Lactic acidosis in vivo: testing the link between lactate generation and $\text{H}^+$ accumulation in ischemic mouse muscle

David J. Marcinek, Martin J. Kushmerick, and Kevin E. Conley

Departments of 1Radiology, 2Physiology and Biophysics, and 3Bioengineering, University of Washington Medical Center, Seattle, Washington

Submitted 20 October 2009; accepted in final form 31 January 2010

Marcinek DJ, Kushmerick MJ, Conley KE. Lactic acidosis in vivo: testing the link between lactate generation and $\text{H}^+$ accumulation in ischemic mouse muscle. J Appl Physiol 108: 1479–1486, 2010. First published February 4, 2010; doi:10.1152/japplphysiol.01189.2009.—The link between lactate generation and cellular acidosis has been questioned based on the possibility of $\text{H}^+$ generation, independent of lactate production during glycolysis under physiological conditions. Here we test whether glycolytic $\text{H}^+$ generation matches lactate production over a physiological pH and lactate range using ischemia applied to the hindlimb of a mouse. We measured the $\text{H}^+$ generation and ATP level in vivo using $^{31}$P-magnetic resonance spectroscopy and chemically determined intracellular lactate level in the hindlimb muscles. No significant change was found in ATP content by chemical analysis ($P > 0.1$), in agreement with the stoichiometric decline in phosphocreatine ($20.2 \pm 1.2$ mM) vs. rise in $P_i$ ($18.7 \pm 2.0$ mM), as measured by $^{31}$P-magnetic resonance spectroscopy. A substantial drop in pH from 7.0 to 6.7 and lactate accumulation to 25 mM were found during 25 min of ischemia. The rise in $\text{H}^+$ generation closely agreed with the accumulation of lactate, as shown by a close correlation with a slope near identity ($0.98; r^2 = 0.86$). This agreement between glycolytic $\text{H}^+$ production and elevation of lactate is confirmed by an analysis of the underlying reactions involved in glycolysis in vivo and supports the concept of lactic acidosis under conditions that substantially elevate lactate and drop pH. However, this link is expected to fail with conditions that deplete phosphocreatine, leading to net ATP hydrolysis and nonglycolytic $\text{H}^+$ generation. Thus both direct measurements and an analysis of the stoichiometry of glycolysis in vivo support lactate acidosis as a robust concept for physiological conditions of the muscle cell.

$^{31}$P-magnetic resonance spectroscopy; hindlimb; stoichiometry; Lohmann reaction; phosphocreatine; adenosine 5’-triphosphate

Cell acidosis is conventionally linked to lactate generation, since glycolysis produces $\text{H}^+$ stoichiometrically with lactate in the process of carbohydrate metabolism in the absence of oxidative phosphorylation (9, 11). Modeling of glycolysis has resulted in quantitative agreement between lactate generation and acidosis in biochemical systems (18, 28), as well as muscle in vivo (17). Recently, the concept of lactic acidosis has been challenged because the reactions generating $\text{H}^+$ differ from those generating lactate (24, 25). In specific, the $\text{H}^+$ generation that comes with ATP hydrolysis is cited as an important source of $\text{H}^+$, independent of the glycolytic pathway. However, ATP level is typically stable under physiological conditions in skeletal muscle (21) and declines only under intense burst activity (7) or prolonged ischemia (27). Thus the debate concerning the link between lactate generation and acidosis may not reside in the biochemistry of glycolysis, but rather the conditions under which lactic acidosis is determined. A direct comparison under physiological conditions is needed to test whether glycolysis underlies lactate production and cellular acidosis. Unfortunately, few studies have measured ATP levels and both $\text{H}^+$ and lactate accumulation to evaluate this possibility and test the basic link underlying lactic acidosis in vivo. Here we report a direct comparison of $\text{H}^+$ and lactate fluxes under physiologically relevant changes in pH and lactate to test whether glycolytic lactate production is responsible for cellular acidosis.

