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The effect of exogenous substrate concentrations on true and apparent metabolism of hyperpolarized pyruvate in the isolated perfused lung

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Abstract

Although relatively metabolically inactive, the lung has an important role in maintaining systemic glycolytic intermediate and cytosolic redox balance. Failure to perform this function appropriately may lead to lung disease progression, including systemic aspects of these disorders. In this study, we experimentally probe the response of the isolated, perfused organ to varying glycolytic intermediate (pyruvate and lactate) concentrations, and the effect on the apparent metabolism of hyperpolarized 1-¹³C pyruvate. Twenty four separate conditions were studied, from sub-physiological to super-physiological concentrations of each metabolite. A three-compartment model is developed, which accurately matches the full range of experiments and includes a full accounting for evolution of agent concentration and polarization. The model is then refined using a series of approximations which are shown to be applicable to cases of physiological relevance, and which facilitate an intuitive understanding of the saturation and scaling behavior. Perturbations of the model assumptions are used to determine the sensitivity to input parameter estimates, and finally the model is used to examine the relationship between measurements accessible by NMR and the underlying physiological parameters of interest. Based on the observed scaling of lactate labeling with lactate and pyruvate concentrations, we conclude that the level of hyperpolarized lactate signal in the lung is primarily determined by the rate at which NAD⁺ is reduced to NADH. Further, although weak dependencies on other factors are predicted, the modeled NAD⁺ reduction rate is largely governed by the intracellular lactate pool size. Conditions affecting the lactate pool can therefore be expected to display the highest contrast in hyperpolarized ¹³C-pyruvate imaging. The work is intended to serve as a basis to interpret both the signal dynamics of hyperpolarized measurements in the normal lung, and to understand the cause of alternations seen in a variety of disease and exposure models.

Keywords

Hyperpolarized; Metabolism; Pyruvate; Lactate Dehydrogenase

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Introduction

Liquid state hyperpolarized NMR contrast agents were first applied to *in vivo*, metabolic measurements almost a decade ago. Since that time, substantial progress has been made in finding disease states which manifest as a modification of apparent metabolic rate (i.e., the rate or level at which metabolite signals appear in the NMR spectrum). Nonetheless, it has become clear that the relationship between apparent rates and true enzymatic activity is complex, and is obscured by agent uptake, saturation kinetics, intracellular metabolite and cofactor pool sizes (possibly modified by the administration of the agent itself), spin-lattice relaxation, and agent inflow/washout.

In the case of hyperpolarized 1-¹³C pyruvate, by far the best-studied agent, several abnormal conditions can lead to a dramatically increased apparent rate of lactate production. Among these are cancer (for which increased intracellular lactate and glycolytic rate are termed the Warburg effect) (1,2), inflammation (3,4) and ischemia (5). The observation that several diverse conditions are characterized by the same apparent metabolic change state implies a common origin. It has been suggested that the apparent flux through the relevant enzyme (Lactate Dehydrogenase, or LDH), is in fact largely exchange of hyperpolarized agent with the endogenous lactate pool (6–9). In this case, the size of the observed hyperpolarized lactate signal would reflect lactate pool size to a much greater extent than LDH concentration, activity, or initial redox status, although the hyperpolarized lactate signal may be significantly affected by transport dynamics (9).

In this paper, we seek to clarify the relationship between the observed signal evolution and underlying transport and metabolic processes in the isolated, perfused lung. As was previously shown (10,11) observed metabolite signal does not scale linearly with the concentration of the infused hyperpolarized agent. By comparison to a multi-compartment model of signal dynamics, we address the extent to which the reduction in relative activity is caused by saturation of uptake, saturation of enzyme activity, or modified cell redox state. Furthermore, by comparison to studies performed at varying perfusate pyruvate and lactate concentrations, we directly address the relationship between intracellular pool sizes and observed metabolism.

In the proposed model of signal dynamics, we use our measurements and, where necessary, previously determined concentrations and rate constants to refine a picture of the true, rather than apparent, biochemical processes. Although complex, some aspects of the model may be simplified without significant loss of fidelity when rate-limiting steps are not involved. In this light, we address the appropriateness of two common simplifying assumptions in our experimental system; that the low NAD concentrations disallows significant metabolic flux through LDH, and that the lactate:pyruvate ratio accurately reflects the NADH:NAD⁺ ratio at all times, regardless of large transient metabolite concentrations during agent administration.

Background

The kinematics of LDH has been well studied and a detailed analysis of both lactate and pyruvate uptake and enzymatic interconversion has been pursued previously. Our treatment

is based closely on the work of Downer, et al. (12), in that each association/dissociation step with metabolites and cofactors is explicitly modeled rather than attempting to predict saturation behavior *a priori*. The advantage of doing so is that apparent LDH saturation kinetics in one situation may in fact arise from lack of cofactor availability, leading to errors if the same kinetics are assumed in other situations. These explicit modeling methods are then generalized for the signal characteristics of hyperpolarized agents in which the detected signal is proportional to the product of the concentration and the degree of alignment (hyperpolarization) of each species. The model must keep track of both quantities.

Figure 1a shows a schematic representation of the three-compartment model that we employ. The compartments represent the perfusate reservoir (which takes the place of blood in the perfused organ study and transiently contains the hyperpolarized substrate), the extracellular/vascular space, and the intracellular space. We note that although methods have been proposed and implemented (13,14), it is generally difficult to accurately separate the latter two compartments by NMR; in the experiments discussed here, hyperpolarized species that are within the sensitive area of the NMR coil contribute to the overall signal in a manner that does not distinguish between intracellular and extracellular compartments. In the discussion below, concentrations are represented by square brackets, and the polarization of species X as $P(X)$. Thus, e.g., intracellular lactate is described by the pair $[Lac_I]$, $P(Lac_I)$.

Initial conditions- Perfusate

As detailed in the methods section, the hyperpolarized agent, as well as any additional (unpolarized) lactate and pyruvate, originate in the buffered perfusate. At $t = 0$, we switch from the steady-state perfusion buffer with concentrations $[Pyr]_0$ and $[Lac]_0$ to buffer containing the same concentration of lactate and, in general, a different concentration $[Pyr]_1$ of hyperpolarized pyruvate with polarization $P(Pyr_P)$. During the injection of duration T , this species reverts to thermal polarization with time-constant T_{1P} . The perfusate compartment is therefore represented by

$$[Pyr_P] = \begin{cases} [Pyr]_0 & t < 0, t > T \\ [Pyr]_1 & 0 < t < T \end{cases} \quad [Lac_P] = [Lac]_0 \quad (1)$$

$$P(Pyr_P) = \begin{cases} 0 & t < 0, t > T \\ P_0 e^{-t/T_{1P}} & 0 < t < T \end{cases} \quad P(Lac_P) = 0 \quad (2)$$

The evolution of species in the other two compartments is governed by perfusate flow, compound extravasation, trans-membrane transport, enzymatic activity and spin-lattice relaxation. Each contribution is discussed below; note that rates of change that originate with different mechanisms, whether of concentration or polarization, are independent. For clarity of presentation, terms are grouped according to their mechanism. The total rate of change of a quantity is the sum of the rates; e.g., $P(Lac_I)$ changes due to enzymatic activity, transport and relaxation, all of which are summed in the final equation.

Perfusate flow

Vascular and extravascular, extracellular space of volume V_E is replaced by fresh perfusate with flow rate F . Thus,

$$\frac{d[Pyr_E]}{dt} = \frac{F}{V_E} ([Pyr_P] - [Pyr_E]) \quad (3)$$

$$\frac{d[Lac_E]}{dt} = \frac{F}{V_E} ([Lac_P] - [Lac_E]) \quad (4)$$

A parallel set of equations can also be derived for the time evolution of the polarization of each species; this is done by keeping track of the subpopulations of species X which are aligned and antialigned with the field (X_+ and X_- , respectively). At polarization $P(X)$,

$$[X_+] = [X] \frac{1+P(X)}{2}; \quad [X_-] = [X] \frac{1-P(X)}{2}; \quad P(X) = \frac{[X_+] - [X_-]}{[X]}$$

Through explicit differentiation of the above expression,

$$\frac{dP(X)}{dt} = \frac{1}{[X]} \left(\frac{d[X_+]}{dt} - \frac{d[X_-]}{dt} - P(X) \frac{d[X]}{dt} \right).$$

In the case of $[Pyr_E]$, substitution of eq. 3 into the above expression gives

$$\frac{dP([Pyr_E])}{dt} = \frac{1}{[Pyr_E]} \frac{F}{V_E} \{ [Pyr_{P+}] - [Pyr_{E+}] - [Pyr_{P-}] + [Pyr_{E-}] - P([Pyr_E]) ([Pyr_P] - [Pyr_E]) \},$$

which can be simplified to eq. 5. The same process is applied to $[Lac_E]$ to yield eq. 6.

$$\frac{dP([Pyr_E])}{dt} = \frac{F}{V_E} \frac{[Pyr_P]}{[Pyr_E]} (P([Pyr_P]) - P([Pyr_E])) \quad (5)$$

$$\frac{dP([Lac_E])}{dt} = -\frac{F}{V_E} \frac{[Lac_P]}{[Lac_E]} P([Lac_E]) \quad (6)$$

Relaxation

Although likely subject to different relaxation processes, we model spin-lattice relaxation within the lung using the same time-constant T_1 in the intracellular and extracellular compartments. In the experiments discussed here, the observed relaxation is substantially

RF-induced, which is identical in the two compartments. Any residual differences are small enough that the model is unable to constrain them independently. Thus,

$$\frac{dP(Pyr_I)}{dt} = -\frac{1}{T_1} P(Pyr_I) \quad (7)$$

and similarly for the other detectable species Pyr_E , Lac_I , Lac_E , Ala and HCO_3^-/CO_2 .

