Tumor xenografts—We also used xenografts of breast cancer cell lines (MCF-7(#4), MDA-MB-468(#5), MDA-MB-231(#6, #7)) in athymic nude mice (10,11) with diameters ranging from 0.7 to 1.5 cm.

Animals were sedated and maintained with 1.5% isoflurane during the experiment. A dual-tuned surface coil (1.1 or 1.4 cm in diameter) was placed over the tumor. The probe was placed in a 9.4-T wide-bore, vertical magnet equipped with a Varian console. Body temperature was monitored via fluoroptic rectal probe (Luxtron) and maintained with heated air. A stock DNP (dynamic nuclear polarization) sample was produced by dissolving 10.7 mg OX063 trityl radical (Oxford Instruments) and 15 μ L 1:10 Dotarem(Guerbet):H₂O in 500 μ L (630 mg) [1-¹³C]pyruvic acid (Cambridge Isotope Laboratories). 28-mg aliquots of this stock were polarized to ~30% in a HyperSense polarizer (Oxford Instruments) at 1.4 K. The sample was dissolved in 4 mL Tris-buffered saline containing 100 mg/L Na₂EDTA and sodium hydroxide to neutralize the pyruvic acid. The dissolution buffer was heated to 190 °C and pressurized to 10 bar, and upon dissolution, yielded a neutral isotonic solution of ~75 mM sodium [1-¹³C]pyruvate. 10 μ L/g body weight of this solution were injected into mice via tail vein over a period of 10–12 sec.

Single-pulse ¹³C-NMR spectra were collected with a 9–15° nominal flip angle every 1–2 seconds for about 2 minutes. The pyruvate peak signal-to-noise ratios (SNR) ranged from 300 to 500 for the transgenic model and 18–122 for the xenografts. Line broadening of 20–30 Hz was applied prior to the Fourier transformation of the FID data. A global fitting was conducted by summing up the spectra of all time points and fitting the sum with Lorentzian functions to obtain an estimate of the peak position and width of the major ¹³C-labeled species pyruvate, lactate, pyruvate hydrate, and alanine. Individual peak areas of metabolites at each time point t were obtained by fitting the individual NMR spectrum at time t with four Lorentzian functions covering aforementioned species. The background was removed for both global and individual spectrum fittings by fitting the background spectra outside the window of interest with second order polynomial functions. With this analysis, the time courses of $P(t)$, $L(t)$, and $R_{lp}(t)$ were obtained. The time course of $R_{lp}(t)$ was then smoothed by a moving average of every three neighboring points and then differentiated to obtain $q(t)$. The linear range of the time course of $q(t)$ versus $R_{lp}(t)$ was identified and then fitted to Eq. 3, and the time course of $R_{lp}(t)$ versus t was fitted to Eq. 4 using the same time window as the $q \sim R$ fit. For the tumor xenografts, the ending time point for the fit was determined by a SNR threshold of 2.5. All fits were performed using a customized program based on the *FIT* or *NLINFIT* function and a modified *NLPARCI* function in Matlab 2011a (MathWorks Inc., Natick, MA). These fits yielded k_p , k_l , their standard errors (SE), the p values on the basis of t distribution, and the correlation coefficients R^2 .

We further performed computer simulations to understand the sensitivity of the ratiometric approach to noise levels and the effects of transport function (combined effects of arterial input function and cell membrane transport process) on the fitting results. We first used a customized Matlab® program to generate $P(t)$ and $L(t)$ time courses from Eq. 2 with assumed values of rate constants (0.005–0.1 s⁻¹) and apparent relaxation rate ρ (0.01–0.05

s^{-1} , including the true relaxation rate plus the signal suppression by RF pulses), and $P(0)=0$ and $L(0)=0$. In Eq. 2, we added a gamma variate function

$$TF(t)=\gamma(t)=C \cdot a(1) \cdot t^{a(2)} \cdot e^{-a(3)t} \quad (5)$$

to the right hand side of the Eq. 2a to approximate the transport function of pyruvate, where $a(1)=10$, $a(2)=2$, and $a(3)=0.1, 0.25$, and 0.5 for a broad (I), medium (II) and narrow (III) TF width, respectively; C is a constant chosen to normalize the area under the TF curve to one. The time range was from 0 to 100s and the time interval between adjacent data points was 0.1s for generating the simulated data.

We selected one-tenth of the simulated data points with time interval of 1s as the original signal data set with zero noise. We then added various levels of noise using the random number generator in Matlab® to the signal time courses. The noise level, or the standard deviations of the random number, was represented by the noise factor ranging from 0 to 10% multiplied by the peak intensity of pyruvate, corresponding to SNR of ~10 and above. The two-parameter RF approach was then used to model the ratio time courses to quantify the reaction rate constants. The time window for the fit was chosen by setting the initial time as the third point after the pyruvate peak and the ending time point was determined by the SNR threshold of 2.5. The simulation was repeated for 30 times for each noise level and the final reported rate constants are the averaged values. The relative errors of the rate constants were calculated by comparing the averaged fit values to the true rate constants used for the simulation. For comparison, the DEF method (direct fitting of $P(t)$ and $L(t)$ to Eq. 2) was used to quantify the rate constants and the apparent T_1 relaxation rate and their relative errors using the same SNR threshold for signal time courses. The simulations were performed under three conditions of transport functions with narrow (I), medium (II), and broad (III) widths combined with three conditions of the rate constant ratios, i.e., $k_p/k_l=5\sim 20$ (A), $k_p/k_l=1$ (B), and $k_l/k_p=5\sim 10$ (C) for multiple times (>3) for each condition.

