Measuring intracellular pH in the heart using hyperpolarized carbon dioxide and bicarbonate: a $^{13}$C and $^{31}$P magnetic resonance spectroscopy study

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Aims

Technological limitations have restricted in vivo assessment of intracellular pH (pHi) in the myocardium. The aim of this study was to evaluate the potential of hyperpolarized [1-13C]pyruvate, coupled with $^{13}$C magnetic resonance spectroscopy (MRS), to measure pHi in the healthy and diseased heart.

Methods and results

Hyperpolarized [1-13C]pyruvate was infused into isolated rat hearts before and immediately after ischaemia, and the formation of $^{13}$CO$_2$ and H$^{13}$CO$_3^-$ was monitored using $^{13}$C MRS. The HCO$_3^-$/$^{13}$CO$_2$ ratio was used in the Henderson–Hasselbalch equation to estimate pH$_i$. We tested the validity of this approach by comparing $^{13}$C-based pH$_i$ measurements with $^{31}$P MRS measurements of pH$_i$. There was good agreement between the pH$_i$ measured using $^{13}$C and $^{31}$P MRS in control hearts, being 7.12 ± 0.10 and 7.07 ± 0.02, respectively. In reperfused hearts, $^{13}$C and $^{31}$P measurements of pH$_i$ also agreed, although $^{13}$C equilibration limited observation of myocardial recovery from acidosis. In hearts pre-treated with the carbonic anhydrase (CA) inhibitor, 6-ethoxycarbonyl-1,2,4,5,6,7,8,9-octahydro-5H-carbazole-3-carboxylic acid (HOE 1093), the $^{13}$C measurement underestimated the $^{31}$P-measured pH$_i$ by 0.80 pH units. Mathematical modelling predicted that the validity of measuring pH$_i$ from the H$^{13}$CO$_3^-$/$^{13}$CO$_2$ ratio depended on CA activity, and may give an incorrect measure of pH$_i$ under conditions in which CA was inhibited, such as in acidosis. Hyperpolarized [1-13C]pyruvate was also infused into healthy living rats, where in vivo pH$_i$ from the H$^{13}$CO$_3^-$/$^{13}$CO$_2$ ratio was measured to be 7.20 ± 0.03.

Conclusion

Metabolically generated $^{13}$CO$_2$ and H$^{13}$CO$_3^-$ can be used as a marker of cardiac pH$_i$ in vivo, provided that CA activity is at normal levels.

Keywords

Magnetic resonance spectroscopy • Hyperpolarization • pH • Ischaemia • Carbonic anhydrase

1. Introduction

The rapid onset of acidosis is a well-documented characteristic of myocardial ischaemia.1,2 Under poor coronary perfusion, anaerobic glycolysis increases in the heart, producing intracellular protons and lactic acid that accumulate in the intra- and extracellular spaces.3 Some of the acid reacts with HCO$_3^-$ to form CO$_2$, which adds to any CO$_2$ generated by residual oxidative metabolism. Accumulation of protons, lactic acid, and CO$_2$ in the ischaemic heart decreases intracellular pH (pHi) from normal levels of around 7.1–7.2.1,2 Transient acidosis during ischaemia may be beneficial, as it decreases the major adenosine triphosphate (ATP) consumer, contractility, and thus conserves ATP for ion transport.4 However, the ATP reduction caused by severe and sustained ischaemia decreases Na$^+$,K$^+$-ATPase activity, which increases myocardial Na$^+$ levels. Increased Na$^+$ inhibits Ca$^{2+}$ extrusion via the Na$^+/Ca^{2+}$ exchanger, thus elevating myocardial Ca$^{2+}$ and damaging the myocardium.5

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31P magnetic resonance spectroscopy (MRS) has long been the gold standard for pHi measurement in the isolated perfused heart, based on the chemical shift of the inorganic phosphate (Pi) peak. However, 31P MRS cannot measure cardiac pH in vivo, because 2,3-diphosphoglycerate (2,3-DPG) in the ventricular blood contaminates the myocardial Pi peak. The pH-dependent equilibrium between bicarbonate and CO₂ has been used to measure extracellular pH (pHo) non-invasively in tumours. By infusing hyperpolarized 13C-bicarbonate intravenously, MR was used to image the distribution of hyperpolarized bicarbonate and CO₂ and a pH map was generated using the Henderson–Hasselbalch equation:

$$pH = pK_a + \log \left( \frac{\left[ \text{HCO}_3^- \right]}{\left[ \text{CO}_2 \right]} \right)$$

where pKa is the acid-dissociation constant of CO₂, which is 6.15 in the Krebs–Henseleit buffer.

For correct application of the Henderson–Hasselbalch equation, the following two conditions must be met:
(i) 13CO₂ to H¹³CO₃⁻ exchange kinetics, catalysed by carbonic anhydrase (CA), must be rapid and
(ii) 13CO₂ and H¹³CO₃⁻ signals must be detected simultaneously from the same cellular compartment.