Transport between vascular/extracellular and intracellular compartments

We note that a comprehensive treatment of signal dynamics and compartmentation would explicitly separate vascular and interstitial space. Unfortunately, little is known about the diffusion or transport of specific compounds from the lung capillary bed to the interstitial space. For that reason, we have chosen to combine extracellular and vascular compartments in this work; thus, the simplified representation of trans-membrane transport that follows may also include the process (in serial) of extravasation. Both cellular uptake and extravasation were previously shown to contribute hyperpolarized signal dynamics in a tumor study (15).

Transport of both pyruvate and lactate between intracellular and extracellular space occurs via four monocarboxylate transporters (MCT1-4) in combination with a contribution from passive diffusion which is small under physiological conditions (16), but may become significant at high concentrations or at reduced pH. MCT activity requires co-transport of a proton (17), and different saturation characteristics have been reported for each MCT and substrate type. The lung is composed primarily of two types of pneumocytes and inflammatory cells, each of which may express transporters differently. Although a simplification, we approximate the aggregate transport with a single saturation curve for each chemical species, and further assume that 1) transport of the species is independent (i.e., saturation of transport by one species does not imply a reduced transport rate for another), 2) trans-membrane transport in one direction is not affected by transport in the opposing direction, and both directions are described by the same pH-dependent maximum flux and saturation concentration, and 3) Trans-membrane flux into(out of) the cell is proportional to the proton concentration on the outside(inside) of the cell. All of these approximations have been commonly employed in previous, successful modeling schemes (9,18,19). The latter assumption comes from the noting that MCT transport and passive diffusion both proceed only when a proton and the relevant anion are present (MCT activity requires the protonated transporter, and diffusion is only significant in the neutral, protonated species). It is possible to use this linear dependence on proton concentration to explicitly model all aspects of the transport process (proton binding and release, substrate binding and release, and translocation) as was recently presented for MCT1 in ref. (19). However, given the multiple transporter-mediated and diffusive processes in our system, we have chosen to approximate transport of each species by a bidirectional Michaelis-Menten function, with maximum transport rate in each direction adjusted as noted above.

In accordance with the notation used in previous treatments (20), we denote the maximum aggregate transport as J_{max} and the characteristic saturation concentration as K_m . The rate of

change of concentration in each compartment is therefore scaled by the compartment volume as:

$$\frac{d[Pyrr_I]}{dt} = \frac{J_{max,P}}{V_I} \left(\frac{[Pyrr_E]}{K_{m,P} + [Pyrr_E]} - \frac{[Pyrr_I]10^{7.4-pH_I}}{K_{m,P} + [Pyrr_I]} \right) \quad (8)$$

$$\frac{d[Pyrr_E]}{dt} = \frac{J_{max,P}}{V_E} \left(\frac{[Pyrr_I]10^{7.4-pH_I}}{K_{m,P} + [Pyrr_I]} - \frac{[Pyrr_E]}{K_{m,P} + [Pyrr_E]} \right) \quad (9)$$

$$\frac{d[Lac_I]}{dt} = \frac{J_{max,L}}{V_I} \left(\frac{[Lac_E]}{K_{m,L} + [Lac_E]} - \frac{[Lac_I]10^{7.4-pH_I}}{K_{m,L} + [Lac_I]} \right) \quad (10)$$

$$\frac{d[Lac_E]}{dt} = \frac{J_{max,L}}{V_E} \left(\frac{[Lac_I]10^{7.4-pH_I}}{K_{m,L} + [Lac_I]} - \frac{[Lac_E]}{K_{m,L} + [Lac_E]} \right) \quad (11)$$

Using a technique identical to that which led to eq. 5 yields:

$$\frac{dP(Pyrr_I)}{dt} = \frac{J_{max,P}}{V_I} \frac{[Pyrr_E]}{[Pyrr_I]} \frac{P(Pyrr_E) - P(Pyrr_I)}{K_{m,P} + [Pyrr_E]} \quad (12)$$

$$\frac{dP(Pyrr_E)}{dt} = 10^{7.4-pH_I} \frac{J_{max,P}}{V_E} \frac{[Pyrr_I]}{[Pyrr_E]} \frac{P(Pyrr_I) - P(Pyrr_E)}{K_{m,P} + [Pyrr_I]} \quad (13)$$

$$\frac{dP(Lac_I)}{dt} = \frac{J_{max,L}}{V_I} \frac{[Lac_E]}{[Lac_I]} \frac{P(Lac_E) - P(Lac_I)}{K_{m,L} + [Lac_E]} \quad (14)$$

$$\frac{dP(Lac_E)}{dt} = 10^{7.4-pH_I} \frac{J_{max,L}}{V_E} \frac{[Lac_I]}{[Lac_E]} \frac{P(Lac_I) - P(Lac_E)}{K_{m,L} + [Lac_I]} \quad (15)$$

Note that in our studies, extracellular is maintained near pH=7.4 by continual washout by buffered perfusate. J_{max} in the above expressions represents the value at that pH, and is adjusted for intracellular pH as appropriate.

In modeling the pH-dependent transport rates, it is important to note that under normal conditions the alveolar epithelium maintains an intracellular pH of approximately 7.2 via a Na^+/H^+ antiport system (21). Thus, steady-state efflux can be expected to exceed influx by approximately 60%. In response to uptake of the protonated hyperpolarized agent or perfusion buffer contents, the intracellular pH will drop further; this effect has been used to measure lactate and pyruvate uptake in a variety of cell types (16,22,23) and the relationship

between the quantity of monocarboxylate transported and the pH change has also been quantified (23,24).

As a first-order modeling of this process, we use the near-linear relationship of ref. (24), in which the intracellular buffering capacity between intracellular pH=6.1 and pH=7.4 is approximately $(0.23 - 0.03 \times \text{pH})$ M/unit (that is, at a pH of 7, uptake of 1mM monocarboxylate causes a decrease of 0.05 units). This yields an approximate time-dependence of intracellular pH during the experiment:

$$\frac{d\text{pH}_I}{dt} = -\frac{M^{-1}}{0.23 - 0.03\text{pH}_I} \left(\frac{d[\text{Pyr}_I]}{dt} + \frac{d[\text{Lac}_I]}{dt} \right) \quad (16)$$

A complete model would also include the pH-normalizing effect of the Na^+/H^+ antiport system. However, since this system operates over a several-minute timescale (21), we expect it to be only minimally effective in restoring pH over the period of a hyperpolarized agent injection, and have therefore omitted it from the model.

Enzymatic interconversion

Upon entry into the cell, hyperpolarized 1- ^{13}C pyruvate is subject to at least three enzymatic processes: transamination with glutamate by alanine aminotransferase (AAT) to form alanine, decarboxylation via pyruvate dehydrogenase (PDH) to form $\text{CO}_2/\text{HCO}_3^-$, and reduction by LDH to form lactate. All three products are observed in all experiments.

Here, we approximate the activity of PDH and AAT simply as rate constants for an irreversible reaction; since the present treatment is intended primarily as a study of uptake and LDH kinematics, their role is as an additional sink for intracellular pyruvate.