Results

A representative time series of the ^{13}C -NMR spectra from the transgenic mouse model including both pyruvate and lactate signals are shown in Figure 2, with the raw signals normalized to the noise of the spectrum background. Figure 3a shows the time courses of relative signal intensities of lactate $L(t)$ and pyruvate $P(t)$, exhibiting similar trends for all three transgenic breast tumors (#1–3). For uniformity of presentation the pyruvate signals were normalized such that $\int P(t)dt = 1$. Fig. 3b shows the time courses of the L/P ratio $R_{lp}(t)$ for these tumors rising monotonically with time. Fig. 3c shows an expanded view of the ratio time-courses in the range of 0–25s. All three time courses bend at $t \sim 12\text{--}14\text{s}$ indicating the ending of blood transport influences and the establishment of a quasi-steady distribution of metabolites. In approximately 40–50s, $R_{lp}(t)$ started to fluctuate significantly due to the low SNR of hyperpolarized signals. $R_{lp}(t)$ appeared to reach a plateau during this period. This is in agreement with the prediction of Eq. 4: when $t \rightarrow \infty$, $R_{lp}(t) = k_p/k_l$, which is a constant. However, the low SNRs make it difficult to accurately constrain the rate constant ratio based only on the plateau.

The $q\sim R_{lp}$ curves justify the near-equilibrium and stable rate constant assumptions only for a time window

Further analysis of the $q(t)\sim R_{lp}(t)$ curves showed linearity conforming to Eq. 3 only for specific time windows. The $q(t)\sim R_{lp}(t)$ curves shown in Fig. 4 become linear with negative slopes only after $t \sim 13\text{--}15\text{s}$. After $t \sim 40\text{s}$ for tumor #1 and $\sim 50\text{s}$ for tumor #2 and #3, the curves start to fluctuate such that meaningful quantitation is impossible, presumably due to

low SNR. The linearity indicates stable rate constants. This analysis suggests that the near-equilibrium assumption and the two-site exchange model are valid only for a certain time window after the pyruvate tracers reached peak values. The beginning of this time window, coming a few seconds after the peak pyruvate signal, is coincident with the bending point on the ratio time courses (Fig. 3c). The linearity after this time point indicates the influence of blood transport on tracer kinetics is negligible thereafter, supporting the assumption of quick establishment of near-equilibrium for the LDH reaction within ~2–4 seconds after the pyruvate signals peaked. The ending point of the time window is determined from the $q(t) \sim R_{lp}(t)$ curves right before the portions of random fluctuations (Fig. 4). The ratio data in this time window was fit to Eq. 3 to obtain rate constants k_p and k_l from the slope and intercept. Fig. 4b and Table 1 show the fit results for all three transgenic tumors. Similar linearity for the $q(t) \sim R_{lp}(t)$ curves was also observed for the four breast cancer mouse xenografts. The fitted rate constants for 4 breast cancer xenografts are also included in Table 1.

Fitting the ratio time course to quantify the rate constants

Better fits and more accurate rate constant results were obtained by the two-parameter modeling method - fitting the $R_{lp}(t)$ time courses to Eq. 3 for the same time windows as determined above. The fitting plots have been included in Fig. 4c for the three transgenic tumors. Fig. 5 (upper row) shows the time courses of the lactate and pyruvate signals for three breast cancer xenografts and the lower row shows the time courses of $R_{lp}(t)$ and the corresponding fits. Rate constant results for both transgenic and xenograft models are summarized in Table 1. The standard errors of the fitted rate constants are comparable to or smaller than those obtained from fitting the $q(t) \sim R_{lp}(t)$ curves whereas the correlation coefficients are significantly larger. These results demonstrated the feasibility of the RF method and the consistency between the $q \sim R$ fit and the two-parameter ratio fit.

Our fitting experience showed that the three-parameter (the T1 relaxation rate difference $\delta\rho \neq 0$) ratio fit yielded $\delta\rho = 0$ within the standard error of the fit, or resulted in an implausible value ($>0.05 \text{ s}^{-1}$) or an insignificant p value (>0.05) for the $\delta\rho$ (data not shown).

Comparison of the RF method to the DEF method

Experimental mouse model data—We applied the DEF method to both the transgenic and xenograft mouse models using the same data time ranges as for the RF method. The fitted rate constants, relaxation rates and standard errors are included in Table 2. The DEF method provided reasonable fitting results except that the k_l is negative for tumor #3. Although the differences ranged from a few percent to ~50%, the k_p values were relatively consistent between the RF and DEF method, typically differing by 10–30%. The mean relative errors of k_p were also quite consistent between the RF and DEF method with ~3% for transgenic models and ~15% for xenografts. The differences in k_l between the two methods were larger; relative differences exceeded 50% for four of the seven tumors. For the transgenic model (#3 tumor excluded), the mean value and SD of the k_l relative errors were $15 \pm 5\%$ and $9 \pm 3\%$ for the DEF and RF method, respectively. For the xenografts, the mean value and SD of the k_l relative errors were $33 \pm 16\%$ and $25 \pm 9\%$ for the DEF and RF method respectively. The relative errors of the k_l results from the RF method tend to be smaller than the DEF method on average.

