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Notes & Tips

A method to measure lactate recycling in cultured cells by edited ^{1}H nuclear magnetic resonance spectroscopy

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Lactate plays pivotal roles in the metabolism of the brain, both as an intracellular energy substrate for oxidation in neural cells and as a shuttle intermediate for the transcellular metabolic coupling between neurons and astrocytes [1–6]. In particular, the classical astrocyte to neuron lactate shuttle hypothesis proposed a flux of lactate from astrocytes to neurons to support the energy demands occurring during glutamatergic neurotransmission [4,7]. However, the reversibility of the lactate dehydrogenase isozymes and the monocarboxylate transporters in the plasma membrane [8,9] indicates that lactate can both leave and return to the intracellular space, in a recycling process driven by the concentration of lactate inside and outside the cell and the transmembrane pH gradient. The lactate recycling process may become particularly important during cerebral activation events or in tumoral tissues, where intracellular production of lactate is thought to play a central role in cell energetics as a switch between glycolysis and oxidation of extracellular monocarboxylates [10,11]. However, despite its importance, lactate recycling through the plasma membrane remains a difficult process to investigate, given the identical nature of recycled and nonrecycled lactate molecules hindering their unambiguous identification.

To overcome these difficulties we proposed previously [10] a novel ^{13}C NMR methodology to investigate lactate recycling. Our method was based on the measurement of the turnover from the H2 proton of lactate because it is exchanged by ^{2}H only in the intracellular space of incubations containing heavy water [10]. We proposed subsequently [12] a more sensitive, nonedited, ^{1}H NMR approach, taking advantage of the greater sensitivity of ^{1}H NMR and the fact that it made unnecessary the use of relatively expensive ^{13}C-enriched substrates. Using this approach with C6 glioma cells metabolizing lactate in the presence of 50% $^{2}H_2O$ (vol/vol), we were able to identify clearly those lactate molecules that had passed through the cellular cytosol, since H2 deuteration was present, a circumstance necessarily involving intracellular lactate dehydrogenase activity and NAD(^{2}H). Unfortunately, the discrimination of these deuterated and nondeuterated lactate molecules by conventional nonedited ^{1}H NMR spectroscopy requires considerable NMR expertise, compromising an accurate quantification and requiring expert advise for its correct interpretation [12]. In this short report, we describe a novel, simple, and useful method to measure lactate recycling, maintaining the increased sensitivity of ^{1}H NMR spectroscopy and providing, an easy discrimination between H2 proton of nondeuterated and deuterated lactate molecules through the use of a specific lactate editing sequence [13,14].

C6 glioma cells (LGC Promochem, Barcelona, Spain) were grown to confluence in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (5%) (Gibco BRL, Gent, Belgium), 100 μg/mL streptomycin, 25 μg/mL gentamycin, 100 U/mL of penicillin and fungizone (1% vol/vol), in sterile petri dishes (10-cm diameter) at 37 °C in a humidified atmosphere containing 5% CO_2–95% O_2. Six independent cultures of C6 cells were used. Confluent cells (five cultures) were incubated (3–30 h, 37 °C) in Krebs–Henseleit Buffer (NaCl, 119 mM; KCl, 4.7 mM; $CaCl_2$, 1.3 mM; $MgSO_4$, 1.2 mM; Hepes, 15 mM; KH_2SO_4, 1.2 mM) containing 50% (vol/vol) $^{2}H_2O$ with 10 mM sodium lactate. Aliquots from the medium (1 mL) were collected after 3, 6, 9, 24, and 30 h of incubation, lyophilized, and resuspended in 0.5 mL $^{2}H_2O$

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(99.9% ^{2}H; Apollo Scientific Ltd., Stockport, Cheshire, UK) prior to high-resolution ^{1}H NMR analysis. Appropriate control experiments were performed with cell-free incubations or incubations carried out under similar cellular conditions (one culture) but in the absence of $^{2}H_2O$. All the substrates and reagents were of the highest purity available commercially from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

