

RESEARCH ARTICLE

Real-time influence of intracellular acidification and Na^+/H^+ exchanger inhibition on in-cell pyruvate metabolism in the perfused mouse heart: A ^{31}P -NMR and hyperpolarized ^{13}C -NMR study

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Abstract

Disruption of acid–base balance is linked to various diseases and conditions. In the heart, intracellular acidification is associated with heart failure, maladaptive cardiac hypertrophy, and myocardial ischemia. Previously, we have reported that the ratio of the in-cell lactate dehydrogenase (LDH) to pyruvate dehydrogenase (PDH) activities is correlated with cardiac pH. To further characterize the basis for this correlation, these in-cell activities were investigated under induced intracellular acidification without and with Na^+/H^+ exchanger (NHE1) inhibition by zoniporide. Male mouse hearts ($n = 30$) were isolated and perfused retrogradely. Intracellular acidification was performed in two ways: (1) with the NH_4Cl prepulse methodology; and (2) by combining the NH_4Cl prepulse with zoniporide. ^{31}P NMR spectroscopy was used to determine the intracellular cardiac pH and to quantify the adenosine triphosphate and phosphocreatine content. Hyperpolarized $[1-^{13}\text{C}]\text{pyruvate}$ was obtained using dissolution dynamic nuclear polarization. ^{13}C NMR spectroscopy was used to monitor hyperpolarized $[1-^{13}\text{C}]\text{pyruvate}$ metabolism and determine enzyme activities in real time at a temporal resolution of a few seconds using the product-selective saturating excitation approach. The intracellular acidification induced by the NH_4Cl prepulse led to reduced LDH and PDH activities (–16% and –39%, respectively). This finding is in line with previous evidence of reduced myocardial contraction and therefore reduced metabolic activity upon intracellular acidification. Concomitantly, the LDH/PDH activity ratio increased with the reduction in pH, as previously reported. Combining the NH_4Cl prepulse with zoniporide led to a greater reduction in LDH activity (–29%) and to increased PDH activity (+40%). These changes resulted in a surprising decrease in the LDH/PDH ratio, as opposed to previous

Abbreviations: AAR, area at risk; ADP, adenosine diphosphate; AON, area of necrosis; AOP, area of perfused myocardium; ATP, adenosine triphosphate; CBE, $\text{Cl}^-/\text{HCO}_3^-$ exchanger; CHE, Cl^-/OH^- exchanger; dDNP, dissolution dynamic nuclear polarization; DO, dissolved oxygen; EVB, Evan's blue; Inj, injection; KH buffer, Krebs–Henseleit buffer; LDH, lactate dehydrogenase; MR, magnetic resonance; NAD⁺, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); NBC, $\text{Na}^+/\text{HCO}_3^-$ co-transporter; NHE, Na^+/H^+ exchanger; PCr, phosphocreatine; PDH, pyruvate dehydrogenase; pH_i , intracellular pH; Pi_{in} , intracellular inorganic phosphate; TTC, triphenyltetrazolium chloride.

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predictions. Zoniporide alone (without intracellular acidification) did not change these enzyme activities. A possible explanation for the enzymatic changes observed during the combination of the NH_4Cl prepulse and NHE1 inhibition may be related to mitochondrial NHE1 inhibition, which likely negates the mitochondrial matrix acidification. This effect, combined with the increased acidity in the cytosol, would result in an enhanced H^+ gradient across the mitochondrial membrane and a temporarily higher pyruvate transport into the mitochondria, thereby increasing the PDH activity at the expense of the cytosolic LDH activity. These findings demonstrate the complexity of in-cell cardiac metabolism and its dependence on intracellular acidification. This study demonstrates the capabilities and limitations of hyperpolarized $[1-^{13}\text{C}]$ pyruvate in the characterization of intracellular acidification as regards cardiac pathologies.

KEYWORDS

ammonium chloride, intracellular pH, lactate dehydrogenase, mitochondrial pH, NHE1, pyruvate dehydrogenase, zoniporide

1 | INTRODUCTION

Intracellular pH (pH_i) plays an important role in the regulation of cellular enzymatic activity¹ and protein stability.² In the heart, it has a profound effect on contractile function, calcium homeostasis, and electrical activity.^{3–5} Hyperpolarized magnetic resonance using the dissolution dynamic nuclear polarization (dDNP) approach is useful in determining intracellular enzymatic activity in a noninvasive and nonradioactive manner. This technology has previously enabled multiple studies of in-cell metabolic reactions determined in vivo, in real time, in preclinical models,^{6–12} and in men.^{13,14} Multiple studies have also shown such in-cell enzyme activities in perfused whole organs from rodents such as heart and liver,^{15,16} tissue slices,^{17–22} and organoids.²³ In the dDNP approach, the magnetic resonance signal of the metabolic substrate can be increased by several orders of magnitude,²⁴ enabling observation of its metabolic fates in real time, as long as those occur before the meta-stable hyperpolarized state has decayed (a few minutes).

Hyperpolarized $[1-^{13}\text{C}]$ pyruvate is the dominant metabolic substrate in dDNP-hyperpolarized magnetic resonance (MR) studies because of its superb polarization properties, and the central role pyruvate metabolism plays in cellular energetics, at the crossroads of aerobic and anaerobic metabolism. Hyperpolarized $[1-^{13}\text{C}]$ pyruvate was useful in the characterization of several conditions in the heart, in preclinical animal models, such as heart failure,⁸ ischemia,^{7,25} ischemia/reperfusion injury,⁹ myocardial infarction,¹⁵ and cardiac hypertrophy.¹¹ Of note, hyperpolarized $[1-^{13}\text{C}]$ pyruvate is approved for testing in humans and has shown utility in clinical cardiac studies.^{13,14}

Pyruvate metabolism may be affected by pH_i , as two of its major fates, the conversion to acetyl-CoA and CO_2 by pyruvate dehydrogenase (PDH) and the conversion to lactate by lactate dehydrogenase (LDH), have both been shown, in vitro, to be pH dependent^{26,27}; a pH drop in the physiological range increased the activity of LDH²⁶ and decreased the activity of PDH.²⁷

As the hyperpolarized MR approach enables monitoring of in-cell enzymatic activities without tissue homogenizations or compartmentalization, it offers the possibility to test the effects of pH_i on these enzyme activities in-cell, in the viable organ. However, we note that a study of the effect of pH_i in the whole heart on these in-cell enzyme activities is complicated, because of the difference in the cellular compartments in which these enzymes reside. While LDH is a cytosolic enzyme, PDH is a mitochondrial enzyme. Because pH_i may be distributed unevenly among the cellular compartments, it is not straightforward to both control and predict the effect of pH_i on these cellular enzymes. In addition, we note that in the intact cell, pH_i changes will always affect a broad variety of intracellular enzymes, and these may involve opposing systems that are activated or inhibited at the same time.²⁸ Such changes may have indirect effects on LDH and PDH activities. Previously, we have reported that the in-cell LDH to PDH activities ratio in the perfused mouse heart is strongly correlated with cardiac pH.²⁹ Here, we aimed at further characterizing the mechanism underlying this observation and its possible utility as a reporter of pH_i for cardiac examinations. The following is a description of some of the unknowns in this field.