In tumours, the Henderson–Hasselbalch equation was applied correctly because of the high CA activity on the surface of tumour cells and within erythrocytes, and the slow, transporter-mediated, cellular uptake of infused bicarbonate.

A similar approach may be useful for measuring pH in the in vivo heart. Infusion of hyperpolarized [1-13C]pyruvate results in mitochondrial production of hyperpolarized 13CO₂ by pyruvate dehydrogenase (PDH, Figure 1). Hyperpolarization by the dynamic nuclear polarization method increases the 13C MR sensitivity of pyruvate, and other 13C-labelled metabolites, more than 20 000-fold. Further, when a hyperpolarized metabolite is infused into tissue, the high-sensitivity 13C label is transferred to the tracer’s metabolic products, enabling unprecedented real-time visualization of the biochemical mechanisms of normal and abnormal metabolism. Only metabolic processes that occur rapidly can be monitored using hyperpolarized 13C MR methods because the hyperpolarized signal decays to thermal equilibrium according to its inherent spin–lattice relaxation time (in the case of [1-13C]pyruvate with a time constant of 50–60 s).

In theory, simultaneous detection of hyperpolarized [1-13C]pyruvate-derived 13CO₂ and H¹³CO₃⁻ could be used to measure pH. However, in cardiac myocytes, the conditions required for the correct use of the Henderson–Hasselbalch equation may not apply. As shown in Figure 1, metabolically generated CO₂ diffuses rapidly from its site of production into the cytosol, and subsequently into the extracellular space. Cardiac myocytes also have HCO₃⁻ transport activity, through proteins such as the Na⁺–HCO₃⁻ co-transporter and the Cl⁻/HCO₃⁻ exchanger. Studies of CA localization and kinetics in cardiac myocytes suggest low intracellular CA activity. Under the acidic conditions, typical of ischaemia, CA activity is expected to be even lower. Further, PDH flux post-ischaemia must be sufficiently high to enable MR detection of 13CO₂ and H¹³CO₃⁻ prior to decay of the hyperpolarized 13C MR signal.
The aim of the present study was to evaluate the potential of hyperpolarized $^{13}$C MR for the non-invasive measurement of pH in the heart. We measured PDH flux and the production of $^{13}$CO$_2$ and H$^{13}$CO$_3^-$ in isolated hearts before and after ischaemia and with CA activity inhibited. We used mathematical modelling to determine whether, and under what conditions, the H$^{13}$CO$_3^-$/$^{13}$CO$_2$ ratio could be used to measure cardiac pH. Finally, we measured pH$_i$ in the in vivo rat heart.

2. Methods

2.1 The isolated perfused rat heart

All investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (HMSO), and to institutional guidelines. Male Wistar rats (≈300 g) were anaesthetized using a 0.7 mL ip injection of pentobarbital sodium (200 mg/mL Euthetal). The beating hearts were quickly removed and arrested in the ice-cold Krebs–Henseleit perfusion buffer, and the aorta was cannulated for perfusion in recirculating retrograde Langendorff mode at a constant 85 mmHg pressure and 37°C temperature.

The Krebs–Henseleit bicarbonate perfusion buffer contained 1.2 mM inorganic phosphate (KH$_2$PO$_4$), 11 mM glucose, and 2.5 mM pyruvate and was aerated with a mixture of 95% oxygen (O$_2$) and 5% carbon dioxide (CO$_2$) for 20 min immediately before and 9 min during ischaemia. Hyperpolarized $^{13}$C pyruvate was infused immediately after ischaemia, such that hearts were repolarized with hyperpolarized tracer. In some hearts (n = 6), $^{31}$P MRS was performed throughout the reperfusion period. In other hearts (n = 6), $^{13}$C MRS was performed for 2 min during initial reperfusion with hyperpolarized $[1-^{13}$C$]$pyruvate, followed by 10 min of $^{31}$P MR spectral acquisition. For details of the $[1-^{13}$C$]$pyruvate preparation and delivery, see Supplementary material online, S2.

2.2 Experimental protocols

2.2.1 Control protocol

Isolated hearts (n = 6) were perfused for ~30 min at 85 mmHg. For the initial 20 min, $^{31}$P MRS was used to measure pH. After this, hyperpolarized $[1-^{13}$C$]$pyruvate was infused and the progress of $^{13}$C-labelled compounds was followed using $^{13}$C MRS.

2.2.2 Inhibition of CA

Hearts (n = 5) were perfused for ~30 min. After 10 min of perfusion in normal buffer, the hearts were switched to buffer containing 100 μM ETZ. $^{31}$P MRS was performed for 20 min (10 min before and 10 min after switch over to ETZ-containing buffer). Ten minutes after the start of ETZ perfusion, hyperpolarized $[1-^{13}$C$]$pyruvate was infused and MRS was switched from $^{31}$P to $^{13}$C.