Magnetic resonance spectroscopy (MRS) offers the unique ability to measure intracellular metabolite levels and determine cellular pH for a test of lactic acidosis in vivo (2, 3, 13). Serial collection of $^{31}$P spectra not only provides measurement of phosphocreatine (PCr) dynamics, ATP level, and pH (2, 3, 14, 19) but also determination of $\text{H}^+$ generation using the stoichiometry of the underlying reactions (16, 28, 29). Using this approach, several studies of energetic and metabolic fluxes resulting from glycolysis have shown agreement with the established stoichiometry of this pathway. For example, isolated and in vivo muscle studies reveal a ratio of PCr synthesis to $\text{H}^+$ production (2, 27), in accord with the classical value of 1.5 for glycogenolysis (9, 19). In addition, a comparison of lactate accumulation in blood to MRS determination of $\text{H}^+$ generation in rattlesnake tailshaker muscle in vivo showed good correspondence (14), in support of the classical stoichiometry of glycolysis [1 $\text{H}^+$:1 lactate (9, 11)]. None of these studies directly tested lactic acidosis and evaluated ATP levels in intact muscle, but it is now possible to quantify metabolic fluxes by glycolysis in vivo to test the link between lactate generation and cell acidosis. Paired comparison of $\text{H}^+$ flux in vivo with lactate determined biochemically from tissue taken from the location of the $^{31}$P determinations allows us to test lactic acidosis.

Here we report using ischemia to deoxygenate muscle in vivo and allow glycolysis to generate a large range of $\text{H}^+$ and lactate levels. Several authors have suggested that conditions from neutral cell pH to moderate acidosis (pH 7.0 to ~6.6) represent the physiological conditions of the cell best suited to test the concept of lactic acidosis (12, 25). Muscle ischemia is a straightforward way to activate glycolysis and achieve a wide physiological range of metabolite levels to permit testing of the relationship between cellular acidosis and lactate generation (2, 20). In this study, we use $^{31}$P-magnetic resonance (MR) spectra throughout ischemia to measure ATP, PCr, and inorganic phosphate ($P_i$) levels and determine $\text{H}^+$ generation in mouse hindlimb muscle. Biochemical assays for lactate and ATP concentrations ([lactate] and [ATP], respectively) are made on the same hindlimb muscles. These paired samples from mouse hindlimb at time points of progressive ischemia are used to...
determine the link between lactate generation and acidosis under physiological conditions of the muscle cell.

MATERIALS AND METHODS

Animal Procedures

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Washington in accordance with National Institutes of Health guidelines on the care and use of laboratory animals. Thirty female Swiss-Webster mice (27.5 ± 1.7 g, mean ± SE) were anesthetized with an intraperitoneal injection of Avertin. Supplemental anesthetic was delivered subcutaneously throughout experiments. During MR experiments, the temperature of the leg was monitored continuously and maintained at 36.5–37.5°C with forced air. For the MR experiments, the mouse hindlimb was mounted in a solenoidal coil, such that the source of the signal was the region of the hindlimb distal to the knee. Ischemia was induced by hanging a weight from a cord wrapped around the leg proximal to the knee to block blood flow. The [PCr] was determined every 26 s from the 31P signal.