Simulations—To further evaluate the sensitivity of fitting methods (RF and DEF) to noise and transport functions, we simulated the signal time courses of pyruvate and lactate and solved the rate constants under various conditions of noise level and transport function shape. Fig. 6 is a typical plot of the time courses of pyruvate, lactate, $R_{lp}(t)$, and $TF(t)$ under representative conditions of TF width, i.e., narrow, medium and broad, in combination with

three rate ratio conditions, i.e., $k_p/k_l = 10, 1$, and 0.1 . $P(t)$, $L(t)$ and $R_{lp}(t)$ exhibited different temporal patterns for each condition. The $L(t)$ and $P(t)$ signals tended to peak earlier for TF with a narrower width. $L(t)$ had a higher peak intensity (the range of change is ~ 10 fold) when k_p/k_l became larger, whereas $P(t)$ peak intensity increased (up to ~ 2 fold) when k_p/k_l became lower.

The relative errors (%) of the fitting parameters including k_p , k_l , and ρ (DEF only) are shown in Fig. 7 for $\rho = 0.03 \text{ s}^{-1}$. Overall, the accuracies of the fitting results for these parameters are dependent on noise, rate ratio, TF width, and the method of modeling. The figure clearly shows greater error in determining the rate constants and relaxation rates as the TF width increases. The DEF method is less dependent on noise than the RF method for most conditions except when $k_p/k_l < 1$ and the TF has medium or broad width. Nonetheless, the RF method generally determines the rate constants with greater accuracy than the DEF method when $k_p/k_l \geq 1$. Similar qualitative results were also found for $\rho = 0.01 \text{ s}^{-1}$ and 0.05 s^{-1} (Fig. 8 & 9).

The picture is mixed for $k_p/k_l > 1$ depending on the relaxation rate ρ . When $\rho = 0.01 \text{ s}^{-1}$, the DEF method is more accurate than the RF method. When $\rho = 0.03 \text{ s}^{-1}$, the DEF method is more accurate when the TF width is narrow or broad; when the TF width is medium, either RF or DEF can be more accurate depending on SNR. When $\rho = 0.05 \text{ s}^{-1}$, the DEF method is more accurate than the RF method for broad and narrow TF widths, while the RF method is more accurate than the DEF method in determining k_p when TF width is medium.

These findings indicate that the accuracy of the reaction rate constant quantifications by HP-NMR depends on various factors such as noise, TF shape, rate constant ratio, T1 relaxation rate, and the method of modeling.

Classification of the mouse tumor data by comparing the experimental data with simulations—Based on our simulation, we classified our tumor datasets using nine conditions, i.e., 3 categories of TF widths (I: broad; II: medium; III: narrow) in combination with 3 categories of rate ratios (A: $k_p/k_l > 1$; B: $k_p/k_l = 1$; C: $k_p/k_l < 1$). Since the rate ratios in our tumor study ranged from 3–20 and ρ is expected to be approximately 0.03 s^{-1} for pyruvate or lactate, we plotted additional error diagrams corresponding to the rate ratios of 5, 15, and 20 and $\rho = 0.03 \text{ s}^{-1}$ in Fig. 10. We examined the diagrams in the 1st and 2nd rows of Fig. 7 and all of Fig. 10 to cover the full range of rate ratios and compare the relative error difference between the RF and DEF fitting results.

As illustrative examples, let us focus on tumors #1, 5, and 6 for which $\rho \approx 0.03 \text{ s}^{-1}$ and the rate ratios are about 20 (#1) and 5. For the transgenic tumor #1, the rate constant k_p from RF was about 10% less than that from DEF, whereas the k_l from RF was about 55% less than that from the DEF. Since this tumor had very high SNR (>300), we excluded A20III (A for $k_p/k_l > 1$, 20 for the rate ratio and III for TF width) that indicates either an opposite trend or insignificant difference between the two fitting methods at such a low noise. They can be classified into A20II rather than A20I based on the signal time course comparison between Fig. 3a and the 1st row of Fig. 6 (The simulated signal time course patterns are similar between when the rate ratio = 10 and 20). Tumors #5 and #6, with SNR ~ 60 and 38, respectively, had the RF results significantly greater than that of the DEF. They should be classified into A5III in Fig. 10. For the other two possible diagrams A5I and A5II, opposite results would be obtained when comparing the relative errors of the RF and DEF methods. Similarly qualitative classifications can be made for the rest 3 tumors (#2 for A15II, #4 for A5II, and #7 for A5 between I & II) by comparing the tumor signal time courses and relative errors of the rate constants from the RF and DEF methods to the signal time courses and rate

error diagrams from computer simulations for $\rho = 0.01 \text{ s}^{-1}$ (#4) or 0.05 s^{-1} (#2 & #7). The tumor #3 was excluded due to the improper fit of k_1 of by DEF.

