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Quantitation of Erythrocyte Pentose Pathway Flux With [2-^{13}C]Glucose and ^{1}H NMR Analysis of the Lactate Methyl Signal

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A simple and sensitive NMR method for quantifying excess ^{13}C-enrichment in positions 2 and 3 of lactate by ^{1}H NMR spectroscopy of the lactate methyl signal is described. The measurement requires neither signal calibrations nor the addition of a standard and accounts for natural abundance ^{13}C-contributions. As a demonstration, the measurement was applied to ~3 μmol of lactate generated by erythrocyte preparations incubated with [2-^{13}C]glucose to determine the fraction of glucose metabolized by the pentose phosphate pathway (PP). PP fluxes were estimated from the ratio of excess ^{13}C-enrichment in lactate carbon 3 relative to carbon 2 in accordance with established metabolic models. Under baseline conditions, PP flux accounted for 7 ± 2% of glucose consumption while in the presence of methylene blue, a classical activator of PP activity, its contribution increased to 27 ± 10% of total glucose consumption ($P < 0.01$). Magn Reson Med 51:1283–1286, 2004.

Key words: lactate; ^{13}C-enrichment; pentose phosphate pathway; isotopomer

In erythrocytes, the sacrificial oxidation of reduced glutathione (GSH) is the primary defense against oxidative stress. Under these conditions, GSH is regenerated by the NADPH-dependent glutathione reductase, while NADPH is replenished via the oxidation of glucose through the oxidative branch of the pentose phosphate pathway (PP). In response to an oxidative insult, PP flux is increased to the extent that it accounts for a significant fraction of glucose consumption. Since NADPH generation via PP is dedicated to the glutathione redox cycle, measurement of PP flux provides a sensitive and direct reflection of erythrocyte glutathione antioxidant activity.

For systematic applications, such as screening an array of compounds for oxidant activity, PP flux measurement has to be fast, robust, and reproducible. While many different tracer methods have been developed to quantify the fraction of glucose consumed by PP relative to glycolysis (1–5), it is generally agreed that tracing the label from [2-^{13}C] or [2-^{14}C]glucose into positions 2 and 3 of lactate is in theory the optimal approach. The main impediment to this method has been the quantification of positional ^{14}C-specific activity or ^{13}C-enrichment of lactate. With [2-^{14}C]glucose, determination of the specific activity in carbons 2 and 3 of lactate requires carbon-by-carbon degradation and is therefore not practical for large-scale applications. Lactate ^{13}C-enrichment from [2-^{13}C]glucose can be quantified by gas-chromatography/mass spectrometry (GC-MS) but the analysis does not provide positional ^{13}C-enrichment information (3). Alternatively, NMR analysis of lactate ^{13}C-enrichment from [2-^{13}C]glucose can be performed either in situ or following minimal sample manipulation while directly providing positional ^{13}C-enrichment information. Previous NMR methods have focused on quantifying ^{13}C-enrichment of lactate by ^{13}C NMR spectroscopy (6–9). This approach has low sensitivity, requires corrections for different T_1 and nOe parameters of the lactate carbons, and the natural abundance ^{13}C contribution (or excess ^{13}C-enrichment) cannot be directly obtained from the lactate ^{13}C NMR signals. A more recent report demonstrated the indirect detection of ^{13}C-enrichment in positions 2 and 3 of lactate by ^{1}H NMR (10). However, that study did not explore the effects of natural abundance ^{13}C contributions to PP estimates nor did it address the effect of ^{13}C isotopomer populations on the quantification of lactate excess ^{13}C-enrichment in carbons 2 and 3 by indirect detection. We present a simple ^{1}H NMR method that accounts for natural abundance ^{13}C contributions and utilizes selective ^{13}C-decoupling to simplify signal distributions and improve the precision of excess ^{13}C-enrichment measurements of carbons 2 and 3 of lactate. The analysis is simple, robust, and can take advantage of the latest improvements in NMR sensitivity (i.e., inverse-detect nanoprobes and cryoprobes) and should therefore be highly amenable to routine screening of oxidative PP flux activation in erythrocyte preparations.