31P-MRS

The MR experiments were performed in a 4.7-Tesla Bruker horizontal bore magnet. B0 field homogeneity was optimized by off-resonance shimming of the proton peak from tissue water. Unfiltered PCr line widths were 20–30 Hz. A high signal-to-noise 31P-MRS spectrum was taken under fully relaxed conditions (128 acquisitions with a 16-s interpulse delay) at a spectral width of 3,500 Hz, consisting of 1,024 data points. During the experimental procedure, spectra were acquired with a standard one-pulse sequence with a 1.5-s interpulse delay. For each spectrum, 16 free induction decays were averaged, resulting in higher signal-to-noise spectra with a resolution of 26 s. The free induction decays were Fourier transformed, baseline corrected, line broadened with a 10-Hz exponential filter, and zero filled. Fully relaxed peak areas were calculated by integration of the processed spectra using the Omega software on a General Electric console. Metabolite concentrations were then absolutely quantified by measuring [ATP] with HPLC from extracts of frozen hindlimbs (20). pH was determined from the chemical shift of Pi relative to PCr using the Fit-to-Standard algorithm (6). Metabolite concentrations were then absolutely quantified by measuring [ATP] with HPLC from extracts of frozen hindlimbs (20). pH was determined from the chemical shift of Pi relative to PCr using the Fit-to-Standard algorithm (6).

Muscle Stimulation for Determination of Buffer Capacity

We performed separate experiments on four mice to measure in vivo cell buffer capacity. Electrical stimulation of the distal musculature of the mouse hindlimb achieved rapid PCr breakdown and accumulation of lactate. Electrical stimulation of the distal musculature of the mouse hindlimb achieved rapid PCr breakdown and accumulation of lactate. Electrical stimulation of the distal musculature of the mouse hindlimb achieved rapid PCr breakdown and accumulation of lactate. Electrical stimulation of the distal musculature of the mouse hindlimb achieved rapid PCr breakdown and accumulation of lactate.

Muscle Extraction

Legs were removed from anesthetized mice under ischemic conditions and frozen between aluminum blocks in liquid nitrogen. Frozen muscle tissue was dissected from skin and bone on an iced aluminum block, transferred to liquid nitrogen, and then stored at −80°C until extracted. Extracts were prepared by pulverizing the muscle tissue to a fine powder at liquid nitrogen temperatures. An aliquot of the powder was weighed and transferred to a frozen 5 × volume of 0.6 M perchloric acid in Teflon tubes. A stainless steel ball cooled in liquid nitrogen was added on top of the powdered muscle and frozen perchloric acid. The tube was covered, and the mixture pulverized using an amalgamator (Crescent Dental Manufacturing, Lyons, IL) with four 20-s bursts. The muscle and perchloric acid were allowed to warm to 4°C with repeated mixing and centrifuged for 10 min at 12,000 rpm. The supernatant was neutralized with the addition of 0.6 M potassium hydroxide containing 40 mM TES buffer. After 10 min, the extract was centrifuged for 10 min at 3,000 g to collect the potassium perchlorate precipitate. The supernatant was stored at −80°C for HPLC and enzymatic lactate analysis.

Lactate Analysis

Tissue [lactate] were determined using a commercial enzymatic lactate analysis kit (Sigma no. 826-B). Each reaction mixture contained 14.4 units of lactate dehydrogenase and 2.2 μM NAD in 0.18 M glycine buffer with excess hydrazine. The reaction was initiated by the addition of 0.1 ml muscle extract to bring the total reaction volume to 1 ml. The reaction was allowed to proceed to completion, and the production of NADH measured at 340 nm was used to quantify the starting [lactate].

Coupled Reactions

Muscle contraction involves two reactions that supply ATP and split PCr. The combination of ATPase (Eq. 1) and creatine kinase (CK) reactions (2) are termed the Lohmann reaction (Eq. 3):

\[
\text{ATPase: } \text{ATP} \rightarrow \text{ADP} + \text{Pi} + \alpha \text{H}^+ \quad (1)
\]

\[
+ \text{CK: } \text{PCr} + \text{ADP} + \beta \text{H}^+ \rightarrow \text{ATP} + \text{Cr} \quad (2)
\]

\[
= \text{PCr splitting: } \text{PCr} + \gamma \text{H}^+ \rightarrow \text{Cr} + \text{Pi} \quad (3)
\]

where the proton stoichiometric coefficient \(\gamma\) of the coupled Lohmann reaction is the algebraic sum of the coefficients of the ATPase and CK reactions (\(\epsilon\) and \(\beta\)). The coefficient \(\gamma\) relates the net uptake or release of H+ to the change in PCr level (Fig. 1). This coefficient is used to determine the buffer capacity of the cell and extent of H+ change due to net decline in PCr (see below). Note that all coefficients on the left side of the equations represent H+ uptake, and those on the right represent H+ generation. Thus \(\beta\) and \(\gamma\) have negative values, while \(\alpha\) has a positive value.