In contrast, LDH activity is modeled in accordance with the well-known mechanics of the reaction and its cofactors. The enzyme catalyzes the reaction by first reversibly associating with the oxidized or reduced cofactor NAD^+ or NADH to form LDH:NAD^+ or LDH:NADH . Next, lactate or pyruvate further associates with the enzyme cofactor complex to form $\text{LDH:NAD}^+:\text{Lac}$ or LDH:NADH:Pyr ; we refer to both equivalent forms below as LDH^* . Finally, lactate or pyruvate is released and the cofactor may be recycled before the next conversion. Along with the other intracellular processes modeled, these details of LDH activity are depicted schematically in Figure 1b.

Each of the above steps is characterized by a rate constant, denoted $k_{\pm 1} - k_{\pm 4}$ below. The additional association with a proton (required for pyruvate to lactate conversion) has not been shown in the figure; as with other proton-dependent processes, we assume that the rate coefficient for the step that requires proton binding (k_{-3}) is proportional to proton concentration. Because these coefficients are reported in a pH-normalized manner (12,25), and intracellular pH is modeled in eq. 15, we simply multiply the reported value of k_{-3} by $10^{7.2-\text{pH}_I}$ at each calculation of LDH activity. In practice, we find that this modification has minimal effect and is not explicitly included in the expressions to follow.

LDH is found in five different isoforms, consisting of homo- or heterotetramers of the subunits LDHA and LDHB. Previous work has shown that the lung primarily expresses LDH-3, which consists of equal concentrations of the two subunits. Thus, lung LDH activity can be well approximated by the average of the rate constants extracted from the LDHA and LDHB homotetramers (12,25).

The rate of enzymatic conversion can therefore be expressed in terms of these rate coefficients, intracellular pyruvate and lactate concentrations $[Pyr_I]$ and $[Lac_I]$, and enzyme and cofactor concentrations. For each species that can carry hyperpolarization (pyruvate, lactate, alanine, CO_2/HCO_3^- and LDH*), the evolution of polarization can be derived by noting that transformation to another species does not affect the polarization of the remaining pool, while the new species' polarization is the average of the newly formed and pre-existing pools' polarization, weighted by their respective concentrations. More formally, for interconversion between compounds X and Y at rates $K_{X \rightarrow Y}$ and $K_{Y \rightarrow X}$,

$$P(Y)(t+dt) = \frac{P(Y)(t)([Y](t) - k_{Y \rightarrow X} dt) + P(X)k_{X \rightarrow Y} dt}{[Y](t) + (k_{X \rightarrow Y} - k_{Y \rightarrow X})dt}$$

leading to

$$\frac{dP(Y)}{dt} = \frac{k_{X \rightarrow Y}}{[Y]} (P(X) - P(Y)) \quad (17)$$

As in previous treatments (26,27), we assume that no polarization is lost as a result of the formation or disassociation of a complex, and that complex lifetime is sufficiently short that spin-lattice relaxation in the complex is negligible.

The system of differential equations depicted in fig. 1b is in general too complex to yield a satisfactory closed-form solution; however, the problem can be greatly simplified by noting that in contrast to lactate, pyruvate and NAD^+ , which exist in ~mM concentrations, all LDH species and NADH are present in very low concentration (~μM). These compounds therefore reach a quasi-steady-state within a few ms of a perturbation. Thus, all relevant fluxes can be well approximated by assuming that:

$$\frac{d[NADH]}{dt} = k_4[LDH:NADH] - k_{-4}[LDH] + G' = 0 \quad (18)$$

$$\frac{d[LDH]}{dt} = k_4[LDH:NADH] - k_{-4}[LDH][NADH] + k_{-1}[LDH:NAD^+] - k_1[LDH][NAD^+] = 0 \quad (19)$$

$$\frac{d[LDH:NADH]}{dt} = k_{-4}[LDH][NADH] - k_4[LDH:NADH] + k_3[LDH^*] - k_{-3}[LDH:NADH][Pyr_I] = 0 \quad (20)$$

$$\frac{d[LDH:NAD^+]}{dt} = k_1[LDH][NAD^+] - k_{-1}[LDH:NAD^+] + k_2[LDH^*] - k_2[LDH:NAD^+][Lac_I] = 0 \quad (21)$$

$$\frac{d[LDH^*]}{dt} = k_2[LDH:NAD^+][Lac_I] + k_{-3}[LDH:NADH][Pyr_I] - \{k_3 + k_{-2}\}[LDH^*] = 0 \quad (22)$$

In addition, the low NADH levels present in all situations assures that $[NAD^+]$ is well approximated by the total free NAD concentration, and we impose the further constraint that the total LDH concentration is unchanged during the experiment:

$$[LDH] + [LDH:NADH] + [LDH:NAD^+] + [LDH^*] = [LDH_{TOT}] \quad (23)$$

In eq. 18, we have introduced G' to represent the aggregate rate at which cytosolic NAD^+ is reduced to NADH by agents other than LDH; in the cytosol, this reduction is predominantly but not entirely attributable to glycolysis. Thus, G' is distinct from G , which in the following refers to the rate at which new pyruvate is generated from glucose.

The system of equations 17–22 can be solved to give the concentrations of ‘active’ enzymatic species:

$$[LDH:NADH] = \frac{[LDH_{TOT}] + G' \left[\frac{1}{k_1[NAD]} + \frac{1}{k_3} + \frac{k_{-2} + k_3}{k_2 k_3 [Lac_I]} \left(1 + \frac{k_{-1}}{k_1[NAD]} \right) \right]}{1 + \frac{k_{-3}}{k_3} [Pyr_I] + \frac{k_{-2} k_{-3}}{k_2 k_3} \frac{[Pyr_I]}{[Lac_I]} \left(1 + \frac{k_{-1}}{k_1[NAD]} \right)} \quad (24)$$

$$[LDH:NAD^+] = \frac{[LDH_{TOT}] + G' \left[\frac{1}{k_1[NAD]} - \frac{1}{k_{-3}[Pyr_I]} - \frac{1}{k_{-2}} \left(1 + \frac{k_3}{k_{-3}[Pyr_I]} \right) \right]}{1 + \frac{k_{-1}}{k_1[NAD]} + \frac{k_2}{k_{-2}} [Lac_I] \left(1 + \frac{k_3}{k_{-3}[Pyr_I]} \right)} \quad (25)$$

Because only these species interact directly with the lactate and pyruvate pools, knowing their concentrations is sufficient to determine the rate of change of intercellular concentration and polarization, as is depicted schematically in Figure 1c. Using eq. 24 and 25 and summing the other relevant processes from Figure 1 leads to expressions describing the evolution of lactate and pyruvate concentrations:

$$\frac{d[Lac_I]}{dt} = G' \quad (26)$$

$$\frac{d[Pyr_I]}{dt} = G - G' - k_A [Pyr_I] \quad (27)$$

In deriving the expressions for the time-dependent polarizations, we substitute eq. 23 and 24 into eq. 17, yielding:

$$\frac{dP(Pyrr_I)}{dt} = (P(Lac_I) - P(Pyrr_I))k_+ \frac{[LDH_{TOT}] + G' \left(\frac{\eta-1}{k_{-1}} - \frac{1}{k_- [Pyrr_I]} - \frac{1}{k_{-2}} \right)}{\frac{k_2 k_3}{k_{-2} k_{-3}} + \frac{k_2}{k_{-2}} [Pyrr_I] + \eta \frac{[Pyrr_I]}{[Lac_I]}} \quad (28)$$

$$\frac{dP(Lac_I)}{dt} = (P(Pyrr_I) - P(Lac_I))k_- \frac{[LDH_{TOT}] + G' \left(\frac{\eta-1}{k_{-1}} + \frac{\eta}{k_+ [Lac_I]} + \frac{1}{k_3} \right)}{\eta \frac{k_{-2} k_{-3}}{k_2 k_3} + \frac{k_{-3}}{k_3} [Lac_I] + \frac{[Lac_I]}{[Pyrr_I]}} \quad (29)$$

In the above expressions, $\eta = 1 + \frac{k_{-1}}{k_1 [NAD]}$, $k_+ = \frac{k_2 k_3}{k_{-2} + k_3}$ and $k_- = \frac{k_{-2} k_{-3}}{k_{-2} + k_3}$ (estimates of these and other parameters appear in Table 1). The last term in eq. 27 has been included to represent additional, but minor, pathways through which intracellular pyruvate may be metabolized and is discussed further below.