1.1 | pH_i change: Cause or effect?

To the best of our knowledge, prior literature does not provide a comprehensive understanding of the relationship between intracellular pH and in-cell LDH and PDH activities in a living system. In states such as ischemia, there is a reduction of available oxygen and nutrients and reduced

washout of metabolites.⁴ The reduction in pH in ischemia is attributed to several factors. First, the reduction in adenosine triphosphate (ATP), which is due to oxygen depletion and nutrient deprivation, reduces the activity of membrane-bound pH regulators of the cell, leading to a lower pH_i .⁴ Second, the reduction in washout of acidic metabolites that occurs in ischemia also reduces pH_i .⁴ Third, oxygen deprivation leads to a decrease in the NAD^+/NADH ratio,³⁰ which in turn stimulates glycolysis and inhibits oxidative phosphorylation in the mitochondria. This metabolic shift is accompanied by increased LDH activity^{20–22} and decreased PDH activity.^{21,22} In one aspect, lactate, the product of pyruvate metabolism by LDH, is capable of lowering the tissue pH due to its low pK_a of 3.78,³¹ and lactate concentration was previously found to be correlated to both extracellular pH (pH_e) and pH_i in the rat brain during ischemia.^{32,33} However, it is not known if increased lactate production under conditions that also involve acidification (such as stroke and ischemia^{20–22}) is the cause of or is driven by intracellular acidification. In another aspect, PDH activity has been shown to decrease under ischemia that was accompanied by acidification in brain slices.^{21,22} However, to the best of our knowledge, changes in PDH activity in muscle are not expected to be followed by changes in pH_i .³⁴ To the best of our knowledge, it is yet unknown whether in-cell cardiac PDH activity is directly affected by pH_i . Therefore, even though LDH, PDH, and pH_i are changing simultaneously in ischemia, the direct effect of pH_i reduction on LDH and PDH activities is not known.

1.2 | pH and high-energy phosphate nucleotides

LDH and PDH activities are both also affected by the ratios of high-energy phosphate nucleotides, such as nicotinamide adenine dinucleotides and adenosine triphosphates and diphosphates (NAD^+/NADH and ATP/ADP).^{30,35} As pH_i may affect these ratios, the interpretation and prediction of the response of LDH and PDH to changes in pH_i is further complicated and may represent a balance between the direct effects of pH on these proteins and the indirect effects of pH_i , which may lead to other intracellular changes that may affect these enzymes. To the best of our knowledge, these global effects of pH_i on cardiac enzyme activities have not been studied before.

1.3 | Clinical relevance

In the clinical setting, acidosis of the blood and the extracellular space may occur in some patients as part of the etiology of several cardiac conditions and may induce a reduction in pH_i as well. In general, a reduction in cardiac pH leads to reduction in a heart contractility.^{3,5} In agreement, global metabolic acidosis also affects the heart. Figure 1 summarizes the knowns and unknowns as regards pH_e , pH_i , and their effects on in-cell metabolic enzyme activities.

The use of hyperpolarized $[1-^{13}\text{C}]$ pyruvate is a promising modality for cardiac imaging and has shown utility in probing various conditions such as diabetic cardiomyopathy,¹² heart failure,³⁶ ischemia and ischemia/reperfusion injury,³⁷ and myocardial hypertrophy.¹¹ The current study may provide a better understanding of the processes that govern pyruvate metabolism in these conditions, with regard to the contribution of pH_i reduction, which accompanies these conditions in some patients. This is key for correct interpretation of such cardiac studies using the production of $[1-^{13}\text{C}]$ lactate and $[^{13}\text{C}]$ bicarbonate as biomarkers.

1.4 | Methodological considerations

The methodology used to investigate the in-cell effect of pH_i on LDH and PDH activities in the isolated heart is briefly described in the following. The pharmacological challenges that were used to induce intracellular acidification are an NH_4Cl prepulse (Figure S1) with or without a Na^+/H^+ exchanger (NHE1) inhibitor. These were administered to the perfused mouse heart ex vivo. This investigatory system was chosen to isolate the effect of intracellular acidification on LDH and PDH activities. The perfused heart system (also known as the Langendorff heart³⁸) enables acidification of the cardiac tissue, which may be less tolerated in vivo, and monitoring it without the effects of other organs' acidification. At the same time, this model system preserves an adequate physiologic cardiac function.

The mouse hearts were perfused inside an NMR spectrometer. This enabled pH determination using ^{31}P -NMR spectroscopy and LDH and PDH activity quantification using ^{13}C -NMR spectroscopy and hyperpolarized $[1-^{13}\text{C}]$ pyruvate. Oxygen consumption and temperature were monitored in tandem with the NMR spectroscopy recordings using NMR-compatible oxygen and temperature sensor systems.

2 | MATERIALS AND METHODS

Information on the materials and methods that were used in the current study and previously described²⁹—including the chemicals; surgical procedure; perfusion system; NMR spectroscopy; hyperpolarized ^{13}C spectroscopy; DNP spin polarization and dissolution; tissue wet weight; LDH

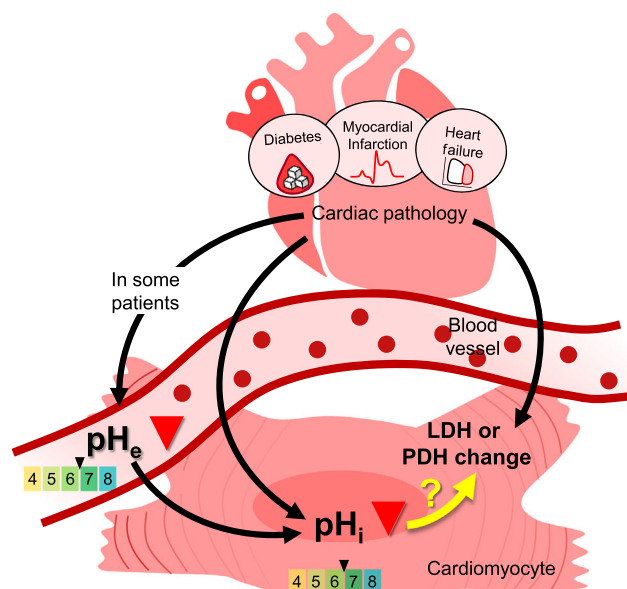


FIGURE 1 Several cardiac conditions capable of damaging the heart consist of a reduction in pH_i and, in some patients, also involve a reduction in pH_e . These conditions are also known to be associated with changes in LDH and PDH activities. However, it is not known whether acidification of the intracellular space alone, without further insults, can lead to changes in LDH and PDH activities. LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; pH_e , extracellular pH; pH_i , intracellular pH.

and PDH rate calculation; pH_i calculation; ATP and phosphocreatine (PCr) content quantification; and normalization of the enzymatic rates to ATP content—is provided in the supporting information (Note S1).