Reversing the Lohmann reaction also involves reactions that supply ATP and then split ATP to form PCr. Thus the result of substrate level phosphorylation, ATP synthesis [reversing the ATPase reaction by glycolysis (Eq. 4)], is coupled with the CK reaction (Eq. 5 written to reverse Eq. 2) to generate PCr by glycolysis (Eq. 6):

\[
\text{ATP synthesis: } \text{N-glycogen} + 3 \text{ADP} + 3\text{Pi} \\
+ 3\epsilon\text{H}^+ \rightarrow 3 \text{ATP} + 2 \text{lact} + (\text{N-1-glycogen} \quad (4)
\]

Fig. 1. Proton stoichiometry coefficient values as a function of pH for the Lohmann reaction (\(\gamma\), dotted curve), reverse Lohmann reaction (\(\delta\), solid curve), and the sum of coefficients (\(\gamma + \delta\), dashed line). [Based on calculations in Ref. 16.]
where the proton stoichiometric coefficient $\delta$ of the reverse Lohmann reaction is the algebraic sum of the coefficients of the ATPase and CK reactions ($\varepsilon$ and $\beta$). Note that the coefficient value for $\varepsilon$ is negative and that for $\delta$ is positive, in accord with the convention of the coefficient position in the equation (see above). The position of coefficient $\beta$ on the right-hand side of Eq. 5 denotes $H^+$ generation in the CK reaction driven by glycolysis (the minus sign in this equation changes the value of the coefficient to positive).

**Experimental Design**

The MRS experiments were carried out as follows: off-resonance shimming followed by the acquisition of resting spectra for ~10 min before inducing ischemia. Ischemia was maintained for varying intervals up to 25 min. Following the MRS acquisitions, the legs were removed under ischemia and immediately frozen between aluminum blocks in liquid nitrogen before the animals were killed with an overdose of anesthetic. These frozen tissues were used for the in vitro analysis of [lactate] and phosphometabolite concentrations.

**Estimation of buffer capacity and fluxes.** The methods and approach for application of the stoichiometric approach to determining glycolytic flux from $^{31}$P-MRS measurements have been published in detail (2). The equations critical to the analysis in this study are summarized below.

**BUFFER CAPACITY.** Protons are buffered in muscle by metabolites, bicarbonate, and nonbicarbonate compounds (e.g., proteins). The stoichiometry of the Lohmann reaction was used to determine this composite buffering capacity ($\beta_a$) using the initial change in PCr ($\Delta$PCr) and alkalinization of pH ($\Delta$PH) at the onset of ischemia. For this experiment, stimulation was used to increase the rate of PCr decline to increase $H^+$ uptake well above any basal glycolysis:

$$\beta_a = \gamma \cdot \Delta$$PCr$/{\Delta}$PH

$\beta_a$ is positive because $\gamma$ and $\Delta$PCr are negative quantities and $\Delta$PH is positive.

The contribution of $P_i$, as an $H^+$ buffer ($\beta_{Pi}$) during stimulation was determined based on the dissociation constant of the buffer ($K_a$) by the standard formula:

$$\beta_{Pi} = 2.303 \cdot [H^+] \cdot K_a \cdot [P_i]/([K_a + [H^+])]^2$$

where $K_a$ is $1.58 \times 10^{-7}$ M. These contributions were subtracted to yield the proton buffering of the tissue alone ($\beta_i$):

$$\beta_i = \beta_a - \beta_{Pi}$$

The buffer capacity of muscle ($\beta_{tot}$) is then the sum of the factors that reversibly bind protons:

$$\beta_{tot} = \beta_i + \beta_{Pi}$$

The component due to bicarbonate buffering is considered to change negligibly in a closed system, such as the ischemic limb, and, therefore, is taken into account in the $\beta$ determination.