Eq. 28 and 29 have been written in this form to emphasize the scaling and saturation behavior. Noting that the observed pyruvate and lactate signal is the product of polarization and concentration, the expressions' denominators describe signal growth of each species with saturation similar to the often-employed Michaelis-Menten curve (28), but with an additional characteristic concentration which depends on the lactate:pyruvate ratio. At physiological concentrations, this saturation term dominates the behavior of eq. 28, but is negligible in eq. 29, leading to the simplified forms:

$$\frac{dP(Pyrr_I)}{dt} = (P(Lac_I) - P(Pyrr_I)) \frac{k_+}{\eta} \left([LDH_{TOT}] + G' \frac{\eta-1}{k_{-1}} \right) \frac{[Lac_I]}{[Pyrr_I]} \quad (30)$$

$$\frac{dP(Lac_I)}{dt} = (P(Pyrr_I) - P(Lac_I)) \frac{k_+}{\eta} \left([LDH_{TOT}] + G' \frac{\eta}{k_+ [Lac_I]} \right) \quad (31)$$

Under these conditions, the rate at which hyperpolarized signal is transferred in either direction between pyruvate and lactate is proportional to the lactate pool size, and is independent of the pyruvate pool size. In the comparison to experimental data, a wide range of concentrations is explored, including those in which eq. 30 and 31 are not valid. Eq. 28 and 29 are therefore used in all modeling unless otherwise noted.

In total, nine quantities are followed over the course of a modeling run, tracking the intracellular and extracellular concentrations and polarizations of pyruvate and lactate, plus the intracellular pH. Initial conditions are unimportant in that a steady state is achieved by simulating a few minutes of exposure to the perfusate before the hyperpolarized sample is introduced. This steady state represents a balance between glycolytically-derived compounds (pyruvate and lactate), and transport to and from the perfusate; no other assumptions are required.

To summarize the resulting model, the final differential equation governing the evolution of extracellular pyruvate concentration $[Pyr_E]$ is the sum of contributions from equations 3 and 9, and its polarization $P(Pyr_E)$ is the sum of contributions from equations 5 and 13. Similarly, $[Lac_E]$ is the sum of eq. 4 and 11, $P(Lac_E)$ the sum of eq. 6 and 15, $[Pyr_I]$ is the sum of eq. 8 and 27, $P(Pyr_I)$ is the sum of eq. 12 and 28, $[Lac_I]$ is the sum of eq. 10 and 26, $P(Lac_I)$ is the sum of eq. 14 and 29, and intracellular pH pH_I is as given in eq. 16. The model also contains several thus-far unspecified parameters which characterize the organ and perfusion system. However, these, time-independent parameters have previously been measured or can be estimated with a reasonable degree of accuracy.

Compartment volumes—The intact lung of a 250g rat contains approximately 1.2 ml blood (29,30) (9% of the total blood supply) of which slightly less than half (0.5 ml) is in the capillary bed (31). This pulmonary blood volume measurement includes the entire vasculature leading to and from the heart, which is intact in the experiments described here. Of the ~0.4g nonvascular tissue wet weight (0.9g pooled lobar weight (29,32) minus the capillary blood), 70% is cellular and the remainder is noncellular interstitium (33). Intracellular volume consists of approximately 30% each of type I epithelial cells and endothelial cells with a smaller volume of cellular interstitium (~22%), type II cells (~12%), and inflammatory cells (6%) (33).

In the perfused lung studies, the blood is entirely replaced by noncellular perfusate. It is therefore reasonable to suppose that the total intracellular volume of the lung is approximately 0.30 cm^3 , and that the extracellular volume is at least 1.2 cm^3 ; additional contributions may also arise from the noncellular interstitium and from perfusate which has flowed out of the pulmonary vein but is still within the sensitive area of the NMR coil.

LDH concentration and rate constants—LDH activity is lower in the lung than in any other organ tissue (34), although published measurements vary substantially (35,36). The most complete set of measurements in rat lung homogenates is that of Wolfe, *et al.* (37), which found an activity of 90 units per gram wet weight of tissue. Thus, the typical lung contains approximately 80 i.u., or 220 i.u./ml intracellular volume.

The LDH rate constants are similar among the mammalian species and as noted previously are well represented by the average of those derived from LDH-1 and LDH-5 (25): $k_1 = 1.10 \times 10^6 \text{ M/s}$, $k_{-1} = 1.18 \times 10^3 \text{ /s}$, $k_2 = 1.24 \times 10^5 \text{ M/s}$, $k_{-2} = 1.43 \times 10^3 \text{ /s}$, $k_3 = 2.40 \times 10^4 \text{ /s}$, $k_{-3} = 3.12 \times 10^7 \text{ M/s}$ (at pH=7.2), $k_4 = 5.49 \times 10^2 \text{ /s}$, $k_{-4} = 1.13 \times 10^8 \text{ M/s}$. These rate coefficients can then be used along with the definition of an LDH unit (1 μmol lactate production per unit per minute in excess NADH and pyruvate (37)) such that the measured activity is reproduced. Thus we find that 1 unit/ml corresponds to an [LDH] of 37 nM, and therefore the average intracellular concentration is approximately 8 μM . Note that the above approximation method is insensitive to an overall scaling error in the rate coefficients; it is, however, sensitive to non-cytosolic LDH in the homogenates, although this fraction is likely to be small (38).

Free NAD concentration and $\text{NAD}^+:\text{NADH}$ equilibrium—Several measurements of total nicotinamide adenine dinucleotide concentration in the lung have been reported (39–

41). When scaled to the lung intracellular volume, these measurements correspond to a cytoplasm concentration of approximately 800 μ M (39,41). However, experimental evidence in many cell types strongly suggests that most of the NADH and in particular NAD⁺ exists in bound form and is unavailable for interaction with LDH and the lactate/pyruvate pools. Estimates of free total NAD exist in the mouse erythrocytes (42) and rat hepatocytes (43), and correspond to approximately 350 μ M in these cell types. Although the total NAD/g dry weight is somewhat lower in the lung (41), we assume that the free NAD concentration is nonetheless approximately consistent across tissue types (44).

Confirmation of this estimate can be found in the ³¹P spectra of the perfused lung. Figure 2 shows an example spectrum with the three peaks corresponding to ATP and the single peak corresponding to NAD⁺/NADH highlighted. Although shimming of the NMR lineshape in the perfused lung is difficult, sufficient separation between α -ATP and NADH/NAD⁺ can be achieved to estimate their relative free concentrations (ATP does not exist in bound form, and we expect that bound NAD is broadened and shifted such that it does not appear in the peak shown in fig. 2). Among the experiments reported here, the average ATP:(NAD⁺ + NADH) ratio was found to be 14.5 $\pm$ 1.3. The total ATP concentration in the rat lung has been measured, and corresponds to 5mM (45,46) when scaled for intracellular volume. We therefore arrive at an independent estimation of the free NAD concentration of 5/14.5 = 340 μ M. As discussed below, the predicted hyperpolarized signal dynamics is fairly insensitive to errors in the estimation of either total LDH or free NAD concentrations.

The free NAD⁺:NADH ratio at equilibrium can be estimated using the technique of Willimason, *et al.* (47), which relies on relatively stable intracellular lactate, pyruvate and total free NAD concentrations, the high turnover rate of the dehydrogenases and LDH in particular, measured intracellular lactate:pyruvate ratios of ~9–13 in the perfused lung (48), and the measured equilibrium constant (47):

$$\frac{[Py_r][NADH][H^+]}{[Lac_l][NAD^+]} = K = 1.11 \times 10^{-11} \quad (32)$$

At a pH of 7.2, this equation yields a NAD⁺:NADH ratio of approximately 500. We note, however, that the nature of the bolus hyperpolarized experiment is such that equilibrium conditions are very much not met; given this large ratio and the many redox-dependent reactions taking place it is not even clear that equilibrium is achieved in the steady state (39).

Pyruvate and lactate transport

Of the quantitative parameters that constrain the present model, the most difficult to estimate *a priori* involve monocarboxylate transport across the plasma membrane. Only recently has the transporter profile in the rat lung been studied; Johnson, *et al.* (49) find significant expression of the transporters MCT1, 2 and 4. At physiological concentrations, approximately half of circulating pyruvate is taken up during transit through the lung (49), although the situation is more complex under the conditions studied here due to saturation of the various transporters at different concentrations and the potential contribution of passive

diffusion at the high lactate and pyruvate levels under study. The widely differing reports of MCT saturation characteristics among different tissues and in cloned transporters (49–51) also suggest variability based on other characteristics of the system that may be difficult to predict in the perfused organ. We have therefore chosen to allow the parameters describing monocarboxylate transport to vary in order to best reproduce the experimental lactate signal data with all other parameters fixed.