2.1 | Animals

Male Institute for Cancer Research (ICR) mice ($n = 30$, 39–45 g) were obtained from the Institutional Authority of Biological and Biomedical Models. The joint ethics committee (Institutional Animal Care and Use Committee, IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare (protocol number MD-19-15827-1). The Hebrew University is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) international-accredited institute. Care was taken to minimize pain and discomfort to the animals. All procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2 | Intracellular acidification with an NH_4Cl prepulse and an NHE1 blocker

The NH_4Cl prepulse is a well-established approach to induce intracellular acidification, which is achieved by adding NH_4Cl and removing it after several minutes³⁹ (Figure S1). NHE1 accounts for approximately 60% of the proton removal capability of cardiac cells during intracellular acidification.⁴⁰ NHE1 on the plasma membrane co-transporters H^+ out of the cytosol and Na^+ into the cytosol. Zoniporide was used here to reduce the pH_i to a level that is lower than what would be achieved using the NH_4Cl prepulse alone. The half maximal inhibitory concentration (IC_{50}) of zoniporide is 14 nM,⁴¹ and it was administered here at a concentration of 1 μM ; therefore, we may assume a complete inhibition of NHE1.

A detailed description on NH_4Cl prepulse methodology and on NHE1 block is provided in Figure S1 and Note S1.

2.3 | Experimental workflow: Prior to the pharmacologic challenge

First, a ^{31}P spectrum of oxygenated Krebs–Henseleit buffer (KH buffer) alone was acquired in the same NMR tube that was used for the rest of the experiment (10 mm diameter), at a flow rate that was used throughout the experiment. Then the mouse heart ($n = 30$) underwent aortic retrograde cannulation in vivo and was then isolated. The heart was then perfused with oxygenated KH buffer, at 37°C, using a peristaltic pump at a

constant flow rate of 7.5 mL/min. After confirming that the heart was beating spontaneously (visual), it was inserted into the NMR tube, then the tube with the beating heart fixed in it (as well as the sensors for temperature and O_2 and the other perfusion lines) was lowered into the bore of the spectrometer.

2.4 | Experimental media

2.4.1 | Solution 1

A modified version of KH buffer⁴² contains 10 mM glucose, 0.5 mM pyruvate, 118 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, $MgSO_4$, 25 mM $NaHCO_3$, and 0.5 mM insulin in water (95/5 v/v double-distilled H_2O/D_2O). For Groups 4, 5, and 6, we used this KH buffer, which also contained 1.2 mM KH_2PO_4 . For Groups 1, 2, and 3, we used a KH buffer that did not contain KH_2PO_4 . This was done to allow clear visualization of the intracellular Pi signal without the overlapping signal of the extracellular Pi signal.

2.4.2 | Solution 2

Same as Solution 1, with the addition of 20 mM of NH_4Cl (without osmotic compensation).⁴³ This solution was used to induce the NH_4Cl prepulse.

2.4.3 | Solution 3

Same as Solution 1, with the addition of 20 mM of NH_4Cl and 1 μM of zoniporide. This solution was used to induce the NH_4Cl prepulse with NHE1 inhibition.

2.4.4 | Solution 4

Same as Solution 1, with the addition of 1 μM of zoniporide. This solution was used as a control to study the possible effects of zoniporide by itself on LDH and PDH activities.

All four solutions were freshly prepared for the various experimental conditions. During the experiments, the relevant solutions were kept in a water bath at 40°C outside the NMR spectrometer and were bubbled for 1 h prior to cardiac perfusion with 95%/5% O_2/CO_2 at a flow rate of 3.5 L/min. The pH of these media was adjusted to be 7.4.

2.5 | Experimental groups according to pharmacologic challenges and data recording

NH_4Cl prepulse and zoniporide administration were used in conjunction or separately in six experimental groups, as described below and in Figure 2.

2.5.1 | Group 1: NH_4Cl prepulse

This condition was induced by switching the perfusion from Solution 1 to Solution 2 for 24 min then switching back to Solution 1. ^{31}P spectroscopy was recorded to determine pH, ATP, and PCr. Dissolved oxygen (DO) was measured throughout.

2.5.2 | Group 2: NH_4Cl prepulse with NHE1 inhibition

This condition was induced by switching the perfusion from Solution 1 to Solution 3 for 24 min, then switching to Solution 4 for 10 min, and then switching back to Solution 1. ^{31}P spectroscopy was recorded to determine pH, ATP, and PCr. DO was measured throughout.