By using the error diagrams of the simulations as reference and classifying the tumors into the different categories based on the TF width and rate ratios, it is possible to estimate the relative errors of the rate constants quantified by both RF and DEF modeling methods from the true rate values. As shown in Fig. 10, the error is up to ~75% for xenografts and ~25% for transgenic models in our study. These estimations can then guide us on selecting/developing the proper modeling approach based on these different experimental/biological conditions regarding the TF width, the rate ratios, and the SNR (see Discussion).

Discussion and Conclusion

In this study, we have first employed the q~R linear fit analysis to investigate whether the assumption about the rate constant stability and the two site exchange model are valid or not for the experimental data obtained on breast cancer mouse models including transgenic models and xenografts. Both the net-flux and the two-site exchange models assumed stable reaction rate constants. The validity of the two-site exchange model also implies a linear relationship between $q(t)$ and $R_{lp}(t)$ with a negative slope and a positive intercept (Eq. 3). In this study, we found that this linear relationship may not be true for the entire time courses of resonance signals. The initial non-linear segment of the $q(t)$ versus $R_{lp}(t)$ curve indicated that the blood transport was dominant over the intracellular two-site exchange and the significant differences among individual tumors might be the result of the complex interplay of various factors, including transport, T_1 relaxation and tissue heterogeneity *in vivo*. However, after the pyruvate signal peaked and thus a quasi-equilibrium was presumably reached among the vasculature, extracellular, and intracellular space, all tumors exhibited a linear segment in the $q(t)$ ~ $R_{lp}(t)$ curves, which also supports the static rate constants assumption, at least for part of the time course. The results indicate that the two-site exchange model and the stable rate constant assumption are consistent with the experimental data, at least for the time window after the peak of pyruvate and before the signals become too noisy to be analyzed reliably. This supports the use of the two-site exchange model for the extraction of the rate constants by the and defines the appropriate time window for doing so.

We then employed the ratio-fit methods to quantify the rate constants in breast cancer mouse models with consistent results and the two-parameter fitting (Eq. 4) appeared to be more precise than the q~R fit method. Our transgenic breast cancer mouse model is known to be heterogeneous with growth rates and biology. We quantified the ratios of their forward and backward rate constants and these values span a range from 13 to 20 (Table 1). The difference could be understood considering the intertumor difference as well as that the equilibrium constant (or the rate ratio) depends on NAD^+/NADH and pH values, which could vary among tumors within a same tumor model too. Similarly we see the intertumor variations of the rate ratio ~3–6 among breast cancer mouse xenografts with tumor size and cell lines as additional contributing factors. Nevertheless, we demonstrated that the ratio fit method is applicable to various tumor models with a relatively wide range of reaction equilibrium constants. Although the time courses of metabolite signal ratios have been reported in previous studies (5,12,13), this is the first report about modeling the ratio time courses in the field of HP-NMR.

The fitting accuracy of the ratiometric method is mainly limited by the experimental SNR, particularly for the later time points. As the initial time courses of signals are dominated by k_p and usually SNR is not a problem, k_p can be determined quite accurately (relative error within 5% for the transgenic model). The k_l carries more significant weight on the signal

courses at later time points. However, the later time period including the plateau is also the period when hyperpolarized NMR signals get significant loss and the SNR is low. Thus, the relative error in k_l was higher than that in k_p for all methods with the two-parameter RF method yielding the least relative error on average. In addition to trying averaging out neighboring points to improve SNR, hyperpolarized tracers with longer lifetimes are needed to improve the accuracy of the determination of rate constants (particularly for the determination of k_l) and also better probe the stability of enzymatic rate constants for a longer time window.

Another limitation of the RF method is the assumption of equal T_1 relaxation rates for ^{13}C -1-pyruvate and ^{13}C -1-lactate, which have been used in some previous studies (5). The kinetics of the signal ratio would deviate from Eq. 4 if the T_1 relaxation rates of pyruvate and lactate are significantly different. It has been reported that although T_1 relaxation rates for ^{13}C -1-pyruvate and ^{13}C -1-lactate were equal in the liver, they appeared unequal in tumor tissues when analyzed using the net-flux model (6). However, because of the dynamic conversion between pyruvate and lactate *in vivo*, these T_1 measurements should significantly depend on the bidirectional rates of LDH reaction and the modeling methods used. Our simulation indicates that the determination of T_1 relaxation rate by the DEF method has relative errors up to over 100% when k_p/k_l (Fig. 7–10). Thus, it might still be an open question whether the T_1 of ^{13}C -1-pyruvate and ^{13}C -1-lactate have been measured accurately *in vivo*. On the other hand, it has also been pointed out that the derived rate constants using the two-site exchange model were insensitive to relaxation rates and the difference $\Delta\rho=\rho_l-\rho_p$ (5,14). Our direct measurement of the T_1 in phantoms (hyperpolarized ^{13}C -1-pyruvate or ^{13}C -1-lactate in PBS solution) indicated no significant difference between them. The linear relationship between $q(t)$ vs. $R_{lp}(t)$ also supports this assumption (note the nonlinear form of Eq. A8 for ρ_p/ρ_l). This assumption is further supported by our results of the three-parameter fitting, in which $\delta\rho$'s were found to be either smaller/comparable to the modeling errors or with insignificant p values. Therefore, the equal relaxation rate assumption may be a good working hypothesis for this study.