High-resolution ^{1}H NMR spectra from 1-mL aliquots of the incubation medium were obtained at 11.7 Tesla (500.130 MHz, 277 K, pH 7.2) with a Bruker Avance 500 wide bore NMR spectrometer using a commercial (5-mm) triple-resonance probe (^{1}H, ^{13}C, ^{2}H). As indicated above, the nonedited ^{1}H NMR spectra of these samples are complex, mainly because H3 proton resonances from both H2 nondeuterated and deuterated lactate molecules overlap at 1.31 ppm [12]. To resolve this overlap, we implemented the lactate editing method described to isolate lactate from the methylene resonance from lipids [14,15]. Briefly, the experiment comprises two separate acquisitions using a regular spin echo sequence with an echo time of 144 ms ($1/J$), which produces a phase modulation (J modulation) of 180° on the H2 and H3 resonances of lactate. In the first acquisition a selective decoupling pulse that destroys the phase modulation is applied on the H2 resonance of lactate. The second spectrum is acquired in the absence of the decoupling pulse and hence lactate resonances appear with a phase modulation of 180°. The summation of the two spectra cancels out the nondeuterated lactate resonances (modulated) whereas it adds the intensities of the other resonances (nonmodulated), including the H3-shifted singlet of H2-deuterated lactate.

^{1}H spectra were line broadened (0.3 Hz), zero filled, Fourier transformed, phased, and referenced to internal TSP[2] (0.0 ppm) at a concentration of 1 mM. Comparing the TSP area with those obtained in the edited spectra, we were able to easily quantify the time course of (2-^{2}H) lactate concentration. The program NUTS (Acorn, Freemont, CA, USA) was used to analyze the isotopic shifts and quantify the relative areas of shifted and nonshifted resonances. Finally, the ^{1}H NMR time course of H2 deuteration as detected with our editing sequence $C_{(t)}$ was fitted to a single exponential of the type $C_{(t)} = C_{(\infty)}(1-\exp(-k.t))$ to obtain the values of the rate constant k and the asymptote $C_{(\infty)}$. For this purpose we used the nonlinear least squares routines of the Sigma Plot program (SPSS Inc., Chicago, IL, U.S.A.).

Fig. 1 depicts representative ^{1}H NMR (500.130 MHz, 277 K, pH 7.2) nonmodulated (1), modulated (2), and edited (1 + 2) spectra obtained from the incubation medium of C6 glioma cells 30 h after the addition of lactate to the incubation buffer. At the beginning of the incubation, the edited lactate resonance from the lactate methyl group ($\delta = 1.31$ ppm) is virtually zero (not shown). The subsequent substitution of the lactate hydrogen of the H2 proton by one deuteron originates an upfield-shifted singlet in the H3 resonance, which can be clearly isolated and resolved in the edited spectrum. When the same experimental design was used in the cell-free incubations or in incubations without $^{2}H_2O$, this H2 deuteration was not observed (data not shown), confirming that this deuteration process is necessarily intracellular and requires $^{2}H_2O$ in excess of the natural abundance level.