2.2.3 Reperfusion following ischaemia

Hearts (n = 12) were perfused for ~30 min, followed by 10 min of total, global ischaemia, and 15 min reperfusion. $^{31}$P MRS spectra were acquired for 20 min immediately before and 9 min during ischaemia. Hyperpolarized $[1-^{13}$C$]$pyruvate was infused immediately after ischaemia, such that hearts were repolarized with hyperpolarized tracer. In some hearts (n = 6), $^{31}$P MRS was performed throughout the reperfusion period. In other hearts (n = 6), $^{13}$C MRS was performed for 2 min during initial reperfusion with hyperpolarized $[1-^{13}$C$]$pyruvate, followed by 10 min of $^{31}$P MR spectral acquisition. For details of the $[1-^{13}$C$]$pyruvate preparation and delivery, see Supplementary material online, S2.

2.3 Magnetic resonance spectroscopy

$^{31}$P MR spectra were acquired at 202.5 MHz using a 30° radiofrequency (RF) pulse and a repetition delay of 0.25 s. The phosphocreatine (PCr) resonance was set at 0 ppm and the chemical shifts of all peaks were referenced to that of PCr. Each spectrum consisted of 120 transients, giving a total acquisition time of 30 s. As these partially saturated spectra had shorter repetition times than the longitudinal relaxation time of $^{31}$P nuclei, an unsaturated spectrum was initially acquired from the hearts using a 90° pulse with repetition time of 15 s and 40 transients, and an acquisition time of 10 min. The unsaturated spectra were used to correct metabolite concentrations for the effects of saturation.

Acquisition of $^{13}$C MR spectra commenced immediately after infusion of hyperpolarized $[1-^{13}$C$]$pyruvate and $[1-^{13}$C$]$pyruvate infusion continued throughout acquisition. Spectra were acquired with 1 s temporal resolution over 2 min (excitation flip angle = 30°, 120 acquisitions). Spectra were centred at 171 ppm and referenced to the $[1-^{13}$C$]$pyruvate resonance at 171 ppm, and 4096 points were acquired over a bandwidth of 100 ppm.

In vivo hyperpolarized $[1-^{13}$C$]$pyruvate MRS experiments were performed as described previously. Briefly, $[1-^{13}$C$]$pyruvic acid was hyperpolarized and dissolved/neutralized in a prototype $^{13}$C polariser system. Each living rat (n = 6) was positioned at the isocentre of a 7 T Variian horizontal bore MR scanner, with a dual-tuned $^{1}$H/$^{13}$C coil localized over the animal’s chest. Aqueous hyperpolarized $[1-^{13}$C$]$pyruvate (80 μmol) was then infused into a living rat via the tail vein over 10 s, and cardiac $^{13}$C spectra were acquired with a low 7.5° flip angle every second for 1 min. For further details of in vivo hyperpolarized $[1-^{13}$C$]$pyruvate MRS experiments, refer to Supplementary material online, S3.

2.4 Data analysis

2.4.1 Carbon-13

Cardiac $^{13}$C MR spectra were analysed using the AMARES algorithm, as implemented in the jMRUI software package. Spectra were D.C. offset corrected based on the last half of acquired points and peaks corresponding with $[1-^{13}$C$]$pyruvate and its metabolic derivatives were fitted assuming a Lorentzian line shape, initial peak frequencies, relative phases, and linewidths.

For spectra acquired from perfused rat hearts, the maximum peak area of each metabolite over the 2 min of acquisition was determined for each series of spectra and expressed as a percentage of the maximum $[1-^{13}$C$]$pyruvate resonance. The rate of signal production for each metabolite, in percent per second (%/s), was measured as the slope of the mean metabolite increase over the first 5 s following its appearance, over which time the metabolite signal increased linearly. Additionally, a first-order exponential signal decay term was fit to each metabolite peak from the point of maximum signal over the course of signal decay. Decay of the hyperpolarized signal depends on the intrinsic spin–lattice relaxation of the nucleus, production and consumption rates of the metabolite, and metabolite washout, and may therefore provide information about metabolite accumulation in the states of no-flow ischaemia and CA inhibition.