Additionally, we have compared the RF and DEF methods using mouse tumor data and computer simulations. We demonstrated with computer simulation that the accuracy of both of the RF and DEF method depends on the noise level, width of TF, the rate constant ratio and T_1 relaxation rate. The RF method tends to be more suitable when $k_p/k_l \approx 1$ whereas the DEF method tends to be better when $k_p/k_l > 1$ and TF width is not medium. When we employed the DEF method to quantify the rate constants in the mouse models where $k_p/k_l > 1$, the DEF method resulted in one negative k_l (tumor #3) and larger relative error for k_l on average compared to the RF method. We do not have a good understanding yet why the DEF method would result in larger relative error in k_l . Note that the relative errors of fitting results are not same as the relative errors from the true values shown in the error diagrams of simulation. Nor do we understand why the DEF method failed to fit the reverse rate for tumor #3 whereas the RF method could. There are a number of experimental and biological factors that can affect the signal time courses and the quality of fitting. There may be some systematic artifacts or deviations (although we cannot see it visually) of the signal time courses from the two-site exchange model, and the RF method might be less sensitive to them with the ratiometric approach. Ratiometric methods have been used in various research fields including optical spectroscopy and imaging as an effective method to cancel out or reduce systematic or instrumental fluctuations and artifacts (15–18). Since the ^{13}C -lactate is converted from the ^{13}C -pyruvate transported into the tissue, taking the ratio of lactate to pyruvate can cancel out or at least reduce the impact of some instrument/protocol-dependent factors including the initial degree of polarization, tracer dosage, RF excitation flip angle, B1 profile inhomogeneity. For example, there is no need to correct or normalize the raw signals by flip angle and degree of initial polarization. Only two variables are used for the

RF method, and the simplified formula can lead to an easier fit and presumably more stable results.

Our simulation results also indicate that both the RF and DEF methods are significantly subject to the influence of transport function except when the TF width is narrow and the SNR is high. Our estimation about the magnitude of error for the rate quantification by both the RF and DEF methods can be up to 75% for our mouse models depending on the TF width. These results suggest the importance of knowing the transport function in order to achieve accurate reaction rate quantifications. The blood transport function may be measured experimentally as in DCE-MRI, or modeled with approximated mathematical functions. The net-flux modeling does consider the blood transport function with a box-car shape as a simple approximation. However, it is unknown if this approach produces more accurate fitting results, since its accuracy is expected to depend on factors such as TF shape, SNR and rate constants. We will compare the RF and DEF methods with the net-flux modeling by simulations in the future.

Using simulated error diagrams and signal time courses as references, we were able to assign the six tumors (except #3) to a specific modeling condition in the simulation, i.e., AI, AII, or AIII corresponding to broad, medium, or narrow TF width, respectively. We were then able to estimate the errors of rate quantification of each modeling method for our tumor data by reading from the simulated error diagrams for that specific condition. With this information, theoretically we may decide which approach, RF or DEF, is more accurate for each tumor dataset. In reality, note that these assignments are qualitative and the true tumor case may fall into conditions in between categories. Future more systematic modeling with finer intervals of TF width, rate constant and relaxation rate is needed. In addition, our modeling of the transport function can be an oversimplification of the *in vivo* situation. In future simulations, we may also need to take into account various factors such as tracer distribution between intracellular and extracellular space, and the possible difference in relaxation rate constants between pyruvate and lactate, etc.

More investigations are needed to identify the best modeling approach for quantifying the HP-MR data. The ultimate way to validate good modeling approaches is to compare the rate constants quantified with a gold standard. Biochemical assays of enzyme activity in tissue extracts may correlate with the *in vivo* measurements, but cannot serve as the gold standard for rate quantification, because the *in vitro* assays can deviate significantly from the *in vivo* condition. For example, the biochemical assay of LDH activity is usually measured by maintaining certain levels of exogenous NAD⁺ and NADH and pH *in vitro*. These factors can affect the kinetics of the LDH reaction differentially under the *in vitro* and *in vivo* conditions. Nevertheless, it might be possible to calibrate the HP-NMR rate quantification method utilizing perfused cell/organ systems under well-controlled biological/biochemical conditions (14).

In summary, using ratiometric approaches we have developed some new data analysis methods for HP-NMR measurements. The RF method is performed by identifying a specific time window during which the two-site exchange model and the basic assumption of stable reaction rate constants are tested to be valid, and then quantifying the reaction rate constants during this time period. We applied the RF methods to the transgenic and xenograft models of breast cancer and were able to quantify both the forward and reverse rate constants with the relative errors in reverse rate constants smaller than those of the conventional DEF method based on the two site exchange model. Our simulations indicated that both the RF and the DEF method may have significant errors in rate quantification, and one method may be more accurate than the other one, depending on the specific experimental/biological conditions including the noise level, the TF width, the rate constant ratios, and relaxation

rate constant. We classified the tumors into groups corresponding to various simulation conditions in order to interpret the experimental results in light of the computer simulations. This is intended to help us to estimate the error of rate quantification, and select/develop proper approaches for HP-MR reaction rate quantifications for future experiments.