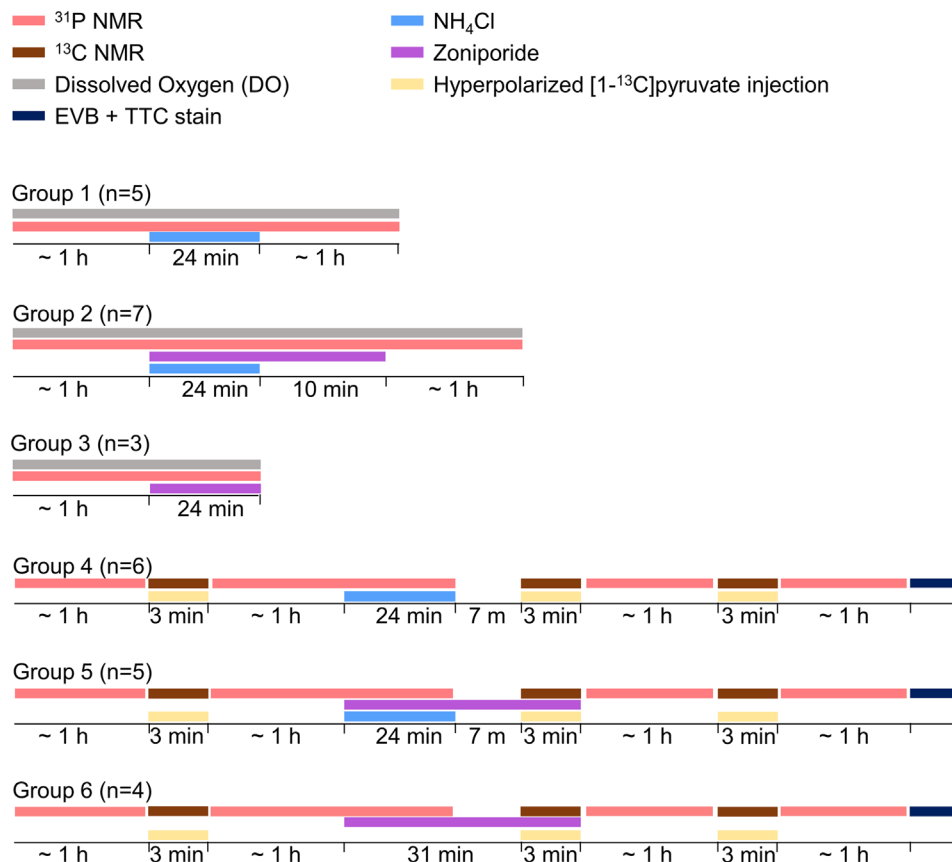


FIGURE 2 The experimental design. In Groups 1–3, ^{31}P spectroscopy and dissolved oxygen measurement were performed in perfused mouse hearts. Baseline data were acquired for approximately 1 h, followed by three different pharmacological conditions, as indicated in the timeline: Group 1, NH_4Cl prepulse ($n = 5$); Group 2, NH_4Cl prepulse combined with zoniporide ($n = 7$); and Group 3, only zoniporide ($n = 3$). In Groups 1 and 2, recovery data were acquired as well for approximately 1 h. In Groups 4–6, ^{31}P and ^{13}C spectroscopy were performed in perfused mouse hearts ($n = 15$). ^{31}P data were acquired for approximately 1 h and this was followed by ^{13}C acquisition of the first hyperpolarized $[1-^{13}\text{C}]$ pyruvate injection to obtain the baseline metabolic activity of LDH and PDH. Then three different conditions were applied before the second hyperpolarized $[1-^{13}\text{C}]$ pyruvate injection, as indicated in the timeline: Group 4, NH_4Cl prepulse ($n = 5$); Group 5, NH_4Cl prepulse combined with zoniporide ($n = 6$); and Group 3, only zoniporide ($n = 4$). A third hyperpolarized $[1-^{13}\text{C}]$ pyruvate injection was then applied to obtain LDH and PDH activity in the recovery phase. ^{31}P spectra were acquired in between, as indicated in the timeline. EVB and TTC stains were obtained at the end of each experiment in Groups 4–6. n = the number of hearts (and animals) investigated in each group. The time durations are not drawn to scale. EVB, Evan's blue; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; TTC, triphenyltetrazolium chloride.

2.5.3 | Group 3: NHE1 inhibition without NH_4Cl prepulse

This condition was induced by switching the perfusion from Solution 1 to Solution 4 for 34 min. ^{31}P spectroscopy was recorded to determine pH, ATP, and PCr. DO was measured throughout.

2.5.4 | Group 4: NH_4Cl prepulse, with real-time in-cell enzymatic activity recording

This condition was induced as described for Group 1. In addition, LDH and PDH activities were recorded 1 h before the switch to Solution 2 and then 7 min and 1 h after the removal of Solution 2.

2.5.5 | Group 5: NH_4Cl prepulse and NHE1 inhibition, with real-time in-cell enzymatic activity recording

This condition was induced as described for Group 2. In addition, LDH and PDH activities were recorded 1 h before the switch to Solution 3, and then 7 min after the switch to Solution 4, and 1 h after the switch back to Solution 1.

2.5.6 | Group 6: NHE1 inhibition without NH_4Cl prepulse, with real-time in-cell enzymatic activity recording

This condition was induced as described for Group 3. In addition, LDH and PDH activities were recorded 1 h before the switch to Solution 4, and then 31 min after the switch to Solution 4, and 1 h after the switch back to Solution 1.

2.6 | Demonstration of flow dynamics

To determine the flow characteristics of the hyperpolarized $[1\text{-}^{13}\text{C}]\text{pyruvate}$ during the injection, we used the decrease in the water ^1H signal during a D_2O injection, as previously described.²² To this end, we injected a volume of 26 mL, composed of 20.8 mL of Solution 1 (80%) and 5.2 mL of D_2O (20%), into the NMR tube, using the same continuous flow system as the hyperpolarized injections. The integrated intensity of the water ^1H signal was inverted then normalized to demonstrate the flow characteristics that would correlate with the administration of the hyperpolarized substrate.

2.7 | Data analysis and statistics

Spectral processing and statistical analysis are provided in Note S1.

2.8 | Measurement of DO

DO in the buffer was measured by O_2 sensor spots (Sticker type: Pst7-10), with an NMR-compatible fiber-optic DO meter (PreSens, Precision Sensing GmbH, Germany). Additional information is provided in Note S1.

2.9 | Retrograde perfused heart staining and analysis

To ensure that the ATP content is a true surrogate for the amount of living cells in the heart, we performed a triphenyltetrazolium chloride (TTC) and an Evan's blue (EVB) staining of the isolated heart at the end of each experiment with Groups 4, 5, and 6. TTC staining is a well-established method to assess tissue viability, as such tissue turns red by mitochondrial dehydrogenases.⁴⁴ The EVB staining is an efficient tool with which to assess tissue perfusion.⁴⁴ The combination of both staining techniques was used to assess the proportion of tissue that is either perfused or not perfused but metabolically active, and thus informs regarding the percentage of the cardiac tissue that participated in $[1\text{-}^{13}\text{C}]\text{pyruvate}$ metabolism. Additional information on the staining procedures and the viability quantification is provided in Note S1.

3 | RESULTS

3.1 | LDH and PDH rate calculation

Using product-selective saturating-excitation pulses,⁴⁵ the hyperpolarized metabolites $[1\text{-}^{13}\text{C}]\text{lactate}$ and $[^{13}\text{C}]\text{bicarbonate}$ were fully sampled (and depolarized) by each selective pulse, and only newly synthesized metabolites were detected on each excitation. This enabled absolute quantification of the LDH and PDH activities on each injection. Figure 3 summarizes the acquisition and processing steps that enabled these enzymatic activity determinations. Further information is provided in Note S1.

3.2 | Demonstration of flow dynamics

To study the flow characteristics of hyperpolarized $[1\text{-}^{13}\text{C}]\text{pyruvate}$ during injection, we utilized the reduction in the water ^1H signal during a D_2O injection (section 2.6). Figure 3C demonstrates one such D_2O injection and 45 ($n = 15$, three injections each) individual effective relaxation time constant (T_{eff}) corrected $[1\text{-}^{13}\text{C}]\text{pyruvate}$ signal time courses. A high similarity is demonstrated in the wash-in and the plateau phases of both flow profiles. In the washout phase, it appears that the T_{eff} correction signal curve is variable, and with less agreement with the profile calculated based