Average time courses for H$^{13}$CO$_3^-$, H$^{13}$CO$_2^-$ and their sum were calculated for all hearts for further data analysis. H$^{13}$CO$_2^-$ plus H$^{13}$CO$_3^-$ was normalized to the maximum pyruvate peak area to allow for any differences in polarization, was used as a qualitative indicator of PDH flux. The average H$^{13}$CO$_3^-$ and H$^{13}$CO$_2^-$ time courses were inserted into an applied form of the Henderson–Hasselbalch equation:

$$pR = 6.15 + \log \left( \frac{[H^{13}CO_3^-]}{[H^{13}CO_2^-]} \right)$$  (2)

The output of Eq. (2) is a variable pR which should, under the two conditions outlined in Section 1, measure pH. Upon the initial arrival of $[1-^{13}$C$]$pyruvate, the relative proportions of $^{13}$CO$_2$ and $^{13}$CO$_3$ (and thus pR) equilibrated over several seconds to reach a
steady-state value. The calculated pR was fit to a first-order exponential equation to determine the steady-state value and time constant.

2.4.2 Phosphorus-31
Cardiac $^{31}$P MR spectra were analysed using the AMARES algorithm in the jMRUI software package. Spectra were corrected for DC offset using the last half of acquired points. The PCr, P, α-, β-, and γ-ATP resonances were fitted assuming a Lorentzian line shape, peak frequencies, relative phases, linewidths, and J-coupling parameters. pHi was calculated from the Pi chemisorption assuming a Lorentzian line shape, peak frequencies, relative phases, and chemical shift. Absolute $^{31}$P metabolite concentrations were calculated using the least-squares method fitted to a first-order exponential equation to determine the steady-state value. The calculated pR was fit to a first-order exponential equation.

2.4.3 Modelling
A system of ordinary differential equations was formulated to test the suitability of using the CO$_2$–HCO$_3^-$ equilibrium to measure pHi. For details of the mathematical model, see Supplementary material online, S4.

2.4.4 Statistical methods
Data are given as mean ± standard error. Statistical significances between pre- and post-ischaemic groups, and pre-ischaemic and ETZ-perfused groups, were assessed using a paired Student’s t-test. Statistical significance was considered at P < 0.05.

3. Results

3.1 Myocardial energetics
Cardiac function and $^{31}$P MR spectra were characteristic of the isolated rat heart during pre-ischaemia, ischaemia, and reperfusion. A description of cardiac function throughout the protocol and an example of a $^{31}$P spectrum of a heart before ischaemia are shown in Supplementary material online, S5. Pre-ischaemia, the average [ATP] was 10.6 ± 0.7 mM and [PCr] was 19.7 ± 0.9 mM (Figure 2). Two minutes after stopping coronary flow, [PCr] decreased to 3.2 mM, to remain at 1.1–2.1 mM for the remainder of ischaemia. The rate of [ATP] hydrolysis during ischaemia was 0.14 ± 0.10 mM/min. Five minutes after reperfusion, PCr had recovered to 17.6 ± 1.9 mM, whereas ATP remained at 8.2 ± 2.5 mM.

Perfusion with ETZ had no effect on [ATP] or [PCr] throughout the perfusion protocol (data not shown). Prior to ETZ perfusion, hearts had an average PCr of 17.8 ± 1.9 mM and ATP of 10.6 ± 0.5 mM. During ETZ perfusion, the average [PCr] was 18.0 ± 1.5 mM and [ATP] was 10.3 ± 0.9 mM.

3.2 PDH flux
A representative spectrum of [1-$^{13}$C]pyruvate in the perfused heart, and the typical kinetic progression of [1-$^{13}$C]pyruvate metabolites, is shown in Figure 3. Following infusion of [1-$^{13}$C]pyruvate into control hearts, [1-$^{13}$C]lactate (183.2 ppm), H$_3^{13}$CO$_2^-$ (160.9 ppm), and [1-$^{13}$C]alanine (176.5 ppm) were clearly detectable with high signal compared with the baseline. A resonance corresponding to $^{13}$CO$_2$ was also visible, with 1 s temporal resolution, at a chemical shift of 124.5 ppm. The initial rates of production and the maximum peak areas for the [1-$^{13}$C]pyruvate-derived metabolites, in pre-ischaemic, ETZ, and reperfused hearts, are given in Table 1.

The maximum peak areas of H$_3^{13}$CO$_2^-$, $^{13}$CO$_2$, and their sum were not significantly different from baseline when [1-$^{13}$C]pyruvate was infused into the myocardium upon reperfusion. However, the initial rate of H$_3^{13}$CO$_2^-$ plus $^{13}$CO$_2$ production was 54% slower upon reperfusion, compared with the pre-ischaemic myocardium, as indicated by the slope of the reperfusion peaks shown in Figure 4. Additionally, the decay rate of hyperpolarized $^{13}$CO$_2$ signal was 30% faster in reperfused hearts than in pre-ischaemic hearts (P < 0.001), indicating enhanced CO$_2$ washout upon re-flow after ischaemia.