MATERIALS AND METHODS

Blood Processing

Blood was obtained following informed consent from a healthy adult volunteer and was drawn by venipuncture into tubes containing citrate as an anticoagulant. Blood samples were immediately centrifuged at 3000 rpm for 10 min and the plasma and buffy coat were removed and discarded. The erythrocytes were then resuspended in an equal volume of Hepes/saline buffer (25.0 mM Hepes, 120.0 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, and 1.0 mM NaH_2PO_4; pH 7.40–7.45) at 37°C, centrifuged as before, and resuspended in fresh buffer. This process was repeated two more times, after which the erythrocytes were

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resuspended at a higher dilution (~10% hematocrit) in the same buffer solution for 60 min to wash out endogenous glucose and lactate. Following this washout period, erythrocyte samples were centrifuged and resuspended at a hematocrit of 45–50% in Hepes/saline buffer containing 11 mM [2-^{13}C] D-glucose (99%, Cambridge Isotope Laboratories, Andover, MA). In experiments designed to stimulate PP flux, a 1 mM stock solution of methylene blue in Hepes/saline was added 5 min after resuspension of the cells in the ^{13}C-labeled glucose buffer to give a final methylene blue concentration of 6.7 μM. All erythrocyte samples were incubated at 37°C for 8 hr, after which they were centrifuged and the supernatants collected for ^{1}H NMR analysis. One hundred μl of supernatant were lyophilized and resuspended in 600 μl D_2O for ^{1}H NMR analysis. In a subset of NMR samples, total lactate levels were measured with an enzymatic lactate assay kit (Boehringer Mannheim, Darmstadt, Germany).

NMR Spectroscopy

NMR spectra were acquired at 11.75 T with a Varian Unity 500 system equipped with a 5-mm broadband "switchable" probe with z-gradient (Varian, Palo Alto, CA). ^{1}H NMR spectra were acquired at 25°C with presaturation of the water signal, a pulse angle of 51°, and acquisition time of 3 sec. The number of acquisitions per spectrum was 512 resulting in a total collection time of 26 min. ^{13}C-decoupling of the carbon 1 signal of lactate was achieved with low-power single-frequency irradiation applied continuously. Deconvolution and quantification of the [2-^{13}C]- and [3-^{13}C]lactate signals was performed with the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR, Fremont CA).

Calculation of [2-^{13}C] and [3-^{13}C]lactate Excess Enrichment and Pentose Cycle Flux From the ^{1}H Lactate Methyl Signal

In the absence of excess enrichment from [2-^{13}C]glucose, the lactate methyl ^{1}H signal under the conditions of the experiment (^{13}C-decoupling of carbon 1) consists of 10 signals. A doublet arising from ^{1}H-^{1}H coupling representing ^{12}C-lactate (D); a quartet arising from a combination of ^{1}H-^{1}H-coupling and one-bond ^{1}H-^{13}C coupling (Q3) arising from natural abundance [3-^{13}C]lactate; and a quartet arising from ^{1}H-^{1}H coupling and 2-bond ^{1}H-^{13}C coupling (Q2) arising from natural abundance [2-^{13}C]lactate. Assuming natural abundance ^{13}C levels of 1.1% and random distribution of ^{13}C among the lactate carbons, the relative intensities of the signals are the following: [^{12}C]lactate (D): 97.8%;* [3-^{13}C]lactate (Q3): 1.1% and [2-^{13}C]lactate (Q2): 1.1%. Based on these values, lactate Q2 and Q3 signals derived from [2-^{13}C]glucose metabolism were corrected for natural abundance contributions as follows:

$$Q2_{corr} = Q2 - (D \times 0.011/0.978)$$

$$Q3_{corr} = Q3 - (D \times 0.011/0.978)$$

$Q2_{corr}$ and $Q3_{corr}$ correspond to excess ^{13}C-enrichment in positions 2 and 3 of lactate. These are equivalent to ^{14}C-specific activities of carbons 2 and 3 of lactate derived from [2-^{14}C]glucose and can be applied to the equation of Schrader et al. (6) for obtaining estimates for the fraction of glucose metabolized by the PP.