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List of Abbreviations

DCE-MRI	dynamic contrast-enhanced
MRI DEF	differential-equation fit method
HP-MR	hyperpolarized NMR
LDH	lactate dehydrogenase
NMR	nuclear magnetic resonance
MCT	monocarboxylic transporter
MMTV	mouse mammary tumor virus promoter
NAD⁺	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
RF	ratio fit or ratiometric fitting
rtTA	reverse tetracycline-inducible transactivator
SE	standard error
SNR	signal-to-noise ratio

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Appendix A. Theoretical Considerations about the Ratiometric Approach for A Two-Site Exchange Reaction

Two metabolites P (e.g. pyruvate) and L (e.g. lactate) are coupled by the following reaction

in which k_p and k_l as the forward and reverse reaction rate constants. The MR signals, $P(t)$ and $L(t)$ can be described by the two-site exchange model (5) using the following differential equations:

$$\frac{dP}{dt} = -\rho_P P - k_P P + k_L L \quad (\text{A2a})$$

$$\frac{dL}{dt} = -\rho_L L + k_P P - k_L L \quad (\text{A2b})$$

where $P(t)$ and $L(t)$ are the NMR polarizations in the z direction, and the thermal equilibrium magnetization is considered negligible, and ρ_l and ρ_p are the relaxation rates for L and P respectively.

If $\rho_l = \rho_p = \rho$, the solution of Eq. A2 is

$$P = P(0)(k_L e^{-\rho t} + k_P e^{-(\rho + k_L + k_P)t}) / (k_L + k_P) + L(0)(k_L e^{-\rho t} - k_L e^{-(\rho + k_L + k_P)t}) / (k_L + k_P) \quad (\text{A3a})$$

$$L = P(0)(k_P e^{-\rho t} - k_P e^{-(\rho + k_L + k_P)t}) / (k_L + k_P) + L(0)(k_P e^{-\rho t} + k_L e^{-(\rho + k_L + k_P)t}) / (k_L + k_P) \quad (\text{A3b})$$

Let us divide Eq. A3b by Eq. A3a, then

$$L(t)/P(t) = \frac{r_{PL}(1 + L(0)/P(0)) + [L(0)/P(0) - r_{PL}] e^{-s_{PL}t}}{1 + L(0)/P(0) + [r_{PL} - L(0)/P(0)] e^{-s_{PL}t}} \quad (\text{A4})$$

where the L/P ratio is independent on or insensitive to the initial degree of polarization, T_1 relaxations, and vascular transport properties. Eq. A4, also confirmed by using MAPLE 14 (Maplesoft Inc, Canada) solving Eq. 2, only has two unknowns, i.e., the ratio and the sum of k_l and k_p . Let us define $r_{pl} = k_p/k_l$, $s_{pl} = k_p + k_l$. If we also generalize the initial time point to $t = t_0$, Eq. A4 becomes

$$R_{lp}(t) = L_z/P_z = \frac{r_{pl}(1 + L(t_0)/P(t_0)) + [L(t_0)/P(t_0) - r_{pl}] e^{-s_{pl}(t-t_0)}}{1 + L(t_0)/P(t_0) + [r_{pl} - L(t_0)/P(t_0)] e^{-s_{pl}(t-t_0)}} \quad (\text{A5})$$

which is Eq. 4 in the main text. Note that when $t - t_0 > 0$, we take the 1st order approximation $e^{-s(t-t_0)} = 1 - s(t-t_0)$, then Eq. A5 becomes

$$R_{lp}(t) \approx \frac{R_{lp}(t_0) + \left[\frac{r - R_{lp}(t_0)}{1+r} \right] s(t-t_0)}{1 - \left[\frac{r - R_{lp}(t_0)}{1+r} \right] s(t-t_0)} \approx R_{lp}(t_0) + [1 + R_{lp}(t_0)] [k_p - R_{lp}(t_0)k_l] (t-t_0) \quad (\text{A6})$$

By taking the time derivative of Eq. A6, we can see below that Eq. A6 is consistent with Eq. A9 or Eq. 3.

A simpler approach to derive Eq. 3 directly from Eq. A2 is as follows. Let us multiply Eq. A2a by $L/(P+L)$ and Eq. A2b by $P/(P+L)$, and then subtract them

$$\frac{P}{P+L} \frac{dL}{dt} - \frac{L}{P+L} \frac{dP}{dt} = \frac{PL}{P+L} (\rho_P - \rho_L) - k_l L + k_p P \quad (\text{A7})$$

The term on the left is $\frac{P^2}{P+L} \left(\frac{dR_{lp}}{dt} \right)$. Replace L/P by R_{lp} , and then Eq. A7 becomes

$$\frac{1}{1+R_{lp}} \left(\frac{dR_{lp}}{dt} \right) = \frac{R_{lp}}{1+R_{lp}} (\rho_p - \rho_l) - k_l R_{lp} + k_p \quad (\text{A8})$$