ETZ had no significant effect on the initial rate of H$_3^{13}$CO$_2^-$ plus $^{13}$CO$_2$ production, or the maximum peak area of the sum of H$_3^{13}$CO$_2^-$ and $^{13}$CO$_2$, compared with pre-ischaemic hearts (Figure 4). However, ETZ increased the maximum $^{13}$CO$_2$ peak area by four-fold, whereas decreasing the maximum H$_3^{13}$CO$_2^-$ peak area by two-fold (Table 1, P < 0.001). Additionally, the decay rate of hyperpolarized $^{13}$CO$_2$ signal was 19% faster in reperfused hearts than in pre-ischaemic hearts (P < 0.05), possibly indicating enhanced CO$_2$ diffusion out of myocytes in the absence of CA activity.

3.3 Measurement of pH$_i$ in the isolated perfused heart
Figure 5A shows the changes in H$_3^{13}$CO$_2^-$ and $^{13}$CO$_2$, both normalized to the maximum [1-$^{13}$C]pyruvate signal, which were used for the
calculation of pR. When hyperpolarized [1-13C]pyruvate reached the isolated heart, metabolically generated H13CO3 and 13CO2 were out of equilibrium for 7.12 ± 0.10 (Figure 5B). Fully relaxed 31P measurements, acquired in the pre-ischaemic heart, gave a pH of 7.07 ± 0.02. The pH measured using 31P MRS and the 95% confidence interval are overlaid on the 13C results in Figure 5B.

31P MRS confirmed that CA inhibition with ETZ had no effect on steady-state myocardial pH (pH of 7.02 ± 0.03 before ETZ treatment and 7.00 ± 0.04 after ETZ treatment). Perfusion with [1-13C]pyruvate and ETZ generated more 13CO2 than H13CO32 (Figure 5C) with no change in total H13CO32 plus 13CO2. The pR, calculated from the H13CO32/CO2 ratio, stabilized within 20 s to a steady-state pR of 6.21 ± 0.13 (Figure 5D). Thus, inhibition of CA activity slowed CO2 –HCO32 conversion, as shown by the lengthening of the out-of-equilibrium period, but also by the steady-state pR which was 0.79 pH units below the pH measured using 31P MRS.

In reperfused hearts, 31P MRS revealed that pH recovered from a value of 6.49 ± 0.04 at the end of ischaemia to 7.04 ± 0.13, at a rate of 0.73 pH units/min during the 45 s immediately after reflow (Figure 6). In hearts reperfused with hyperpolarized [1-13C]pyruvate, the pR from the H13CO32/CO2 ratio was the same as pH, from 31P MRS after 15 s of reperfusion, when averaged into 30 s segments that corresponded with acquisition of 31P spectra. After 45 and 75 s, both 13C and 31P measurements gave almost identical pH measurements (13C: 7.01 ± 0.01 at 45 s and 6.98 ± 0.02 at 75 s; 31P: 7.04 ± 0.13 at 45 s and 7.00 ± 0.04 at 75 s, Figure 6).

### 3.4 Mathematical modelling of experimental results

Results of the mathematical model of 13CO2 production, efflux, and hydration to H13CO3 by CA are depicted in Figure 7. Figure 7A shows the output of the model that best-fits the experimental data presented in Figure 5. Constants KCO2 (10^-6.15 M), k_f (0.14 s^-1), and k_r (k_f/KCO2) were obtained from published values21 and other parameters were obtained by least-squares fitting: P_pyr (0.2 s^-1), P_CO2 (0.2 s^-1), p (0.006 s^-1), α (1/33 s for pyruvate, 1/6 s for CO2 and HCO3^-). The best simulation of our experimental 13CO2 and H13CO32 results (Figure 7A) indicated that CA activity (γ) enhanced the conversion rate of 13CO2 into H⁺ + H13CO32 by 10-fold in pre-ischaemic hearts. This value was in line with an in vitro study that reported CA-enhanced CO2 hydration by five-fold in isolated myocytes.21

Figure 7B shows the value of pR [Eq. (2)] derived from the simulations in Figure 7A. At normal CA activity (γ = 10), pR approached 6.8 within 17 s, giving a reasonable approximation to the real pH of 7.1. However, in the absence of CA activity (γ = 1), pR approached a significantly lower asymptote of 6.1 within 24 s.

Apart from CA activity, another factor that can disturb the equilibrium between H⁺, HCO3^- and CO2 is HCO3^- transport. Figure 7C and D illustrates the implications of HCO3^- extrusion and uptake, respectively, on the steady-state value of pR. In the presence of ± 5 mM/min transmembrane HCO3^- flux, the value of pR was not greatly altered, compared with a model with no net HCO3^- transport.
It is noteworthy that hyperpolarized H\textsuperscript{13}CO\textsubscript{3} is only a small fraction of total HCO\textsubscript{3}, and only a minor fraction of transmembrane HCO\textsubscript{3} efflux would be labelled with hyperpolarized 13C.