Steady-state lactate and pyruvate concentrations in the cell

Unusual among organs, the end product of glucose metabolism in the lung is primarily lactate and pyruvate, even at high O_2 concentrations (48,52). We approximate the rate of intracellular lactate and pyruvate production via glycolysis using previous measurements of glucose consumption ($\sim 13.5 \mu\text{mol/hr/gram wet weight}$ (48) in the estimated $350 \mu\text{l}$ intracellular volume. Thus, we assume a continuous intracellular production of $G = 13.5 \mu\text{mol/hr/gww} \times 0.9 \text{gww} / (3600 \text{ s/hr}) / (350 \mu\text{l}) \times 2 \text{ pyruvate/glucose} = 19.3 \mu\text{M pyruvate/s}$. Steady state lactate and pyruvate concentrations (i.e., those present before the hyperpolarized agent is injected) are determined by the balance of this production and transport to and from the extracellular space. The rate of glucose consumption is significantly affected by intracellular lactate and pyruvate concentrations, as well as their ratio (48,53,54); however, we do not find it necessary to include these effects in the present treatment because at high metabolite concentrations, where the effect is most pronounced, glycolytic activity becomes increasingly irrelevant to the overall rate of NAD oxidation and reduction.

During glycolysis, an identical concentration G of NADH is produced via reduction of NAD^+ , but additional processes affect NADH concentration as well. Although a detailed modeling of the many redox-coupled reactions inside the cell is beyond the scope of this work, we approximate the aggregate reduction rate of free NAD^+ as $G' \approx 0.8G$, based on estimates described in ref. (55), and the condition that the $\sim 10:1$ intracellular lactate:pyruvate ratio observed in studies of the perfused organ (46,52) is reproduced in this model. We expect perfused organ measurements to be most relevant to the relationship between G , G' and transport, because general considerations based on measured LDH activity and monocarboxylate transport in many tissues suggest that cytosolic lactate:pyruvate ratios and redox state are strongly linked among organs and cell types by blood lactate and pyruvate concentrations (16); thus *in vivo* measurements are likely to be strongly affected by the presence of other tissues. Although in even the isolated lung, NADH is also generated in the mitochondria, it is primarily used for generation of ATP and is unlikely to contribute substantially to cytoplasm $\text{NAD}^+:\text{NADH}$ balance (55).

Importantly, the model is simplified by the relative inactivity of the TCA cycle in the lung; the measured rate of glucose-derived CO_2 arising from pyruvate dehydrogenase flux is approximately an order of magnitude less than that of glucose-derived pyruvate and lactate leaving the organ (55). Carbon incorporation into protein occurs at a somewhat larger rate, although this still represents a minor pathway for intracellular pyruvate (54). In this model, both pathways are represented as an additional decay of the intracellular pyruvate

concentration (eq. 27); this rate is chosen to best reproduce the hyperpolarized alanine peak integrals. We find the best fit for $k_A = 0.035 \text{ s}^{-1}$.

A summary of all of the parameters used in the model appears as Table 1.

Materials and Methods

General animal handling

All animal experiments were performed under an IACUC-approved protocol at the University of Pennsylvania. 55 male Sprague-Dawley rats (250–300 g) were utilized in this study. The subjects were anesthetized with i.p. pentobarbital, tracheostomy was performed, and 200 U heparin were administered via tail vein to prevent alterations in lung perfusion due to clotting. The lungs were prepared for NMR study according to the previously reported method of “degassing” (56,57); the animals were ventilated with pure O₂ (50 bpm, 11–14 cm H₂O PIP) for 10 minutes in order to remove N₂ from the airways. After ventilation, the trachea was sealed (end exhalation) with a suture, allowing residual O₂ to be absorbed by the circulating blood and perfusate. Thoracotomy was quickly started, the heart was cut transversely, and the pulmonary artery was cannulated via the right ventricle. After perfusion was started, the lungs were rapidly excised and placed in a 20-mm NMR tube. The lungs were perfused at 10 ml/min with a modified Krebs-Henseleit buffer: 119 mM NaCl, 25 mM NaHCO₃⁻, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 4.7 mM KCl, 10 mM glucose, 3% (w/v) bovine serum albumin (BSA) and varying concentrations of unlabeled lactate (0–40mM) and pyruvate (0–20mM) as described below. The perfusate was passed through an oxygenating column under a constant flow of 1 atm 95:5 O₂/CO₂, and warmed via passage through water-jacketed tubing.

Hyperpolarized [1-¹³C]pyruvate injection

28.5 mg [1-¹³C]pyruvic acid (Cambridge Isotope Laboratories) were polarized to ~30% at 1.42 K and 94 GHz with a HyperSense DNP system (Oxford Instruments). Before insertion into the DNP system, the neat pyruvic acid was mixed with 1.7% by weight OX063 trityl radical (Oxford Instruments) and DotaremTM Gd chelate (Guerbet) was added to achieve a total concentration of 15 mmol per mole pyruvate. 4 mL Tris-buffered saline was heated to 190°C at 10 bar, and was used to rapidly dissolve the frozen sample. This solution was diluted using sufficient oxygenated Krebs-Henseleit buffer, as above but without BSA, to yield the desired hyperpolarized pyruvate concentration. The solution was mixed thoroughly and injected using syringe pump in lieu of the steady-state perfusate until the hyperpolarized solution was gone (32 mM) or until 20 ml had been injected (all other hyperpolarized pyruvate concentrations). Note that the dilution buffer was prepared with additional lactate such that when mixed with the hyperpolarized solution the resulting injectate maintained the same lactate concentration as the steady-state perfusion buffer.

NMR spectroscopy

All NMR spectroscopy on perfused lungs was performed with a 20-mm ¹H-X probe (Varian, Inc., Palo Alto, CA) on a wide-bore 9.4-T vertical magnet. ³¹P spectra were acquired before and after the hyperpolarized experiment with the following parameters: flip

angle = 75°, repetition time = 1 s, 512 scans, acquisition time = 8.5 min, spectral window = 39 kHz. Individual ^{13}C spectra were acquired with a 1 s repetition time. The scans were acquired with a nominal 12° flip angle (calibrated once for typical sample loading) and 24.5-kHz spectral window. Peak integrals were determined by fitting the spectral peaks to Lorentzians in a custom MatLab (Mathworks, Inc., Natick, MA) routine.

Typical experiment timing

Following the 10 minutes of degassing, the lungs were inserted into the NMR tube and ^1H -imaged to verify placement (5 minutes), shimmed (10–20 minutes), and the probe was tuned and matched to ^{31}P (2 minutes). The 8.5-minute ^{31}P spectrum was acquired, and the probe was retuned to ^{13}C (2 minutes). The sample was melted (5 minutes) and ^{13}C spectra were acquired (5 minutes). Finally, the probe was retuned to ^{31}P (2 minutes) and an additional spectrum was acquired (8.5 minutes) for a typical total post-surgery experiment duration of approximately one hour.

Model fitting

The model, as specified in eq. 3–16, and eq. 26–29, was fit to all of the experimental data jointly using a custom MatLab program. Each set of spectra was normalized such that the integrated pyruvate signal was 1, and the times were individually shifted to minimize the rms difference between the measured and modeled pyruvate signals. Finally, the lactate time-series were fit, allowing $J_{\max,P}$, $J_{\max,L}$, $K_{M,P}$, $K_{M,L}$, and T_1 to vary and minimizing the rms difference between measured and modeled lactate signals, and in a separate optimization, k_A was allowed to vary to minimize the rms difference between measured and modeled alanine signals.

Results

^{31}P spectroscopy

Figure 2 shows a typical ^{31}P spectrum and Lorentzian lineshape fit, in this case from a perfused lung without lactate or pyruvate added to the perfusate. The spectral peaks show well-resolved resonances from α , β , γ -ATP, PCR, and Pi, as well as the NAD⁺/NADH resonance which is adjacent to α ATP but is nonetheless quantifiable. Additional resonances correspond to unresolved phosphomono/diesters, and diphosphodiester. These spectra allow verification of normal high-energy phosphate status of the lung, as well as estimation of the (NADH + NAD⁺):NTP ratio in the organ. Averaged over all 108 ^{31}P spectra in this study, this ratio was 14.5 $\pm$ 1.3 implying an intracellular free NAD concentration of approximately 340 μM .