$$Q3_{corr}/Q2_{corr} = 2PC/(1 + 2PC)$$

PC is the fraction of glucose-6-P consumed by the pentose pathway relative to the total (glycolysis + pentose pathway). Due to the recycling of pentose and hexose phosphates, for every glucose molecule metabolized by the pentose cycle, the flux of glucose-6-P through the oxidative branch of the PP pathway is three times that of PC (2). Each molecule of glucose-6-P that is oxidized to ribose-P generates two of NADPH, therefore the flux of NADPH generation and glutathione recycling is six times that of PC.

RESULTS AND DISCUSSION

As shown in Fig. 1 and inset (a), the methyl signal of lactate has well-resolved signals from ^{13}C-isotopomers formed as a result of [2-^{13}C]glucose metabolism through the glycolytic and PP pathways. Given the near-quantitative glucose conversion to lactate (data not shown) and the amount of supernatant (0.1 ml) harvested for analysis, we estimate that each NMR sample contained about 2 μmol of lactate. Contributions from [3-^{12}C]lactate (D), [2-^{13}C]lactate (Q2), and [3-^{13}C]lactate (Q3) are all present. In the absence of ^{13}C-decoupling of the lactate carbon 1 signal, the Q3 resonances showed additional splitting as a result of long-range coupling between carbon 1 and the methyl hydrogens (Fig. 1b). This confirms the generation of the [1,3-$^{13}C_2$]lactate isotopomer via transketolase-mediated exchange of ribose-5-phosphate and xylulose-5-phosphate (3). After accounting for the contribution from natural abundance ^{13}C-lactate, the contribution of [1,3-$^{13}C_2$]lactate and [3-^{13}C]lactate signals are approximately equal, indicating similar populations of these two isotopomers and twice the enrichment of lactate carbon 3 relative to carbon 1. This enrichment distribution is consistent with that of the theoretical lactate ^{14}C-specific activity from [2-^{14}C]glucose, as predicted by the classical PP model of Wood et al. (2). Also, the abolition of long-range ^{13}C-^{1}H splittings by carbon 1 decoupling indicates that the [2,3-$^{13}C_2$]lactate isotopomer was not formed in any significant quantities.** This in accord with the ^{13}C NMR study of Schrader et al. (6), where they found very low signal contributions from [2,3-$^{13}C_2$]lactate relative to [3-^{13}C]lactate. Resolution of [1,3-$^{13}C_2$]lactate and [3-^{13}C]lactate isotopomers could provide information on nonoxidative fluxes through the pentose cycle (3,6). However, this has to be balanced against the lower signal/noise and reduced precision of the lactate

*The methyl hydrogen of lactate is coupled to ^{13}C in all three positions. With decoupling of carbon 1, there is splitting from 1.1% natural abundance ^{13}C in positions 2 and 3 only, therefore D represents 100 – 2.2%, or 97.8% of the total ^{1}H methyl signal.

**Lactate carbons 1 and 2 have equal coupling constants to the lactate methyl protons. Therefore, in the absence of carbon 1 decoupling, [1,3-$^{13}C_2$]lactate and [2,3-$^{13}C_2$]lactate isotopomers have a common ^{1}H lactate methyl signal.