Further modelling explored the relationship between pH\textsubscript{i} and the time required for equilibration pR, 1 s in vivo spectra were averaged in groups of two to yield a set of spectra with 2 s temporal resolution and the SNR improved to 16.9 ± 3.5 and 2.0 ± 0.4 for H\textsubscript{13}CO\textsubscript{3} and \textsuperscript{13}CO\textsubscript{2}, respectively. Using the averaged spectra, pR reached a steady-state value of 7.20 ± 0.03, as shown in Figure 8B.

### 4. Discussion

#### 4.1 PDH flux before and after ischaemia

To study the CO\textsubscript{2}/HCO\textsubscript{3} equilibrium, PDH flux must be sufficient to generate MR-detectable levels of \textsuperscript{13}CO\textsubscript{2}. Therefore, our first aim was to determine the effect of ischaemia on pyruvate oxidation. Others have studied PDH flux upon reperfusion of the ischaemic myocardium, with diverse results depending on the ischaemic model and the perfusion conditions.\textsuperscript{17,24,29-31} Kobayashi and Neely\textsuperscript{29} observed that pyruvate plus glucose perfusion largely maintained PDH activity in the isolated reperfused heart, and in vivo PDH activity was maintained following reduction of coronary flow in swine.\textsuperscript{32} However, in the isolated rat heart perfused with pyruvate alone or pyruvate and fatty acids, ischaemia decreased PDH activity and glucose oxidation for several minutes following reperfusion.\textsuperscript{17,30,31}

Here, 10 min of total global ischaemia decreased the rate of production of H\textsubscript{13}CO\textsubscript{3} plus \textsuperscript{13}CO\textsubscript{2} from 0.57 ± 0.06 to 0.26 ± 0.05%/s, indicating a decrease in the initial rate of pyruvate oxidation, and thus inhibition of PDH activity in reperfusion. However, a significant decrease in the total H\textsubscript{13}CO\textsubscript{3} and \textsuperscript{13}CO\textsubscript{2} produced was not observed, suggesting that PDH flux recovered to control levels within 30 s. Most importantly, sufficient \textsuperscript{13}CO\textsubscript{2} was produced at the start of reperfusion to allow the H\textsubscript{13}CO\textsubscript{3}/\textsuperscript{13}CO\textsubscript{2} ratio to be measured, which, under appropriate conditions, may be used to estimate pH\textsubscript{i}.

#### 4.2 CO\textsubscript{2}/HCO\textsubscript{3} equilibrium as a measure of cardiac pH\textsubscript{i}

We converted the H\textsubscript{13}CO\textsubscript{3}/\textsuperscript{13}CO\textsubscript{2} ratio in the isolated perfused rat heart, and in the in vivo rat heart, into a variable, pR, using the Henderson–Hasselbalch equation. At steady-state, pR in the isolated perfused rat heart was 7.12 ± 0.1, similar, within the noise inherent in

### Table 1 Metabolite levels and kinetic parameters from \textsuperscript{13}C MR spectra in pre-ischaemia, reperfused, and ETZ-perfused isolated hearts

|            | [\textsuperscript{1-13}C]Lactate |            | [\textsuperscript{1-13}C]Alanine |
|------------|---------------------------------|------------|---------------------------------|
|            | Pre-ischaemia                    | Reperfusion| ETZ                            |
| Maximum metabolite/pyruvate (%) | 6 ± 1                           | 31 ± 3\textsuperscript{3} | 7 ± 2                           |
| Initial production rate (%/s)   | 0.7 ± 0.1                       | 4.4 ± 0.4\textsuperscript{2} | 0.7 ± 0.2                       |
| Decay, \(\tau\) (s)             | 35 ± 4                          | 22.3 ± 0.2\textsuperscript{2} | 41 ± 5                          |

|            | [\textsuperscript{13}CO\textsubscript{2}] |            |
|------------|-------------------------------------------|------------|
|            | Pre-ischaemia                             | Reperfusion| ETZ |
| Maximum metabolite/pyruvate (%) | 4.7 ± 0.6                      | 3.8 ± 0.4   | 2.1 ± 0.2\textsuperscript{2} |
| Initial production rate (%/s)   | 0.49 ± 0.06                      | 0.21 ± 0.00\textsuperscript{2} | 0.14 ± 0.02\textsuperscript{2} |
| Decay, \(\tau\) (s)             | 43 ± 4                           | 35 ± 2      | 44 ± 4                           |

Data are expressed means ± SEM. All metabolite levels, and initial production rates, are expressed as a percentage of maximum [\textsuperscript{1-13}C]pyruvate signal. Significant difference from pre-ischaemic hearts: *\(P < 0.05\) and †\(P < 0.001\).
each measurement, to the pH of 7.07 ± 0.02 measured using 31P MRS. When measured in rat hearts in vivo, pR was 7.20 ± 0.03. These values are similar to those of Merritt et al.,17 and with pH measured by others using 31P MRS.8–10,20,28 Thus, hyperpolarized [1-13C]pyruvate can be used to obtain an accurate, non-invasive measurement of cardiac pH in vivo in healthy hearts.17

As a first test of the suitability of pR to measure pH, we inhibited cardiac CA activity in perfused rat hearts, without altering pH. We found a significant difference between the steady-state pR of 6.21 and the pH of 7.01 determined using 31P MRS. Low pH, such as that observed during myocardial ischaemia, inhibits CA activity.22,23 Thus, the H13CO3−/13CO2 ratio would not be a good measure of pH in the ischaemic/reperfused heart without correction for low CA activity.