When $\rho_l = \rho_p$, it becomes Eq. 3, i.e.

$$q(t) = \frac{1}{1+R_{lp}} \left(\frac{dR_{lp}}{dt} \right) = -k_l R_{lp} + k_p. \quad (\text{A9})$$

The general solution to Eq. A2 when $\rho_l \neq \rho_p$ (confirmed by MAPLE 14) is

$$P_z = e^{\omega t} \left[P(0)(k_2 + k_1 e^{-\lambda t}) + L(0)(k_l - k_l e^{-\lambda t}) \right] / \lambda \quad (\text{A10a})$$

$$L_z = e^{\omega t} \left[P(0)(k_p - k_p e^{-\lambda t}) + L(0)(k_1 + k_2 e^{-\lambda t}) \right] / \lambda \quad (\text{A10b})$$

where

$$\omega = [\lambda - (\rho_l + k_l + \rho_p + k_p)] / 2 \quad (\text{A11a})$$

$$\delta\rho = \rho_l - \rho_p \quad (\text{A11b})$$

$$\lambda = \sqrt{(\delta\rho + k_l - k_p)^2 + 4k_l \cdot k_p}, \quad (\text{A11c})$$

$$k_1 = \left(k_p - k_l - \delta\rho + \sqrt{(\delta\rho + k_l - k_p)^2 + 4k_l \cdot k_p} \right) / 2 = (k_p - k_l - \delta\rho + \lambda) / 2, \quad (\text{A11d})$$

$$k_2 = (\delta\rho + k_l - k_p + \lambda) / 2, \quad (\text{A11e})$$

$$k_1 + k_2 = \lambda. \quad (\text{A11f})$$

Let us divide Eq. A10b by Eq. A10a, then

$$L_z / P_z = \frac{(k_p - k_p e^{-\lambda t}) + L(0)/P(0) (k_1 + k_2 e^{-\lambda t})}{(k_2 + k_1 e^{-\lambda t}) + L(0)/P(0) (k_l - k_l e^{-\lambda t})} = \frac{(k_p + L(0)/P(0) k_1) + (L(0)/P(0) k_2 - k_p) e^{-\lambda t}}{(k_2 + L(0)/P(0) k_l) + (k_1 - L(0)/P(0) k_l) e^{-\lambda t}} \quad (\text{A12})$$

When $\rho_l = \rho_p$, $k_1 = k_p$, $k_2 = k_l$, Eq. A12 becomes Eq. A4. Ratio data can be fit with Eq. A12 by using three unknown variables k_p , k_l , and $\delta\rho$.

Fig. 1.

Outline of this study. DEF: differential-equation fit; RF: ratio fit; R : the ratio $R(t)=L(t)/P(t)$; q : $q(t)=dR(t)/dt/(1+R(t))$; k_p : the forward rate constant of LDH reaction; k_l : the reverse rate constant of LDH reaction; ρ : apparent T_1 relaxation rate constant for pyruvate and lactate.

Fig. 2. Representative time courses of the ^{13}C -NMR spectra for pyruvate and lactate obtained from tumors in vivo.

Fig. 3. Raw data and ratio plots for three transgenic mammary tumors ('o' for #1, '+' for #2; 'Δ' for #3). (a) Time courses of pyruvate $P(t)$ and lactate $L(t)$; (b) $R_{lp}(t)(=L(t)/P(t))$ versus time; (c) An expanded view of $R_{lp}(t) \sim$ time for 0–25s.

Fig. 4. Ratio fits for all three tumors ('o' for #1, '+' for #2; ' Δ ' for #3). a) q versus R_{lp} ; b) fit of q vs. R_{lp} in linear regions; The time courses of R_{lp} were smoothed before calculating $q(t)$. c) fit of the ratio R_{lp} vs. time (Eq. 4).

Fig. 5.

Ratio fits for the breast cancer mouse xenografts. Upper row: time courses of the metabolites in mouse xenografts of MCF-7 (left #4), MDA-MB-468 (middle #5), and MDA-MB-231(right #6); bottom row: the time courses and their fits of the corresponding tumor's lactate/pyruvate ratios $R(t)$. SNR threshold =2.5.

Fig. 6. The time courses of transport function ($TF(t)$, black solid line), pyruvate (red), lactate (blue) and $L(t)/P(t)$ (dash line) under 3 rate constant conditions: $k_p = 10k_l = 0.08 \text{ s}^{-1}$ (A), $k_p = k_l = 0.02 \text{ s}^{-1}$ (B), and $k_p = k_l/10 = 0.008 \text{ s}^{-1}$ (C); with 3 TF widths: broad (column I), medium (column II) and narrow (column III). The apparent T_1 relaxation rate constant $\rho = 0.03 \text{ s}^{-1}$.

Fig. 7.

The relative errors (%) of the fitting parameters resulted from the RF and DEF method under various TF widths, k_p and k_l rates as described in the legend of Fig. 6. $\rho=0.03\text{s}^{-1}$. The noise level is represented by the percent of the pyruvate peak. Dotted lines for RF, + for the DEF, red for k_p , blue for k_l , black for the apparent T_1 relaxation rate constant ρ .