**GLYCOGENOLYTIC AND GLYCOLYTIC PRODUCTION.** A second role of the Lohmann reaction in determining glycolytic flux was to quantify $H^+$ uptake by net PCR splitting during ischemia. Glycolytic $H^+$ production was determined using the principle of mass balance to measure the extent of proton generation using the change in protons in the tissue ($\Delta$PH; $\beta_{tot}$) and those consumed in the CK reaction ($\gamma \cdot$PCr$_e$) at each experimental time point. This method relies on measurements of both PCr and pH in each spectrum:

$$\Delta H^+ = \Delta$PH$ \cdot \beta_{tot} + \gamma \cdot$PCr$_e$$

The right-most term ($\gamma \cdot$PCr$_e$) is positive because both variables are negative.

**COMPARISON OF MR GLYCOLYSIS MEASUREMENT WITH BIOCHEMICAL MEASUREMENT OF LACTATE ACCUMULATION.** The accumulation of lactate in the muscle was compared with the MR measurement of glycolysis in the same muscle tissue. Immediately following the final MR acquisition, the mouse was removed from the magnet and probe while maintaining ischemia in the hindlimb. The ischemic leg was immediately removed and freeze-clamped in liquid N$_2$. This process was then repeated on the contralateral leg. The time it took to remove the mouse from the magnet and freeze the leg was recorded for each experiment (4–7 min). The MRS measurement of glycolysis for each individual was extrapolated to account for the time lapsed between the final MR acquisition and the freezing of the leg muscles. This allowed us to directly compare the MRS measurement of glycolysis with the biochemical measurement of lactate generation within an individual leg and explains why MR data in Fig. 3 only goes to 20 min of ischemia.

**RESULTS**

**Resting Metabolites**

Figure 2 shows the spectra for resting mouse hindlimb muscle under aerobic conditions and at the end of 18 min of ischemia. The symmetrical $P_i$ line shape at the low pH at the end of prolonged ischemia indicates a uniform response by the underlying muscles and little apparent heterogeneity in metabolism. Thus there is no evidence for the split $P_i$ peaks, indicative of compartments differing in pH that result from distinct...
populations of fiber types, as seen in intense exercise in human muscle (23). The analysis of the fully relaxed spectra in resting muscle yielded a PCr/ATP of 3.54 ± 0.06 (mean ± SE; n = 20) and P/ATP of 0.45 ± 0.02. These determinations were combined with the HPLC analysis of hindlimb muscles isolated from the same animals ([ATP] = 7.8 ± 0.2 mM and total [creatinine] = 38.2 ± 0.5 mM) to yield the [PCr] and [P] for each animal, as shown in Fig. 3 below. These metabolite levels are consistent with a mixed fiber population with values intermediate between the fast-twitch extensor digitorum longus and slow-twitch soleus muscle (15).

**Metabolite Dynamics in Ischemia**

**Initial dynamics.** Figure 3 shows the metabolite and pH levels in hindlimb muscles through the experiment. The levels in hindlimb muscle before tourniquet application are shown up to time = 0. The onset of ischemia is shown by the vertical dotted line, which is followed by a brief period of continued oxidative phosphorylation, after which PCr breakdown, P accumulation, and a brief pH alkalinization are apparent. The change in PCr (20.2 ± 1.2 mM) was stoichiometric with the increase in P (18.7 ± 2.0 mM). There was a significant change in the phosphomonoester (PME) peak during ischemia (4.42 ± 0.8 mM, n = 7), but no significant difference in the decrease in PCr (19.54 ± 1.1 mM) vs. the increase in P and PME (22.0 ± 2.4 mM) for the seven experiments that permitted analysis of the PME peak. The P in resting muscle is too low for a reliable measurement of pH, but the rise in P with ischemia permits determining pH and reveals a rising level soon after ischemia that continues through the subsequent five spectra, followed by acidification of pH until the end of ischemia.