By modelling our H13CO3− and 13CO2 results, we identified the conditions in which pR was not a valid measure of pH. Provided that sufficient 13CO2 is generated via PDH flux, factors that alter the rate of CO2 production (Ppyr, p) will not alter steady-state pR. Likewise, CO2 efflux (Pco2) does not affect steady-state pR when the heart is perfused to the extent that extracellular 13CO2 is washed away rapidly. Changes to CO2 permeability will alter CO2 and HCO3− levels in parallel and will not affect steady-state pR.

A discrepancy between pR and pH occurred with changes in the rate of CO2 hydration and events related to HCO3− and H+. The
The rate of CO₂ hydration depends on CA activity. CA activity in cardiac myocytes is modest 21 and, furthermore, CA is inhibited by low pHi 22,23 and by pharmacological membrane-transport inhibitors. 33 Consequently, CO₂ hydration kinetics have an impact on the suitability of pR as a measure of pHᵢ. The importance of CA activity was tested using a mathematical model (Figure 7). At low CA activity, pR attained a steady-state that could be very different from pHᵢ, and the equilibration time could be a significant fraction of the lifetime of hyperpolarized ¹³C or of recovery from ischaemia-induced acidosis.

Figure 7  The results of mathematically modelling our experimental data, acquired from control hearts and hearts perfused with ETZ. (A) Results from the model which best-fit our experimental data from Figures 4A and 5A. Experimental data were best reproduced with a 10-fold catalytic activity of CA. (B) The model simulation of pR based on the time courses from (A). With moderate levels of CA activity, as may be expected in the heart, the H¹³CO₃⁻/¹³CO₂ ratio indicates a steady-state pR that closely approximates the physiological values. However, with lower CA activity, the model reproduced our experimental finding of pHᵢ underestimation. (C) The relationship between CA activity and steady-state pR, in the presence (dashed grey) and absence (black solid) of HCO₃⁻ efflux or (D) influx. CA activity has a significant effect on the size of the pR–pHᵢ discrepancy, but HCO₃⁻ transport has a much smaller impact on the discordance. (E) Relationship between steady-state pR and pHᵢ, simulated for different levels of CA activity. (F) Relationship between equilibration time and pHᵢ, simulated for different levels of CA activity. Equilibration time was estimated as the time taken for pR to approach steady-state pR within 0.05 U.

4.3 Limitations of the study

To measure pHᵢ using [¹³C]pyruvate, it is essential that the metabolically generated H¹³CO₃⁻ and ¹³CO₂ resonances can be accurately quantified above the baseline noise. At a physiological pH of ~7, the H¹³CO₃⁻ resonance is 10-fold larger than the ¹³CO₂ resonance, so ¹³CO₂ quantification requires efficient hyperpolarization, high Pdh flux, and careful data acquisition. An increase in achievable polarization, from the ~30% observed here to ~60%, has recently been reported 19 and will aid ¹³CO₂ quantification. Also, strategic data acquisition after the ¹³C equilibration period, over a shorter duration, and with a higher excitation flip angle may further increase the ¹³CO₂ signal.
A second limitation of this study is the fact that the hyperpolarized label cannot directly distinguish between metabolites located in the intra- and extracellular spaces. We can be certain that due to high cardiac oxidative rates, which are more than an order of magnitude higher than any neighbouring tissue (i.e. liver, resting skeletal muscle, adipose tissue, diaphragm, or blood), virtually all of the detected H13CO3\(^+\) and 13CO2 signal was produced within the myocardium. However, it is possible that trace amounts of 13CO2 may have diffused out of the myocardium and were subsequently hydrated to form H13CO3\(^+\) either spontaneously, by extracellular cardiac CA, or in vivo, by CA in red cells. However, we believe that the contribution of the extracellular H13CO3\(^+\) and 13CO2 signal to our pH measurement was small because: (i) in vivo spectroscopic 13C images acquired of the heart\(^{34}\) have indicated that H13CO3\(^+\) is confined to the myocardium, a region dominated by the intracellular space; (ii) in the perfused heart, high coronary flow rates (~20 mL/min) would have rapidly removed hyperpolarized metabolites, and in vivo association of 13CO2 with haemoglobin would have caused rapid decay of hyperpolarized MR signal; and (iii) the close agreement between pR measured with 13C and pH measured with 31P in the perfused heart indicated minimal contamination from extracellular H13CO3\(^+\) and 13CO2, as these species would have equilibrated according the pHo of 7.4.