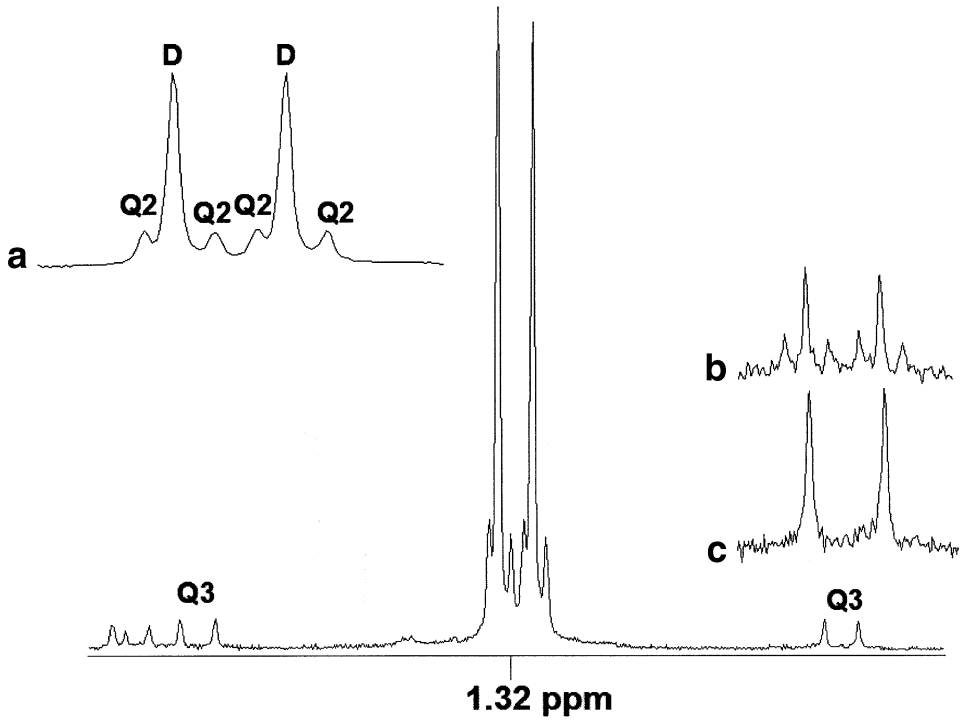

FIG. 1. ^{1}H NMR spectrum of the lactate methyl signal from 0.1 ml of supernatant that was obtained from an erythrocyte preparation treated with methylene blue. The spectrum features contributions from ^{12}C lactate (D), [2-^{13}C]lactate, (Q2) and [3-^{13}C]lactate, (Q3). **a:** An expansion of the D and Q2 components of the spectrum. Also shown is an expansion of the upfield Q3 components in the absence and presence of ^{13}C-decoupling of the carbon 1 signal of lactate (**b,c**, respectively).

carbon 3 ^{13}C-enrichment estimates under these conditions. Since our aim was to quantify oxidative PP flux with the maximum precision, we routinely applied ^{13}C-decoupling of lactate carbon 1, resulting in a common Q3 signal for both [1,3-$^{13}C_2$]lactate and [3-^{13}C]lactate (Fig. 1c).

In quiescent erythrocytes, enrichment of lactate carbon 3 relative to carbon 2 (C3/C2) was low, consistent with a basal PP activity in erythrocytes. Under these conditions, PP flux accounted for 7% of glucose consumption, in accord with previous reports of 6% (6) and 10% (8) from nonoxygenated erythrocyte preparations of similar hematocrit and glucose concentrations. Excess enrichment of lactate carbon 2 was ~25% instead of the expected 40–45%, indicating a dilution of ^{13}C-enrichment presumably due to incomplete washout of endogenous glucose and/or lactate during the preparation. The presence of endogenous lactate was also indicated by the finding that the total amount of lactate per NMR sample (~3 μmol) was in excess of the theoretical yield of ~2.2 μmol from glucose metabolism. In the presence of methylene blue, a classical activator of erythrocyte NADPH oxidation, lactate C3/C2 was substantially higher, reflecting a significant activation of PP flux and generation of NADPH (see Table 1). Under these conditions, PP flux accounted for 27% of total glucose consumption. This is in good agreement with an estimate of 24% obtained from erythrocytes incubated with 0.5 μmol methylene blue (8), but is substantially less than the 53% reported for oxygenated erythrocytes in the presence of 6.7 μmol methylene blue (6). The larger degree of PP flux stimulation and NADPH consumption in oxygenated erythrocyte preparations is likely due to the spontaneous recycling of methylene blue to its active oxidant form by oxygen (6).