**ATP level.** Figure 4 shows the chemical determination of [ATP] in muscle from hindlimb fast frozen in liquid N2 with the tourniquet constricted at the end of each ischemic time period. The slope of the regression of [ATP] as a function of the period of ischemia was not significant (P > 0.1), indicating that [ATP] did not change with up to 25 min of ischemia.

**Buffer capacity.** Figure 5 shows the average PCr and pH changes during electrical stimulation for the four animals used to determine the in vivo buffer capacity. The PCr dropped during the period of alkalinization of pH at an ATPase rate of 0.046 ± 0.01 ATP mM/s, which consumed 0.01 H m/s, according to the Lohmann reaction (Eqs. 1–3). This change in [H+] and pH rise yielded an effective buffer capacity of 20.3 ± 4.0 slykes (Eq. 7). The change in P during this period represented 4.4 ± 1.2 slykes buffer capacity (Eq. 8). The difference in the effective and phosphate buffer capacities resulted in a nonphosphate (tissue) buffer capacity of 15.9 ± 3.6 slykes (Eq. 9).

The nonphosphate or tissue buffer capacity represented 89% of the total buffer capacity (Eq. 10) in resting muscle before ischemia. The rise in P during the ischemic period increased the buffer capacity from 17.8 to 28.0 slykes by the end of ischemia. Thus the relative contribution of the tissue buffer capacity progressively decreased to a final value of 56% of the total by the end of ischemia.

**Glycolysis**

**Lactate.** The lactate level in muscle before inflation of a tourniquet and at intervals up to 25 min of ischemia is shown in Fig. 6. Each datum represents one experiment and one mouse. The levels at time = 0 represent the contralateral legs without ischemia and averaged 1.8 ± 0.2 mM (n = 26). We determined the change in lactate generation (Δ[lactate]) as a function of time by subtracting the lactate values in the aerobic contralateral hindlimb from the lactate level in the ischemic muscles to yield the change in lactate generation during the MR determination of glycolytic H+ generation.
Glycolytic $H^+$ generation. Figure 7 shows the production of $H^+$ determined by $^{31}$P-MRS as a function of time in mouse hindlimb muscle exposed to prolonged ischemia ($n = 8$).

$H^+$ vs. lactate production. A direct comparison of the MR determination of muscle $H^+$ accumulation in the same muscle for which a biochemical assay of muscle lactate was performed is shown in Fig. 8. The subsequent rise in $[H^+]$ and in $[lactate]$ during ischemia were closely correlated over a range that reached $~25$ mM, with a slope near identity ($0.98; r^2 = 0.86$). No difference ($P > 0.9, n = 25$) was found in a paired comparison of $[H^+]$ (mean = $11.2$ mM) vs. $[lactate]$ (mean = $11.3$ mM). This correspondence between $^{31}$P-MRS determination of $H^+$ production and biochemical assay of lactate in the same hindlimb muscles points to a quantitative link between glycolytic $H^+$ generation and lactate accumulation in the intact muscle in vivo.

DISCUSSION

This study tested the link between lactate generation and cellular acidosis under physiological conditions for the muscle cell in vivo. Our first finding was no significant change in ATP (Fig. 4), but a stoichiometric decline in PCr ($20.2 \pm 1.2$ mM) vs. rise in Pi ($18.7 \pm 2.0$ mM; Fig. 3) by the end of 25 min of ischemia in mouse hindlimb muscle. Thus the lack of net ATP hydrolysis eliminated nonglycolytic ATP hydrolysis as a source of $H^+$ generation during the process of lactic acidosis. The absence of a change in ATP level also showed the validity of the Lohmann reaction, which maintains ATP level by varying PCr level ($Eqs. 1–3$). Our second finding was quantitative agreement between the glycolytic $H^+$ production determined by $^{31}$P-MRS and the measured lactate level at discreet time points during ischemia over a range of pH (7.0–6.7) and up to $25$ mM [lactate], as shown by a regression slope near identity (Fig. 8). This close correspondence and the absence of significant ATP hydrolysis support the link between lactate production and $H^+$ generation, leading to acidosis under conditions that substantially elevate lactate and drop pH.