### 4.4 Significance of this work

Currently, the non-invasive measurement of cardiac pH\(i\) in humans is impossible, because blood 2,3-DPG signal overlies the myocardial P\(i\) signal.\(^{6,9,10}\) Here, we have shown that in the presence of endogenous CA activity, the H13CO3\(^+\)/13CO2 ratio accurately measured pH\(i\) in the isolated perfused heart. Further, we have demonstrated that following infusion of hyperpolarized [1-13C]pyruvate into healthy rats in vivo, the MR signal corresponding to H13CO3\(^+\) and 13CO2 could both be quantified, and that their ratio indicated a pH\(i\) value of 7.20, which is in line with invasive measurements.\(^{9,10}\) Consequently, it seems that metabolically generated H13CO3\(^+\) and 13CO2 may offer the first technique for the non-invasive measurement of pH\(i\), in normal hearts, and in diseased hearts with normal or elevated CA activity.\(^{35}\) Measuring in vivo pH\(i\) also implies that other analyses of myocardial energetics may be performed in vivo, including calculation of free ADP concentrations and the free energy available from the hydrolysis of ATP, \(\Delta G_{ATP}\).\(^{5,10}\) Future work will involve correlating in vivo measurements of the H13CO3\(^+\)/13CO2 ratio with pH\(i\) measurements made using invasive, blood-removed or open-chest techniques.\(^{9,10}\)

Since acidosis is a characteristic feature of ischaemia, assessment of ischaemic heart disease in humans is another potentially useful application of a non-invasive pH\(i\) measurement using the H13CO3\(^+\)/13CO2 ratio. We observed excellent agreement between pH\(i\) measured using 31P MRS and pR measured using 13C MRS in the reperfused myocardium when pH\(i\) was \(\geq 6.74\) (Figure 6). However, multiple factors shift pR relative to pH\(i\), including inhibition of CA activity at low pH\(i\),\(^{22,23}\) and stimulation of membrane transport during reperfusion following ischaemia.\(^{5,26}\) Pharmaceutical agents, such as cariporide, inhibit membrane ion transport and have been used clinically to reduce ischaemia–reperfusion injury,\(^{37}\) but also block CA.\(^{33}\) Therefore, the use of the H13CO3\(^+\)/13CO2 ratio to measure pH\(i\) may not be valid in ischaemic, acidic hearts, and in patients with ischaemic heart disease who use drugs that inhibit membrane ion transport. Additionally, it is reasonable to expect that intracellular CA expression and activity may be either reduced or increased in other forms of cardiomyopathy.\(^{35}\) Correction of pR to pH\(i\) will require...
full characterization of CA activity in each pathophysiological state, and mathematical deconvolution of the $^{13}$C equilibration period from the true measured pH$_i$ changes. Eventual translation of the $\text{H}^{13}\text{CO}_3^-/\text{H}^{13}\text{CO}_2$ ratio to measure pH$_i$ in the clinic will require considerable technological advances, in terms of improved methods and hardware for acquisition of $^{13}$C images, and access to affordable hyperpolarization tools and $^{13}$C-labelled compounds. In order to identify focal regions of ischaemia using pH$_i$ measurements from hyperpolarized $[1-^{13}\text{C}]$pyruvate, for example, three-dimensional images of $^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$ with relatively high spatial resolution across the area at risk will be required. The feasibility of acquiring such data across the myocardium of large animals, and therefore patients, has been demonstrated. Further, although the eventual cost of clinical application of the hyperpolarized $^{13}$C MR technology is not clear, it does not appear set to be prohibitive. Clinical polarizers could be operated as standalone systems, placed within existing clinical MR facilities and interfaced to existing MR scanners. Further, the cost of $[1-^{13}\text{C}]$pyruvic acid, as used here, is not excessive and would be in line with contrast agents used in other imaging modalities, such as positron emission tomography.

In summary, we have demonstrated in the perfused heart that the $\text{H}^{13}\text{CO}_3^-/\text{H}^{13}\text{CO}_2$ ratio offers an accurate method to measure cardiac pH$_i$ in hearts with normal or elevated CA activity. Further, the technique appears set to become the first clinically relevant measure of in vivo cardiac pH$_i$, although future work is warranted to characterize CA activity and the response of the $\text{H}^{13}\text{CO}_3^-/\text{H}^{13}\text{CO}_2$ ratio in ischaemia and other cardiomyopathies, and to improve the sensitivity and spatial resolution of $^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$ detection.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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