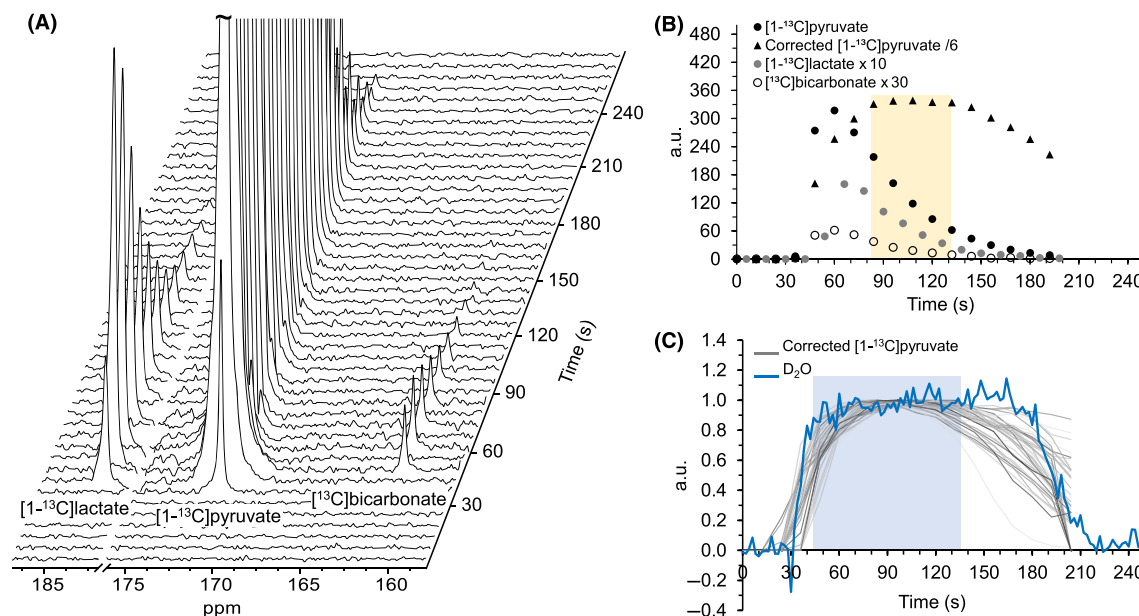


FIGURE 3 Demonstration of processing and analysis of hyperpolarized $[1-^{13}\text{C}]$ pyruvate metabolism. (A) Representative ^{13}C NMR spectra of 14 mM hyperpolarized $[1-^{13}\text{C}]$ pyruvate (171 ppm) injection to a heart and subsequent $[1-^{13}\text{C}]$ lactate (183.2 ppm) and $[^{13}\text{C}]$ bicarbonate (161.1 ppm) formation. The $[1-^{13}\text{C}]$ pyruvate signal is truncated for better visualization of the smaller signals. The $[1-^{13}\text{C}]$ pyruvate-hydrate signal (179.4 ppm) is not shown for clarity of the display. Time 0 is the time of dissolution of the hyperpolarized material. (B) Time courses of the denoised signal integrals of the signals in (A). Black circles, $[1-^{13}\text{C}]$ pyruvate; black triangles, T_{eff} corrected $[1-^{13}\text{C}]$ pyruvate, reduced 6-fold for display; gray circles, $[1-^{13}\text{C}]$ lactate, multiplied 10-fold for display; empty circles, $[^{13}\text{C}]$ bicarbonate, multiplied 30-fold for display. The temporal time window that contains the first four datapoints of constant $[1-^{13}\text{C}]$ pyruvate concentration in this specific experiment, is highlighted. Only $[1-^{13}\text{C}]$ lactate and $[^{13}\text{C}]$ bicarbonate datapoints from this time frame were used for the calculation of LDH and PDH activities. (C) Flow dynamics in the continuous perfusion system demonstrated using a D_2O injection. Each gray line corresponds to an individual, T_{eff} corrected $[1-^{13}\text{C}]$ pyruvate signal time course, normalized to its maximal value (altogether, 45 time courses, $n = 15$ hearts, three hyperpolarized injections to each heart). The blue line corresponds to the inversed and normalized integral of the ^1H signal during the D_2O injection. The highlighted time frame (48–132 s) is the time from dissolutions in which the T_{eff} corrected $[1-^{13}\text{C}]$ pyruvate signals in all the injections (45) were constant (plateau). Within this time frame, in each experiment, the first four time points that showed a signal that was no less than 10% of the maximal corrected $[1-^{13}\text{C}]$ pyruvate signal were used for the calculation of LDH and PDH rates. LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; T_{eff} , effective relaxation time constant.

on the D_2O injection. This variability could reflect the cumulative effects of RF pulsation, variable T_1 decay inside and outside the magnet, and possibly also substrate consumption. Nevertheless, this part of the experimental dataset was not used for enzymatic rates determinations.

3.3 | NH_4Cl prepulse induces intracellular acidification

Figure 4 shows typical changes in ^{31}P NMR spectra during an NH_4Cl prepulse, which was performed in Group 1. As expected, the cardiac intracellular Pi (P_{i}) signal shifted downfield (to the right) during the NH_4Cl removal, indicating intracellular acidification.

In Group 1, the NH_4Cl prepulse led to an intracellular acidification of 0.18 ± 0.09 units at time T_{inj} (indicated by yellow stripes in Figure 5A; $p = 0.004$ compared with baseline, two-tailed, paired, Student's t -test, $n = 5$). Combining the NH_4Cl prepulse with NHE1 inhibition (zoniporide) in Group 2 led to a substantial drop in pH_{i} of 0.43 ± 0.07 units at the same time (T_{inj} , $p = 0.001$, compared with baseline, two-tailed, paired, Student's t -test, $n = 7$; Figure 5D). These results are in line with previous studies that showed a more pronounced drop in pH_{i} upon NH_4Cl prepulse with NHE1 inhibition compared with the NH_4Cl prepulse alone.⁴⁶ At the same time, we could not detect any changes in ATP or PCr levels (Figure 5B,C,E,F). Addition of zoniporide alone (which did not induce intracellular acidification) did not lead to changes in the levels of either ATP or PCr (Figure S3).

The second hyperpolarized $[1-^{13}\text{C}]$ pyruvate injection, which was aimed at investigating metabolism under the intracellular acidification condition, was performed during the time T_{inj} , which showed maximal intracellular acidification for both conditions (as indicated by the yellow stripes in Figures 4 and 5).

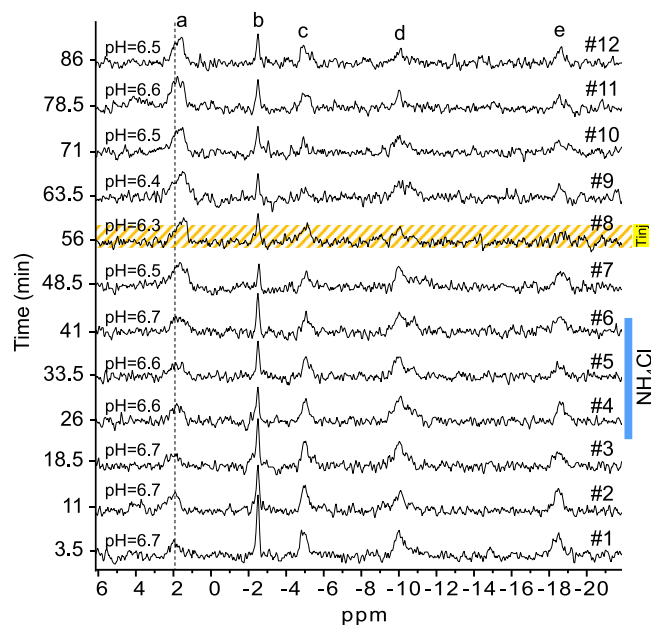


FIGURE 4 Typical ^{31}P NMR spectra of an isolated heart during an NH_4Cl prepulse challenge (Group 1). Each spectrum was acquired within 7.5 min and shows the following metabolites: a, intracellular $\text{Pi}_{(i)}$; b, PCr; c, $\gamma\text{-ATP} + \beta\text{-ADP}$; d, $\alpha\text{-ATP} + \alpha\text{-ADP}$; e, $\beta\text{-ATP}$. Spectra #1–3 were acquired before applying the NH_4Cl prepulse (baseline). Spectra #4–6 were acquired during perfusion with a medium that contained 20 mM NH_4Cl . Spectra #7–12 were acquired further to the removal of NH_4Cl . The dashed line marks the center of the baseline cardiac $\text{Pi}_{(i)}$ signal to assist in visualizing the tissue acidification during the NH_4Cl removal period. For each spectrum, the calculated pH value is provided on the left. The spectrum highlighted in yellow stripes was acquired at the time when the pH_i was the lowest (maximal acidification). This time is marked T_{inj} and is referred to in Figure 5. ADP, adenosine diphosphate; ATP, adenosine triphosphate; PCr, phosphocreatine; pH_i , intracellular pH.