If the natural abundance ^{13}C contribution was not subtracted from the measured ^{13}C-enrichments, fractional PP flux estimates were systematically inflated by ~10% (mean PP flux estimates based on lactate C3/C2 derived from uncorrected lactate ^{13}C-enrichment data were 0.09 ± 0.03 and 0.30 ± 0.11 for baseline and stimulated cells, respectively). Corrections for natural abundance will be more significant in cases where dilution of excess ^{13}C-enrichment by endogenous lactate is higher. Note that

Table 1
Lactate ^{13}C-enrichment and Relative Oxidative Pentose Phosphate Fluxes from H NMR Analysis of the Lactate Methyl Signal of Erythrocytes Incubated with [2-^{13}C]glucose under Baseline Conditions and Following Stimulation of Pentose Phosphate Pathway with Methylene Blue. Means and Standard Deviations Represent Five Measurements for each Condition

	Positional lactate ^{13}C-enrichment				Flux estimates (relative to glucose consumption of 1.0)	
	carbon 2	carbon 3	Corrected carbon 2	Corrected carbon 3	Fraction of glucose utilized by pentose pathway (PC)	NADPH generation
Baseline	0.24 ± 0.03	0.03 ± 0.01	0.23 ± 0.03	0.03 ± 0.01	0.07 ± 0.02	0.42 ± 0.12
Stimulated	0.20 ± 0.03	0.07 ± 0.01	0.19 ± 0.03	0.06 ± 0.01	0.27 ± 0.10*	1.62 ± 0.60*

*$P < 0.01$ compared to baseline.

while levels of excess lactate enrichment from [2-^{13}C]glucose are directly obtained from analysis of the ^{1}H methyl signal, the corresponding ^{13}C NMR signals would require additional calibration to account for natural abundance ^{13}C-contributions (6). Meanwhile, for applications beyond those described in this report, the method has some important limitations that need to be considered. Extensive suppression of the water signal is essential so that the relatively small metabolite signals can be maximally amplified. Also, the linewidths of the lactate methyl resonances need to be narrow (~0.5–0–7 Hz) to ensure adequate resolution of the [2-^{13}C]lactate and [^{12}C]lactate components. While these requirements are easily met with lyophilized extracts dissolved in D_2O, they cannot be realistically accomplished for in vivo lactate signals. Furthermore, the analysis can be compromised by the presence of signals from other metabolites whose chemical shifts coincide with either unlabeled lactate or its ^{13}C-isotopomer satellite signals. Depending on the field strength, this could include fairly ubiquitous metabolites such as alanine (CH_3 ^{1}H signal at 1.46 ppm) and β-hydroxybutyrate (CH_3 ^{1}H signal at 1.19 ppm). Under these conditions, either the lactate and interfering metabolites need to be chromatographically separated or the NMR measurement needs to be performed at a different magnetic field (not necessarily higher) where the lactate signals are resolved. Finally, regardless of the analytical method, PP flux estimates from [2-^{13}C]glucose will be erroneous to the extent that activities of other metabolic pathways such as gluconeogenesis and pyruvate recycling contribute to the randomization of ^{13}C between positions 2 and 3 of lactate.

In summary, a simple, sensitive, and robust method for analysis of lactate positional ^{13}C-enrichment is presented. This allows routine screening of pentose phosphate flux activity in small volumes of erythrocyte preparations incubated with [2-^{13}C]glucose.

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