Fig. 8. The relative errors (%) of the fitting parameters resulted from the RF and DEF method under various TF widths, k_p and k_l rates as described in the legend of Fig. 6. $\rho=0.01s^{-1}$. The noise level is represented by the percent of the pyruvate peak. Dotted lines for RF, + for the DEF, red for k_p , blue for k_l , black for the apparent T_1 relaxation rate constant ρ .

Fig. 9.

The relative errors (%) of the fitting parameters resulted from the RF and DEF method under various TF widths, k_p and k_l rates as described in the legend of Fig. 6. $\rho=0.05\text{s}^{-1}$. The noise level is represented by the percent of the pyruvate peak. Dotted lines for RF, + for the DEF, red for k_p , blue for k_l , black for the apparent T_1 relaxation rate constant ρ .

Fig. 10.

The relative errors (%) of the fitting parameters resulted from computer simulations under various TF width and k_p/k_l ratios: $k_p=20k_l=0.1 \text{ s}^{-1}$ (A20); $k_p=15k_l=0.1 \text{ s}^{-1}$ (A15); $k_p=5k_l=0.1 \text{ s}^{-1}$ (A5); and the apparent T_1 relaxation rate constant $\rho=0.03\text{s}^{-1}$. The noise level is represented by the percent (0–3.6%) of the pyruvate peak. Dotted lines for RF, + for DEF, red for k_p , blue for k_l , black for ρ .

Table 1

Results (rate constants $\pm$ SE) of the radiometric fittings for the lactate/pyruvate ratio time courses.

Model	Tumor (SNR)	Time range (s)	Fitting the q - R_{lp} relationship (Eq. 3)			Two-parameter fitting (Eq. 4) $\delta p=0$		
			k_p (s ⁻¹)	k_t (s ⁻¹)	R ²	k_p (s ⁻¹)	k_t (s ⁻¹)	R ²
Transgenic	#1 (335)	15.4~37.9	0.103 $\pm$ 0.001	0.0056 $\pm$ 0.0004	0.91	0.098 $\pm$ 0.002	0.0051 $\pm$ 0.0004	19.2 0.999
	#2 (525)	13.5~48.0	0.081 $\pm$ 0.002	0.0056 $\pm$ 0.0009	0.65	0.079 $\pm$ 0.002	0.0058 $\pm$ 0.0004	13.6 0.997
	#3 (375)	15.0~48.0	0.060 $\pm$ 0.002	0.0034 $\pm$ 0.0009	0.38	0.059 $\pm$ 0.002	0.0040 $\pm$ 0.0005	14.8 0.997
xenografts	#4 (18)	6.0~50.0	0.103 $\pm$ 0.018	0.023 $\pm$ 0.005	0.55	0.110 $\pm$ 0.027	0.024 $\pm$ 0.007	4.6 0.750
	#5 (60)	20.0~70.0	0.045 $\pm$ 0.011	0.014 $\pm$ 0.005	0.24	0.042 $\pm$ 0.010	0.011 $\pm$ 0.004	3.8 0.814
	#6 (38)	16.0~64.0	0.050 $\pm$ 0.010	0.017 $\pm$ 0.005	0.36	0.050 $\pm$ 0.006	0.016 $\pm$ 0.003	3.1 0.930
	#7 (122)	1.0~47.0	0.074 $\pm$ 0.015	0.017 $\pm$ 0.005	0.20	0.060 $\pm$ 0.005	0.011 $\pm$ 0.002	5.5 0.906

Note: For fitting Eq. 3, we first smooth $R_{lp}(t)$ and then calculate $q(t)$ and then plot q versus smoothed R_{lp} . R_{lp} were not smoothed for fitting Eq. 4.

Results (rate constants $\pm$ SE) of the differential equation fitting (DEF) for the lactate and pyruvate time courses using the same time intervals for the RF method in Table 1.

Table 2

Tumor	Time range (s)	DEF			R ²
		k_p (s ⁻¹)	k_l (s ⁻¹)	ρ (s ⁻¹)	
#1	15.4~37.9	0.114 $\pm$ 0.006	0.011 $\pm$ 0.002	0.0368 $\pm$ 0.0006	0.997
#2	13.5~48.0	0.086 $\pm$ 0.002	0.009 $\pm$ 0.001	0.0455 $\pm$ 0.0003	0.998
#3	15.0~48.0	0.052 $\pm$ 0.001	-0.002 $\pm$ 0.001	0.0566 $\pm$ 0.0003	0.999
#4	6.0~50.0	0.17 $\pm$ 0.06	0.043 $\pm$ 0.020	0.011 $\pm$ 0.002	0.921
#5	20.0~70.0	0.033 $\pm$ 0.002	0.0059 $\pm$ 0.0022	0.038 $\pm$ 0.0004	0.996
#6	16.0~64.0	0.037 $\pm$ 0.003	0.0074 $\pm$ 0.0028	0.034 $\pm$ 0.0006	0.992
#7	1.0~47.0	0.071 $\pm$ 0.003	0.019 $\pm$ 0.002	0.043 $\pm$ 0.0005	0.990