3.4 | TTC and EVB staining correlate to the ATP content of the hearts

The combined staining method of TTC and EVB was used to discriminate between the metabolically active and nonactive regions of the heart (Note S1). Figure 6 shows the correlation between the metabolically active regions (area of perfused myocardium [AOP] + area at risk [AAR]) and the ATP content of the heart (determined during 52 min prior to the staining). This result suggested that the ATP content of the heart indeed provides an adequate surrogate to the amount of viable tissue in the heart.

3.5 | Pyruvate metabolism during intracellular acidification and NHE1 inhibition

The product-selective saturating-excitations acquisition approach was used for acquiring the hyperpolarized data and calculating LDH and PDH rates.⁴⁵ In Groups 4–6, hyperpolarized $[1\text{-}^{13}\text{C}]\text{pyruvate}$ was injected three times into each perfused heart. The first injection was used as a baseline, the second injection was performed during the pharmacological challenge or control, and the third injection was used as a second control further to recovery from the pharmacological challenge.

The NH_4Cl prepulse challenge (moderate intracellular acidification) led to a 16% reduction in LDH activity ($p = 0.04$, $n = 6$; Figure 7A) and to a 39% reduction in PDH activity ($p = 0.001$, $n = 6$; Figure 7B). In agreement, the LDH/PDH activities ratio increased by 41% ($p = 0.02$, $n = 6$; Figure 7C). The combined NH_4Cl prepulse and zoniporide challenge (severe intracellular acidification) led to a 29% reduction in LDH activity ($p = 0.01$, $n = 5$; Figure 7A) and to a surprising 40% increase in PDH activity ($p = 0.007$, $n = 5$; Figure 7B). In agreement, the LDH/PDH activities ratio was reduced 55% ($p = 0.04$, $n = 5$; Figure 7C). The zoniporide challenge alone (no change in intracellular acidification) did not lead to any changes in LDH or PDH activities or their ratio ($n = 4$; Figure 7A–C).

We note that there was no change between injections 1 and 3 in any of the groups (Figure 7). This is reassuring in two aspects: (1) that the hearts have indeed recovered from the pharmacological challenges; and (2) that the standardization of the enzymatic activity results to the ATP content, which was measured throughout the experiment, was a useful tool to correct for the loss of viable regions in each heart over the experimental day. For comparison, the data are presented without this standardization to ATP content in Figure S4.

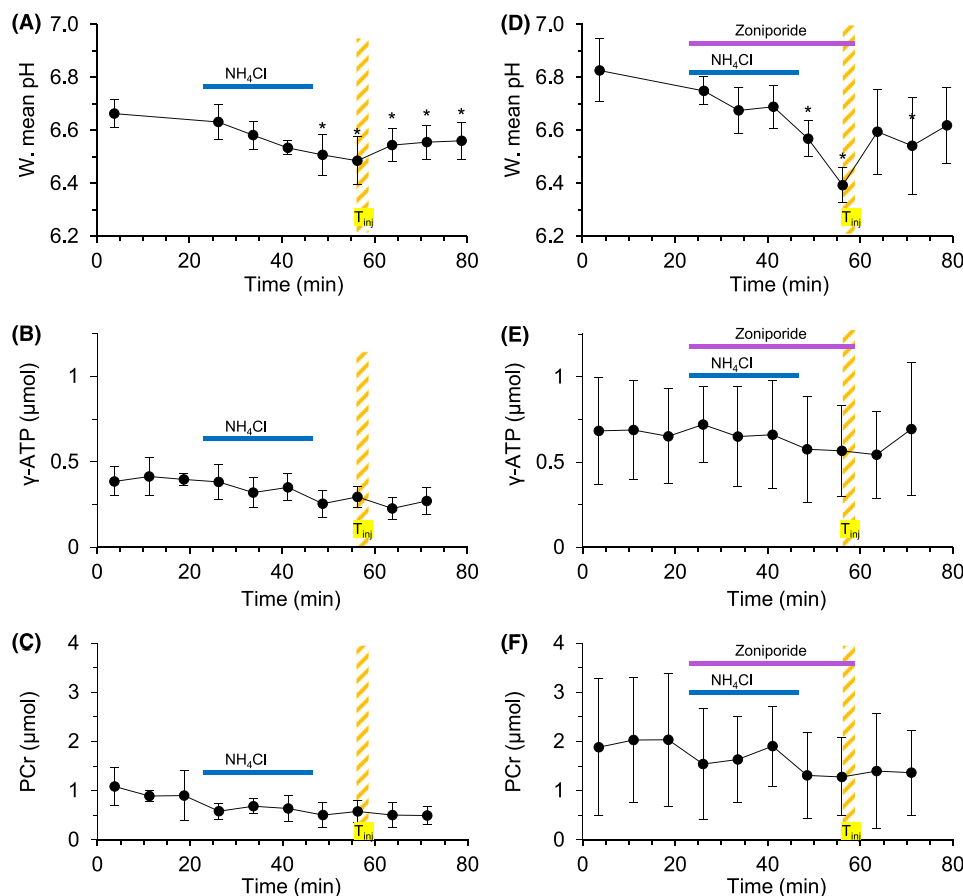


FIGURE 5 Changes in pH_i, ATP, and PCr during an NH₄Cl prepulse with or without zoniporide. (A–C) Weighted mean pH_i, ATP, and PCr, respectively, during the experiment with Group 1, which consisted of an NH₄Cl prepulse. (D–F) Weighted mean pH_i, ATP, and PCr, respectively, during the experiment with Group 2, which consisted of an NH₄Cl prepulse combined with zoniporide. In (A,C,E), each datapoint is an average of $n = 4$ experimental days; in (B), each datapoint is an average of $n = 5$ experimental days; and in (D,F), each datapoint is an average of $n = 7$ experimental days. Each datapoint was acquired for 7.5 min using ³¹P spectroscopy. Values are expressed as mean ± standard deviation. The yellow stripes show the time duration T_{inj}, which indicates the time of second hyperpolarized [1-¹³C]pyruvate injection in Groups 4–6. Significance was tested compared with the baseline values (the first point on each plot), with a two-tailed, paired, Student's *t*-test (* $p \leq 0.05$; individual values for T_{inj} are provided in the text). ADP, adenosine diphosphate; ATP, adenosine triphosphate; PCr, phosphocreatine; pH_i, intracellular pH.