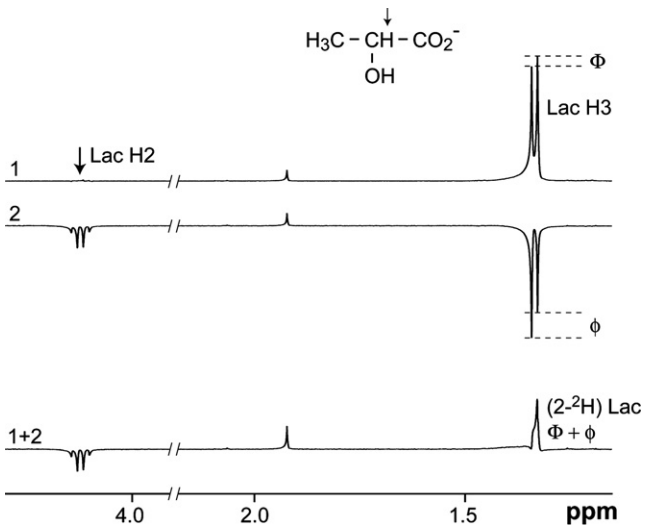

Fig. 1. Representative ^{1}H NMR spectra (500.130 MHz, 277 K, pH 7.2) nonmodulated (1), modulated (2) and edited (1 + 2) from the medium of C6 glioma cells incubated for 30 h with 10 mM lactate in Krebs–Henseleit buffer containing 50% $^{2}H_2O$ (vol/vol). The lactate H3 resonance doublet ($J = 6.9$ Hz) is observed at 1.31 ppm in spectrum 1. Note that the upfield moiety of the lactate doublet shows higher intensity (Φ) because of the overlap with the isotopically shifted methyl resonance of $^{2}H2$ lactate. The arrow symbolizes the selective decoupling pulse applied on the H2 proton resonance at 4.08 ppm (resonance not observable because of the saturation caused by the decoupling pulse). An echo time of 144 ms results in a 180° phase modulation on the nondeuterated lactate H2 and H3 resonances (inverted peaks in spectrum 2), which is not present in the non-modulated spectrum (1). Note that the upfield moiety of the lactate doublet depicts now lower intensity (ϕ). By adding spectra 1 and 2, we can readily observe a peak that represents the double of the concentration of (2-^{2}H) lactate ($\delta \approx 1.3$ ppm, $\Phi + \phi$).

Fig. 2 shows the time course of H2 deuteration in lactate, as detected with this spectral edition protocol in five experiments with different cultures of C6 cells. Since we used 1 mM TSP as an internal reference, it became possible to calculate quantitatively the increase in deuterated lactate concentration. The kinetics for the production of (2-^{2}H) lactate were fitted to a single exponential, providing a value for the rate constant k of 0.09 ± 0.01 h^{-1} and an asymptote value $C_{(\infty)}$ of 1.59 ± 0.07 mM (mean ± SE). These values are similar to those obtained by nonedited ^{1}H NMR [12].

In summary, this report shows how to distinguish nondeuterated lactate, from the (2-^{2}H) lactate, using a robust and easy to implement ^{1}H NMR editing method that allows the turnover of the H2 lactate hydrogen to be quantitatively investigated. Using this sequence, we were able to

[2] *Abbreviation used:* TSP, 2,2′,3,3′-tetradeutero trimethylsilyl propionate sodium salt.

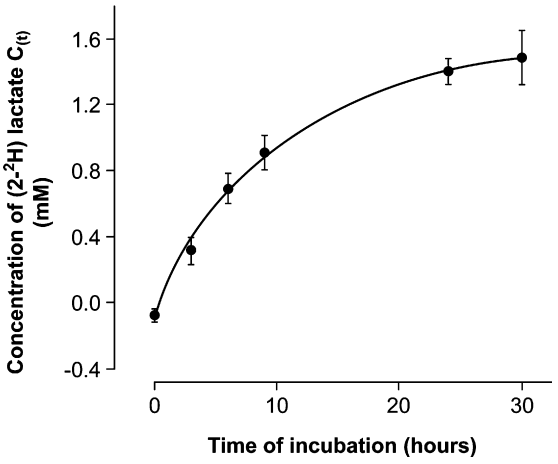

Fig. 2. Turnover of the lactate H2 during incubations with 10 mM lactate in Krebs–Henseleit buffer containing 50% ^2H_2O (vol/vol). Concentrations of (2-^{2}H) lactate for each time point were determined as described in the text. The experimental points $C_{(t)}$ were fitted nonlinearly to a single exponential $C_{(t)} = C_{(\infty)}$ $(1-\exp(-k.t))$ to determine the optimized $C_{(\infty)}$ and k parameter values. Results are the mean ± SE of five experiments with different cell cultures.

eliminate the spectral overlap in the H3 lactate doublet, allowing the H2 deuteration measurement with greater accuracy than previously possible with nonedited ^{1}H NMR. The proposed method may be of potential interest for *in vivo* studies of lactate turnover, where other available methodologies, such as ^{13}C NMR spectroscopy, are not only more expensive but also much more difficult to implement.

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