^{13}C spectroscopy

Figure 3 depicts stacked spectra from two of the hyperpolarized ^{13}C experiments, representing extremes of the parameter space studied. The bottom series (**3a**) shows spectra acquired during injection of 32 mM hyperpolarized agent and 20 mM pyruvate in the steady-state perfusate. The top series (**3b**) shows spectra acquired during injection of 4 mM hyperpolarized agent with 20 mM lactate in the perfusate. The series differ in the overall signal level of pyruvate (171.1 ppm, highlighted red in each series) and pyruvate hydrate

(179.4 ppm), and most dramatically in the overall lactate (183.2 ppm, highlighted blue) signal. Scaled to integrated pyruvate signal, the former series' lactate labeling exceeds that of the latter series by a factor of 24. The Figure insets depict the pyruvate (red, $\div 30$) and lactate (blue) line integrals from corresponding series as a function of time. These time series exemplify the methods of spectrum analysis and display used in subsequent figures.

Figure 4 summarizes the results of the complete set of hyperpolarized perfused lung experiments, acquired in 55 rat subjects under 24 different lactate and pyruvate concentrations in the perfusate. A quantitative version of these results appears as Table 2. Each condition was tested either two or three times, and the time-series shown are the average of the individual series of peak integrals. The plots are displayed in a manner identical to the fig. 3 insets. Each is normalized such that the sum of pyruvate peak integrals is 1, and the plots placed in a roughly 3-dimensional arrangement to emphasize the clearly observed trends as the perfusate contents are changed: 1) the amount of pyruvate label appearing as lactate *increases* strongly with exogenous lactate concentration, 2) lactate labeling *decreases* strongly with hyperpolarized pyruvate concentration, and 3) lactate labeling *decreases* more weakly (but still significantly) with exogenous pyruvate concentration. Superimposed on each time-series plot are the results of the model introduced in eq. 1–25, which is shown to reproduce the observed dependencies quite well; with the exception of the measurements at 4mM hyperpolarized pyruvate, 2/0.2 mM exogenous lactate/pyruvate and at 32 mM hyperpolarized pyruvate, 4 mM exogenous pyruvate, the integrated lactate signal duplicates all experimental results to within 20%.

As noted previously, the parameters in eq. 6–13 describing trans-membrane monocarboxylate transport were allowed to vary to fit the entirety of the experimental lactate data jointly, yielding $J_{max,P} = 5.6 \times 10^{-8}$ mol/s, $K_{M,P} = 3.9$ mM, $J_{max,L} = 7.6 \times 10^{-8}$ mol/s, $K_{M,L} = 28.8$ mM. k_A from eq. 27 was also allowed to vary in order to fit the experimental alanine line integral data (data not shown) yielding $k_A = 0.035$ s $^{-1}$. Although the model was found to be minimally sensitive to it, agent relaxation rate T_1 from eq. 7 was also allowed to vary, yielding a best fit value 15.9s. This value represents the combined effects of true intracellular spin-lattice relaxation and RF-induced relaxation; the flip-angle-adjusted best fit value is $(\frac{1}{19.3s} - (1 - \cos(12^\circ)))^{-1} = 33.4s$.

Agent relaxation time in the injection syringe was chosen to reproduce the injection signal profiles of the hyperpolarized pyruvate. $T_{IP} = 65s$ was found to reproduce the profiles well in all cases except the single instance of 32 mM hyperpolarized pyruvate and no pyruvate or lactate in the perfusion buffer. In that case, agent relaxation was noticeably faster (mostly likely either due to differing syringe pump placement or a paramagnetic impurity in the pyruvate), and $T_{IP} = 45s$ was used.

Discussion

The purpose of performing the experiments described here was to develop and constrain the three-compartment model of hyperpolarized signal dynamics in the lung, and to determine the extent to which the model may be simplified while retaining its essential features. Central to the analysis of the signal dynamics was a detailed accounting of the time-

dependence of agent and metabolite polarization, which is unique to hyperpolarized experiments, but generally usable within that class. We then sought to use the model to answer several questions that have been incompletely addressed in the literature thus far: under what conditions are the assumptions of quasi-steady state LDH and NADH concentrations valid? Is the assumption of label exchange and negligible flux between the pyruvate and lactate pools universally valid? Is the observed signal primarily dependent on lactate and pyruvate pool sizes, transport, LDH or NAD concentration? Are rate coefficients derived simply from the signal dynamics using coupled first-order differential equations (58,59) a meaningful representation of the true rate of intracellular pyruvate and lactate interconversion? And perhaps most importantly, what is the relationship between observed signal dynamics, steady-state lactate:pyruvate signal ratios and NADH:NAD⁺ ratio, and to what extent is that ratio perturbed by the bolus administration of hyperpolarized pyruvate?

Before addressing these questions, we consider the plausibility of the transport fit parameters determined above. As previously noted, it is undoubtedly an oversimplification to represent transport saturation using a single Michaelis-Menten curve. Nonetheless, the derived parameters are at least consistent with previous measurements. Johnson, *et al.* (49) measured lung pyruvate uptake in rats of similar size to be between 75 and 400 µg/min, which is in the same range as $J_{max,P} = 5.6 \times 10^{-8} \text{ mol/s} \times 88 \text{ g/mol} \times 60 \text{ s/min} = 296 \text{ µg/min}$. Similarly, the pyruvate and lactate K_M 's of 3.9 and 28.8 mM, respectively, are consistent with measurements of rat MCT4 (lactate $K_M = 17\text{--}34 \text{ mM}$ (49,60)) and at least one measurement of rat MCT1/4 (pyruvate $K_M = 4.4 \text{ mM}$ (35)) although the latter value is not consistent throughout the literature (49) and several reports are 3–4 times lower (49,61).

Nonetheless, the model appears to be sufficiently consistent with the literature and experimental data to warrant further exploration of the assumptions and the ways in which observed signal levels depend on the metabolic parameters of interest inside the cell. To establish the validity of the underlying 'quasi-steady state' assumption (eq. 18–22), we ran the model twice at each point in the parameter space studied experimentally; first, the quasi steady-state form of eq. 26–29 was used (in which NADH and LDH species mirror the lactate and pyruvate concentrations with negligible time delay), and in an independent run the complete set of differential equations shown schematically in fig. 1b was followed over the entire period. A comparison of the two versions appears as Figure 5a. Although a small deviation is visible between the quasi steady state (blue) and full numerical (green) curves, particularly under conditions of high perfusate lactate, the time-dependence is very similar and the overall deviation is always less than 2% of total lactate signal. Because the parameter space explored here includes both higher and lower concentrations than is likely to be encountered *in vivo*, this establishes the validity of the quasi-steady state approximation for perfused lung experiments; similar arguments are likely applicable to hyperpolarized pyruvate and lactate studies in all cell, organ, and *in vivo* systems.

Figure 5b shows a similar comparison, between modeling results using the quasi-steady state approximation and the further approximation of a sufficiently low lactate:pyruvate ratio leading to the simplified form of enzymatic activity given in eq. 30 and 31. Unlike the previous example, a significant deviation is evident, of up to 20% of the predicted lactate signal, although only at high perfusate lactate concentration. Further comparison of the

modeled results shows that eq. 30 and 31 are valid (i.e., yield signal levels always within 5%) for perfusate lactate concentrations 5mM or less. However, we note that the effect of increasing perfusate lactate concentration is to increase the intracellular lactate pool size before and during the injection; thus, a similar discrepancy is to be expected in other tissues with abnormally large lactate pools, such as cancer.

It is also instructive to use the model to investigate sensitivity to the accuracy of the estimates that constrain it. Figure 6a–e show the change in modeled lactate signal as assumed total free cytosolic NAD, cytosolic LDH, glucose consumption, maximum pyruvate transport rate, and maximum lactate transport rate are either halved (dotted blue line) or doubled (dashed blue line) with respect to the values assumed in the discussion following eq. 31 (solid blue line). We find that the observed lactate signal is very robust with respect to incorrect assumptions about NAD and LDH (**6a, b**). This result is somewhat unexpected in the case of LDH, but indicates that even in the lung, label exchange through LDH occurs faster than processes which affect lactate and pyruvate pool sizes; further increases in the exchange rate do not change this condition.

The signal dynamics are similarly independent of glucose consumption rate G (**6c**), except when the perfusate lactate concentration is very low or zero (**6c**, leftmost plot). Under these circumstances, the intracellular lactate pool is determined by the balance between lactate/pyruvate generation from glucose and its subsequent transport out of the cell. The predicted sensitivity to G therefore arises due to a change in lactate pool and the consequent NADH regeneration rate. At physiological blood lactate levels of ~1mM, this sensitivity is expected to be relatively small (< 20% of lactate signal for all perfusate and hyperpolarized pyruvate concentrations in response to a double or halving of G).