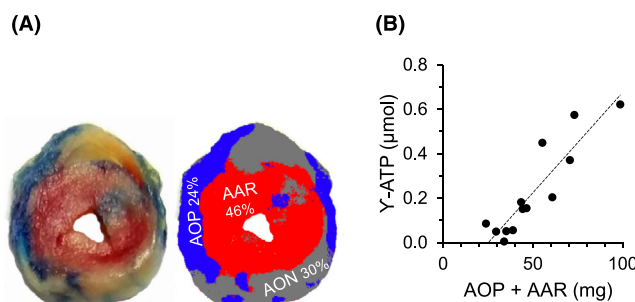


FIGURE 6 TTC and EVB staining of a typical isolated heart, and the correlation to γ-ATP. (A) Left: a typical axial slice of an isolated heart that was stained with TTC and EVB. Right: viability quantification performed by contouring the three-color subsets: blue, AOP; red, AAR; gray, AON; the white area is the ventricle lumen. The relative areas of the blue, red, and gray subsets were obtained using a pixel count tool and the weight of each region was calculated as described in the Methods. (B) Linear correlation ($R^2 = 0.82$) between the AOP + AAR weight to the γ-ATP content of the heart. AAR, area at risk; AON, area of necrosis; AOP, area of perfusion; ATP, adenosine triphosphate; EVB, Evan's blue; TTC, triphenyltetrazolium chloride.

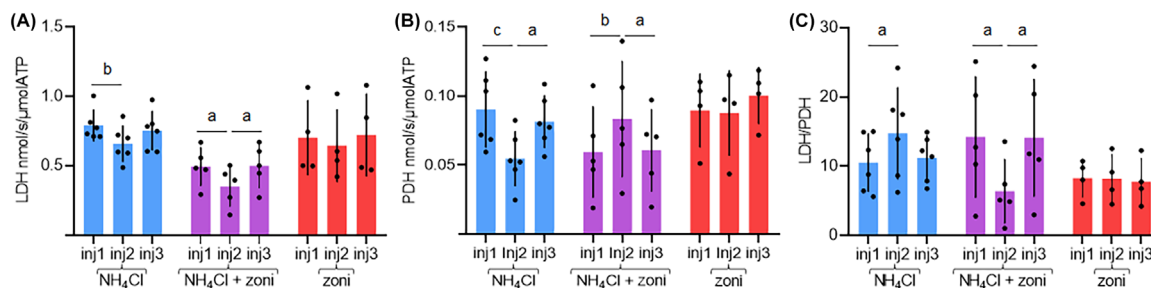


FIGURE 7 The effect of NH_4Cl prepulse and zoniporide on LDH, PDH, and LDH/PDH activities. Data were obtained from ^{13}C spectroscopy of three hyperpolarized $[1-^{13}\text{C}]$ pyruvate injections (inj). The second injection of each heart was performed under three different conditions: Group 4, NH_4Cl prepulse (blue, $n = 6$); Group 5, NH_4Cl prepulse combined with zoniporide addition (purple, $n = 5$); and Group 6, zoniporide addition only (red, $n = 4$). The first and third injections to each heart were performed under regular buffer perfusion without NH_4Cl or zoniporide. (A and B) LDH and PDH activities, respectively, normalized to the ATP content before each injection, expressed in $\text{nmol/s}/\mu\text{mol ATP}$. (C) LDH/PDH activities ratio. Values are shown as mean $\pm$ standard deviation. Significance was tested by one-way ANOVA with repeated measures followed by Fisher's LSD post hoc test (a, $p \leq 0.05$; b, $p < 0.01$; c, $p < 0.005$; individual values are provided in the text). The LDH, PDH, and ATP values comprising the analysis shown here are provided in Table S1. ANOVA, analysis of variance; ATP, adenosine triphosphate; LDH, lactate dehydrogenase; LSD, least significant difference; PDH, pyruvate dehydrogenase; zoni, zoniporide.

3.6 | DO concentration during NH_4Cl prepulse with and without zoniporide

The DO that was associated with myocardial consumption ($\Delta\text{DO}_{\text{heart}}$) was determined, as described in Note S1 and in Figure S5. Briefly, $\Delta\text{DO}_{\text{heart}}$ was determined in three experimental groups (Figures 2 and S5): (1) in Group 1, before the NH_4Cl prepulse (control, $n = 3$); (2) in Group 1, during the NH_4Cl prepulse ($n = 5$); and (3) in Group 2, during NH_4Cl prepulse with zoniporide ($n = 7$). We could not detect significant changes in the $\Delta\text{DO}_{\text{heart}}$ in any of the conditions; however, we note that some of these measurements were noisy. The data are shown in Figure S5.

4 | DISCUSSION

4.1 | Clinical relevance

During states of myocardial dysfunction, the metabolic activity of the heart is changing, and consequently, LDH and PDH may be impacted. pH_i may be impaired as a result of intracellular mechanisms,⁴ and because of a systemic reaction to the cardiac dysfunction, which is followed by metabolic acidosis. In addition, metabolic acidosis may affect the heart by mechanisms that are unrelated to pH_i . There are several clinical scenarios that entangle heart conditions and acidosis. For example, heart failure is usually accompanied by metabolic alkalosis due to drug use and neurohumoral mechanisms; however, metabolic acidosis can develop in end-stage heart failure because of reduced plasma renal flow and renal failure⁴⁷; during acute myocardial infarction, the drop in cardiac output could cause metabolic acidosis as a result of tissue hypoxia^{48,49}; genetic hypertension could cause hypertrophy and at the same time can be associated with metabolic acidosis⁵⁰; and diabetes could lead to metabolic acidosis through diabetic nephropathy⁵¹ and diabetic ketoacidosis.⁵²

Metabolic acidosis was associated with an increased risk for ischemic heart disease and heart failure. Multiple factors may contribute to this association, although the exact mechanism is uncertain. The possible factors are decreased cardiac output, inflammation, activation of the renin-angiotensin-aldosterone system, an increase in intracellular calcium that leads to proteolysis, hormones such as adrenaline, cortisol, and glucagon that cause supply-demand mismatch, and arrhythmias.^{53–56} Therefore, it is important to understand the in-cell effects of pH_i reduction on cardiac metabolic enzyme activities.

4.2 | New enzyme activity characteristics

In vitro studies in cell-free systems and purified enzymes report conflicting results as regards the dependence of LDH activity on pH. A study by Winer and Schwert showed that a pH drop within the range of approximately 10–5.5 increased LDH activity,⁵⁷ while a study by Javed et al.⁵⁸ reported that the LDH activity decreased with decreasing pH in the range of 7–4, and a study by Vesel et al. reported on two opposite trends for the LDH isozymes LDH1 and LDH5 in the pH range of 6.9–7.9.²⁶ For PDH, a pH drop in the range of approximately 8–7 was shown to reduce its activity.²⁷ Here, a drop of 0.2 units in pH_i (from $\text{pH} = 6.67$) resulted in a modest decrease in LDH activity and a substantial decrease in PDH

activity. A larger drop in pH_i (from $\text{pH} = 6.83$) led to a more pronounced decrease in LDH activity and the PDH activity increased rather than decrease. Therefore, in both cases (the modest and the substantial pH decrease), the LDH activity decreased. However, PDH activity decreased on one challenge (NH_4Cl prepulse), but increased on the other challenge (NH_4Cl prepulse and zoniporide). These results could not be expected based on isolated enzyme studies. Therefore, first and foremost, this study demonstrates the importance of in-cell studies in real time. LDH and PDH act within a cellular microenvironment, and their real-time activities may be affected by multiple factors that act within that microenvironment and may be missing in cell-free systems.