A similar effect accounts for the model's prediction of the dependence of hyperpolarized lactate signal on lactate transport (**6e**). At very low perfusate lactate concentrations, intracellular, glucose-derived lactate is extracted more rapidly as $J_{max,L}$ is increased, resulting in the strong inverse dependence exemplified by the leftmost plot of **6e**. This dependence is also reduced as perfusate lactate concentration is increased, although not as dramatically as the sensitivity to G shown in **6c**; near the value of $J_{max,L}$ determined by the model fit, significant sensitivity to $J_{max,L}$ remains until perfusate lactate is increased to approximately 2.5 mM. If $J_{max,L}$ is smaller, the sensitivity persists to higher perfusate lactate concentrations.

We note that changes in the observed hyperpolarized lactate signal with local glucose consumption and lactate transport is expected only to the extent that the intracellular lactate pool is determined by that cell's glycolytic rate. In that sense, perfused organ studies with no perfusate lactate represent an artificial situation not encountered *in vivo*. In most organs, including the lung, cytosolic lactate is in near-equilibrium with the blood, and thereby indirectly with other organ systems (16). We therefore do not expect that situations which display a large dependence on G and $J_{max,L}$ are relevant to *in vivo* studies, or to perfused organ or cell studies performed at physiological lactate and pyruvate concentrations (e.g., 1/0.1 mM lactate/pyruvate).

In contrast, the dependence on pyruvate transport is persistent and unavoidable; across all conditions studied, we predict that the observed hyperpolarized lactate signal scales nearly as the transport rate (**6d**). This indicates that transport is likely the rate-limiting step in generating hyperpolarized lactate signal, and that errors in its estimation will have a large effect on the fidelity of the model. It is therefore advisable to allow this parameter to vary in order to reproduce experimental data. We note that because $J_{max,P}$ is expressed as mol/s, it changes not only with transporter density, activity and pH but also organ size and cellularity (e.g., inflammatory cell density).

Relationship between measured lactate:pyruvate ratio and redox state

As previously noted, the small NADH concentration ensures that the NADH:NAD⁺ ratio is always very nearly in equilibrium with the intracellular lactate and pyruvate concentrations. However, there are three obstacles when interpreting measured lactate:pyruvate ratios using hyperpolarized agents in this manner. First, the true intracellular ratio is obscured by signal arising from hyperpolarized extracellular metabolite and, in particular, agent. Second, even the MR signals arising from intracellular species may not accurately reflect pool sizes. And third, the bolus administration of high-concentration hyperpolarized agent may perturb the NADH:NAD⁺ ratio significantly. All three of these effects are explored in Figure 7, which shows the modeled intracellular lactate:pyruvate ratio (red) in comparison to both the total hyperpolarized signal (blue) and the signal contribution from intracellular agent and metabolite (green). As expected, the observed hyperpolarized signal ratio does not accurately reflect the intracellular concentrations or, by extension, the NADH:NAD⁺ ratio. This is due to the large signal contribution from the hyperpolarized agent in the extracellular and vascular space. Although the lung is particularly difficult in this regard, due to the large vascular space, a significant error is likely introduced in other organs as well. However, for situations in which the intracellular signals may be separately interrogated (13,14) the resulting signal ratio is a much more accurate representation of intracellular concentrations, as long as enough time is allowed for the hyperpolarization to achieve equilibrium between the two pools. Examination of eq. 31 shows that the relevant time constant is approximately $k_+ [LDH_{TOT}] / \eta$. It is important to note, however, that the intracellular concentrations themselves are expected to be greatly affected by the hyperpolarized agent bolus. This can be seen in the large deviation of the red trace from equilibrium (the value for $t < 0$ and $t \rightarrow \infty$) in each of the plots in fig. 7. While it is therefore possible to evaluate lactate pool size using hyperpolarized pyruvate, information about the preexisting pyruvate pool size, and therefore the NADH:NAD⁺ ratio, is obscured by the agent itself, unless agent concentrations can be kept the same before and during the experiment (e.g., tracer concentrations *in vivo*). In principle, it is possible to accurately evaluate metabolite ratios by waiting for a return to equilibrium after the bolus administration, although we find both theoretically and experimentally that the hyperpolarized signal is lost before equilibrium is regained, making this strategy very difficult in practice.

On the other hand, the evaluation of total lactate signal (summed over all spectra) and its relationship to total pyruvate signal can be done in these experiments at high signal-to-noise of >100 in each peak in all experiments described. We therefore believe that instrument noise is an insignificant contributor to the variability in the values shown in Table 2. We

expect that the largest source of uncertainty arises from inconsistency in the quality of perfusion in the lung samples. We have previously shown that ischemia leads to significantly increased hyperpolarized lactate signal (62), and similar effects may have occurred undetected in some of the experiments described. Conversely, complete absence of flow to a region of the lung at the time of agent injection would lead to a loss of the corresponding fraction of expected lactate signal. Although qualitative visual inspection of both the lungs themselves and ^{31}P spectra were used to verify that the samples were not grossly compromised, we cannot quantify the influence of undetected imperfections on the data.

Conclusions

We have presented a three-compartment model of hyperpolarized pyruvate signal dynamics in the perfused lung, which is likely applicable to other biological systems as well. Because the time constants of flow, transport, relaxation and enzymatic interconversion combine to determine these dynamics, we use experimental results under a variety of perfusate conditions to constrain the model, and then explore the robustness to model parameter estimate error and the relationship to cytosolic pool sizes and redox state. Numerical integration of the model is very straightforward, and the intention is to use it to determine the root cause of altered apparent LDH activity in disease-related alterations in the lung. Several quantitative models have been previously reported that are applicable to hyperpolarized ^{13}C measurements in cancer (26,63–66) and the present treatment is fundamentally similar, although it differs in the study of the approximations and scaling behavior in the unusual parameter regime found in the lung and at the relevant high metabolite concentrations.

We derive differential equations which predict the exchange of hyperpolarization between compartments and through lactate dehydrogenase, including a very simple form of LDH activity on polarized species (eq. 30, 31) which can be used unless the lactate:pyruvate ratio is unusually high. Notably, the predicted signal dynamics is only weakly dependent on LDH activity, indicating that equilibration of hyperpolarization between the intracellular lactate and pyruvate pools is not the rate-limiting step, despite the lung's relatively low enzymatic activity. Furthermore, consideration of LDH rate constants under physiological conditions shows that in all but the most extreme circumstances, the rate of exchange of hyperpolarized signal through LDH in either direction is proportional to the cytosolic lactate concentration and is independent of the pyruvate concentration.

These results suggest that hyperpolarized signal dynamics depends primarily on the density of cells in relation to perfused extracellular space, lactate pool size (which is to a large extent determined by blood/perfusate lactate concentration), and transport kinetics. Other potential dependencies can be minimized by ensuring that the perfusate contains physiological concentrations of lactate and pyruvate. However, pyruvate transport kinetics remains the most difficult to determine *a priori*. The approximation presented here, of bidirectional, independent, saturable transport, is the simplest which can be made to fit the data presented. While undoubtedly missing much of the complexity of multiple transporters, their saturation and different expression in multiple cell types, such an approximation is a

necessary step toward a quantitative, physiological interpretation of hyperpolarized signal dynamics. Additional studies are necessary to fully characterize metabolite transport and its potential dependence on blood/perfusate contents and disease state.

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Abbreviations

AAT	Alanine Aminotransferase
LDH	Lactate Dehydrogenase
PDH	Pyruvate Dehydrogenase
DNP	Dynamic Nuclear Polarization
EDTA	Ethylenediaminetetraacetic acid
GPC	Glycerophosphocholine
GPE	Glycerophosphoethanolamine
gww	Gram wet weight
LDH	Lactate Dehydrogenase
MCT	Monocarboxylate Transporter
NADH/NAD⁺	Oxidized/reduced Nicotinamide adenine dinucleotide
NTP	Nucleoside 5'-triphosphate
PCr	Phosphocreatine
Pi	Inorganic phosphate
PME	Phosphomonoester

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Figure 1.