Several factors may explain the results obtained in the current study. LDH is a cytosolic enzyme, and its activity is mainly influenced by the type of isozymes present and substrate availability. In the current (forward) reaction, the relevant substrates are pyruvate and NADH. As pyruvate is given in excess, the decrease in LDH activity may be explained by (1) the presence of LDH isozymes that decrease in activity upon pH reduction; and (2) a decrease in NADH or its binding affinity or by an excess of NAD^+ .

As regards PDH activity, reduced contractile function was previously observed during intracellular and extracellular acidification and was attributed to the increase in sarcolemma-free Ca^{+2} ions.^{59–61} Reduced contractile function may reduce the ATP usage and the ATP/ADP ratio may increase. The latter may activate pyruvate dehydrogenase kinase, which in turn will deactivate PDH.⁶² This process may, in addition to the response of the isolated PDH enzyme to pH reduction, explain the decrease in PDH activity observed further to the NH_4Cl prepulse.

However, these mechanisms cannot explain the increased PDH activity observed under the lower pH_i , which was induced by the combination of the NH_4Cl prepulse and zoniporide. We propose the following explanation for this observation. In the inner mitochondrial membrane, NHE1 transports H^+ into the mitochondrion and co-transport Na^+ to the cytosol. This is opposed to its activity in the plasma membrane, which transports H^+ out of the cytosol into the extracellular space and transports Na^+ into the cytosol⁶³ (Figure S2). Because of NHE1 inhibition by zoniporide, H^+ is likely to accumulate in the cytosol, but not in the mitochondrion. Therefore, the use of the NH_4Cl prepulse acidification approach combined with NHE1 inhibition likely leads to an increased proton gradient across the mitochondrial membrane. This gradient may enhance the activity of other co-transporters that rely on this proton gradient. One such co-transporter is the mitochondrial pyruvate carrier.^{64–67} In this case, pyruvate concentration will increase in the mitochondrion and decrease in the cytosol. The increased mitochondrial pyruvate concentration will provide more substrate to the PDH reaction and therefore will lead to increased PDH activity (manifesting as increased production of hyperpolarized $[^{13}\text{C}]$ bicarbonate), in agreement with the current results. This increased transport of pyruvate into the mitochondria may lead to reduced availability of pyruvate in the cytosol (compared with the same state without NHE1 inhibition). This may lead to reduced LDH activity (manifesting as reduced hyperpolarized $[1-^{13}\text{C}]$ lactate production), in agreement with the current results. This proposed mechanism is illustrated in Figure S6.

Previously, we have reported on a correlation between the cardiac tissue pH and the LDH/PDH activities ratio in the pH range of approximately 6.6–7.2, where a decrease in pH led to an increase in this ratio.²⁹ Such a trend was also demonstrated previously in an ischemia/reperfusion injury model.³⁷ The advantages of using the LDH to PDH ratio are mainly attributable to its independence of quantification factors, such as the amount of viable tissue.²⁹ However, we now show that the use of this ratio may mask the events that underlie it and even lead to opposite conclusions. Here, we have shown that upon mild acidification, the LDH/PDH ratio indeed increased. However, both enzymatic activities decreased, with PDH decreasing more than the decrease in LDH. In the case of severe acidification, the LDH/PDH ratio showed an opposite trend to the previously observed correlation.²⁹

4.3 | Significance of this study

The findings of this study could be used in the future to discriminate states of intracellular acidification from other cardiac conditions. For example, it might be implemented to differentiate between heart regions that suffered an ischemic injury, characterized by increased LDH and reduced PDH,³⁷ from the rest of the heart, which might be exposed to a systemic metabolic acidosis as a result of systemic tissue hypoxia that is secondary to the reduced cardiac output.^{48,49}

There are indications that NHE1 inhibition might be beneficial in the treatment of cardiac injury caused by sepsis, hypovolemic circulatory shock, and certain forms of metabolic acidosis.^{68–70} In this study, we have shown that NHE1 inhibition during intracellular acidosis is characterized by increased PDH activity, which likely increases aerobic respiration. This may provide an explanation for the beneficial effects of NHE1 inhibition.

4.4 | Limitations

4.4.1 | $\Delta\text{DO}_{\text{heart}}$ determination

In this study, we could not detect changes in $\Delta\text{DO}_{\text{heart}}$ during the NH_4Cl prepulse with or without zoniporide, although oxygen consumption is expected to decrease following the reduction of pH_e to 6.8.⁷¹ However, our inability to detect changes in $\Delta\text{DO}_{\text{heart}}$ could be related to the noisy data that were obtained.

4.4.2 | Nonworking heart

The current study was performed in a Langendorff heart preparation, which does not work against resistance. Olson et al.⁷² have conducted a study that investigated metabolic changes in a working (i.e., against resistance) versus Langendorff mouse heart. In the working heart preparation, the inflow goes through the left atrium, and the outflow goes through the aorta; left ventricular pressure is measured through the apex; and the preload and afterload are controlled. The Langendorff preparation used by Olson et al.⁷² was similar to the Langendorff preparation used in our study, except for measurement of left ventricular pressure through the apex. The working heart showed higher oxygen consumption, which likely indicates increased PDH activity, along with increased glycolysis.⁷² Nevertheless, it is unclear whether the balance between LDH and PDH activity is different in the working heart compared with the Langendorff heart. In addition, it is interesting to note that Park et al.⁷³ have demonstrated increased PDH activity in a working skeletal muscle compared with a resting muscle. Based on these two studies, we believe that is reasonable to assume that the base PDH and LDH activities are higher in the working heart compared with the Langendorff heart; however, the relative activities are not likely to be different. In this context, we believe that the results obtained here in the Langendorff heart, as regards changes in the relative activities of LDH and PDH upon intracellular acidification of various degrees, will hold in the working heart as well.

4.4.3 | Determination of regional perfusion and the effects on pH_i determination

The determinations of AOP, AAR, and AON were performed at the end of each experimental day, after several hours of heart perfusion in the NMR spectrometer. A typical example is shown in Figure 6 and demonstrates relatively low AOP (24%) and AAR (46%). However, because these measurements were made at the end of the day, they do not reflect the perfusion status and tissue viability throughout the entire duration of the experiment. The perfusion and viability typically degrade over time, as evidenced by the ATP content. This is because vessel occlusion may occur over time. In the experiments that were used to measure the pH changes (Groups 1–3), there was no P_i in the medium during the acidification protocol. Therefore, the pH measured by the ^{31}P signal of P_i , primarily reflected the intracellular environment. In addition, it is important to note that regions of the heart that were not perfused were also not expected to exhibit pH_i changes after NH_4Cl prepulse, as the NH_4Cl was not delivered to these regions. Therefore, it is important to consider the change in pH_i rather than the absolute pH_i values in this study when interpreting the results.

5 | CONCLUSIONS

In-cell pyruvate metabolism was monitored under intracellular acidosis with and without NHE1 inhibition. Our findings highlight the effect of intracellular acidification on LDH and PDH activities, which should be considered when interpreting hyperpolarized MRI cardiac results. We believe that understanding the relationship between intracellular acidification and LDH and PDH activities will enable better use of these enzymes as biomarkers and may also improve the diagnosis of additional cardiac conditions. This work may also pave the way for further understanding and improvement in NHE1 inhibitor treatment.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

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