A schematic representation of the three-compartment model of hyperpolarized agent dynamics. **a)** Exogenous pyruvate and lactate originate in the perfusate reservoir in concentrations Pyr_P and Lac_P , are exchanged into and out of the perfused organ extracellular and vascular space to with concentration Pyr_E and Lac_E , and are further exchanged into and out of the cellular cytoplasm compartment yielding concentrations Pyr_I and Lac_I . Both concentration and hyperpolarization level (represented by the red segment of each block) are exchanged between compartment and between species in the cytoplasm. **b)** The complete set of processes and species comprising the intracellular compartment, expanded to include LDH association with NADH/NAD⁺ cofactor, further association with intracellular lactate and pyruvate, as well as pyruvate generation and NADH regeneration through glycolysis. All association / dissociation rate constants k_1 - k_4 have been measured. Note that all enzyme behavior including saturation is implied by the processes shown, and no additional assumptions are required apart from total enzyme and cofactor concentrations. **c)** the quasi-steady state assumption allows simplification of the model in terms of the concentrations of

active species LDH:NADH and LDH:NAD⁺. Each glycolytic intermediate associates with the appropriate enzyme:cofactor, and exchanges to the other pool according to the LDH* dissociation branching ratio, yielding the bidirectional flux expressions shown. Note that an in balance in polarization between the pools results in flux of polarization even in the absence of concentration flux, as described by eq. 28–31.

Figure 2.

^{31}P spectra of the lung (blue dots) are used to qualitatively verify the energy status of the organ before and after ^{13}C spectroscopy. Each spectrum was fit to a series of Lorentzian lines centered at -16.5 ppm (β -ATP), -8.5 (NAD^+/NADH) -7.78 (α -ATP), -2.58 (γ -ATP), 0 (PCr), 4.6 (Pi), $6.3/6.9$ (PMEs) relative to PCr. Additional, broader lines were included at -9 , -3.6 and 3.2 ppm to represent unresolved, mostly PDE species and improve the quality of the fit. Lungs were considered sufficiently healthy for experimentation if α -ATP was taller than Pi; poor health usually indicated incomplete perfusion of the organ after surgery. The relative height of the total NAD peak (individual species were never resolved), relative to total NTP (ATP/ADP/AMP were also not resolved) provided an estimate of total free NAD available as an LDH cofactor.

Figure 3.

Two series of stacked spectra representing extremes of the parameter space studied; **a)** 32 mM HP pyruvate with 0 mM lactate, 20 mM pyruvate in the perfusate; **b)** 4mM hyperpolarized pyruvate with 20 mM lactate, 0 mM pyruvate in the perfusate (a 2mM hyperpolarized pyruvate dataset was acquired as well, but is not exemplified here because of poorer signal-to-noise). The juxtaposed figures show the large change in apparent lactate labeling (a factor of 24) as the perfusate conditions are changed. The inset figure (c) shows the integrated pyruvate ($\div 30$, red) and lactate peak (blue) integrals as a function of time. All plots are scaled such that the integrated pyruvate signal is 1.

Figure 4.

Summary of scaled pyruvate and lactate peak integrals (in the manner of the fig. 3 insets, but with superimposed model results; modeled pyruvate signal appears in black and lactate in yellow) for all twenty-four perfusate conditions studied. Each plot is labeled in terms of its (hyperpolarized pyruvate concentration):(perfusate lactate concentration):(perfusate pyruvate concentration). The plots are arranged to emphasize the dramatic increase in apparent lactate labeling at high perfusate lactate concentration and low hyperpolarized pyruvate concentration, the smaller but significant decrease at high perfusate pyruvate concentration, and the general success of the model in duplicating the experimental data across a large range of perfusate conditions.

Figure 5.

a) A comparison of the modeled lactate signal using the quasi-steady state (blue) and full numerical treatment (green, almost entirely obscured) shows negligible difference across the full range of perfusate conditions studied. The traces shown are for all combinations of 2 and 32 mM hyperpolarized pyruvate, 0 and 20 mM perfusate lactate and pyruvate (8 combinations in all; individual conditions corresponding to particular traces are not specified because none show appreciable differences). **b)** A similar comparison between the quasi-steady state approximation and the further low lactate:pyruvate approximation of eq. 30 and 31 shows poorer agreement. The largest disagreement, of about 20%, occurs with a 2/20 mM hyperpolarized pyruvate/lactate injection combined with a 20/20 steady-state pyruvate/lactate perfusion (2:20/20, as shown). Nonetheless, the latter approximation is applicable in cases not characterized by an abnormally large lactate pool, and provides useful insight into the scaling of hyperpolarized signal dynamics.

Figure 6.

Series a)–e) show the modeled lactate signal (blue) scaled to pyruvate signal (red) for the same set of limiting cases described in fig. 4. In each case, a model parameter is halved (dotted blue) and doubled (dashed blue) to investigate the robustness of the model to misestimation of the parameters specified. In most cases, near-physiological conditions or elevated perfusate lactate or pyruvate imply a minimal dependence on the model parameter, with the exception of $J_{max,P}$; sensitivity to this parameter is expected under all circumstances.

Figure 7.

Plots **a–g**) show the modeled relationship between the modeled lactate:pyruvate ratio (which is in rapid equilibrium with the NADH:NAD⁺ ratio and is depicted in red) and the total (blue) or intracellular-only (green) hyperpolarized signal. Modeled conditions for each case are specified as described in fig. 4. The intracellular-only hyperpolarized signal is predicted to be a reasonably accurate (usually within a factor of 2) reflection of the corresponding pool size ratios after a few time-constants $\tau = k_+ [LDH_{TOT}] / \eta$. However, the true intracellular lactate:pyruvate ratio is seen to be substantially altered during the hyperpolarized injection (deviation of red trace) except in cases for which the preexisting perfusate pyruvate and hyperpolarized agent concentrations are similar. This situation is not practical for *in vivo* experiments.

Table 1

A summary of all parameters used in the proposed three-compartment model

Model parameter	symbol	value
LDH activity	k_1	$1.10 \times 10^6 \text{ M/s}$
k_2	$1.24 \times 10^5 \text{ M/s}$
	k_3	$2.40 \times 10^4 \text{ s}$
	k_4	$5.49 \times 10^2 \text{ s}$
	k_{-1}	$1.18 \times 10^3 \text{ s}$
	k_{-2}	$1.43 \times 10^3 \text{ s}$
	k_{-3}	$3.12 \times 10^7 \text{ M/s}$
	k_{-4}	$1.13 \times 10^8 \text{ M/s}$
	k_+	1.17×10^5
	k_-	1.76×10^6
Free NAD	$[NAD]$	$350 \mu\text{M}$
	η	$3.92^{(*)}$
pyruvate transport Vmax	$J_{max,P}$	$0.056 \mu\text{mol/s}$
pyruvate transport saturation	$K_{M,P}$	3.9 mM
lactate transport Vmax	$J_{max,L}$	$0.076 \mu\text{mol/s}$
lactate transport saturation	$K_{M,L}$	28.8 mM
<i>Ex vivo</i> pyruvate relaxation time	$T_{1,P}$	65 s
glycolysis rate	G	$19.3 \mu\text{M/s}$
non-LDH NADH reduction rate	G'	$15.4 \mu\text{M/s}$
total LDH concentration	$[LDH_{TOT}]$	$8 \mu\text{M}$
AAT + PDH activity rate constant	k_A	0.035 s^{-1}
total extracellular volume	V_E	1.2 ml
total intracellular volume	V_I	0.3 ml
perfusate flow rate	F	10 ml/min
intracellular + RF relaxation	T_I	19.3 s^{-1}

Table 2

Measured and modeled integrated lactate signal for all experimental conditions studied.

concentration (mM) Hyperpolarized pyruvate	concentration (mM) Perfusate lactate	concentration (mM) Perfusate pyruvate	signal ratio (measured) Integrated lactate:pyruvate	signal ratio (modeled) Integrated lactate:pyruvate	Model % error
2	0	0	.0368	.0393	6.8%
2	20	0	.1127	.1040	-7.8%
4	0	0	.0230	.0238	3.2%
4	2	0.2	.0208	.0353	69.7%
4	20	0	.0719	.0737	2.4%
8	0	0	.0123	.0137	11.2%
8	20	0	.0460	.0494	7.5%
16	0	0	.0092	.0078	-16.1%
32	0	0	.0055	.0045	-18.9%
32	.5	0	.0064	.0053	-16.2%
32	1	0	.0060	.0062	2.7%
32	2	0	.0089	.0076	-14.6%
32	4	0	.0098	.0102	4.1%
32	8	0	.0118	.0131	11.0%
32	20	0	.0220	.0180	-18.5%
32	40	0	.0253	.0221	-12.5%
32	0	0.5	.0048	.0044	-9.4%
32	0	1	.0071	.0041	-42.2%
32	0	2	.0029	.0035	18.3%
32	0	4	.0038	.0032	-15.8%
32	0	20	.0010	.0008	-19.7%
32	20	2	.0157	.0161	2.6%
32	4	0.4	.0112	.0099	-11.6%
32	8	0.8	.0132	.0123	-7.1%