Physiological Conditions

The broad but physiological range of cell metabolites and pH required for a rigorous test of the concept of lactic acidosis was achieved in this experiment (12, 25). The pH drop from 7.0 to 6.7 provides a span of values frequently seen in muscle exposed to a strong perturbation, but still within conditions considered to be “physiological” for the muscle cell. The robust glycolysis underlying this cellular acidification generated [lactate] that reached the limits of the physiological range reported in muscle, such as in strenuous exercise in human muscle (25 mM) (7). Also found was a substantial reduction in

![Fig. 4. ATP level as a function of time during ischemia, as measured by HPLC analysis of hindlimb muscle. Correlation of ATP vs. time is not significant ($P > 0.1$).](http://jap.physiology.org/)

![Fig. 5. PCr and pH changes during ischemia induced by electrical stimulation in a single mouse hindlimb as an example of the measurement used to determine the cell buffer capacity. The time at which electrical stimulation is applied between acquisitions is noted by Stim on the plot. Data are presented as means ± SE; $n = 4$.](http://jap.physiology.org/)

![Fig. 6. Lactate level as a function of time during ischemia. The values at time = 0 are the lactate levels in the contralateral muscle under resting, aerobic conditions. Data points represent lactate determinations in a single hindlimb from 25 mice.](http://jap.physiology.org/)

![Fig. 7. Glycolytic production of $H^+$ ($\Delta[H^+]$) measured by $^{31}$P-MRS spectroscopy as a function of time during ischemia. Values are means ± SE; $n = 8$.](http://jap.physiology.org/)
the net accumulation of H\(^+\) in the cell. Two factors are important to accurately determine the H\(^+\) accumulation (Eq. 11): 1) that the chemical shift of the Pi peak reflects the true intracellular pH; and 2) that all metabolites remain in the muscle during the assays. Graham et al. (5) reported a careful analysis of the pH determination by Pi chemical shift in vivo and found minimal error if intracellular pH was close to the Pi pK\(_a\) and heterogeneity of intracellular pH was small among the underlying muscle fibers. Only small errors in the pH determination are expected in this study, because the pK\(_a\) of Pi (6.8) is <0.3 units from the intracellular pH throughout the experiment (6.70–7.05). Also, the symmetrical and narrow Pi peak in the \(^{31}\)P-MR spectrum at rest and through ischemia indicates little underlying pH heterogeneity (Fig. 2). Thus the physiological range of pH generated by this ischemic bout is well suited for accurate determination of cell pH and quantification of glycolysis.

Comparison of glycolytic H\(^+\) and lactate production is possible in our experiment because an ischemic tourniquet ensured that both metabolites are retained in the muscle for analysis. This retention of metabolites in the muscle means that neither biocarbonate buffering nor strong ion effects (Stewart analysis) need to be taken into account in our analysis (11). The extensive exchange of ions between muscle and blood that occurs with open circulation changes bicarbonate buffering due to CO\(_2\) exchange, and this ion exchange is thought to be the basis of the Stewart strong ion effect in blood pH buffering. Those processes are expected to occur to a negligible extent in our experiment, because the extracellular space is on the order of 10% of total muscle volume. Thus the lack of substantial ion movements during ischemia means analysis of H\(^+\) generation based on the stoichiometry of the Lohmann reaction, cell metabolites, and pH is sufficient to quantify glycolysis in ischemic muscle and test the coupling of lactate generation to H\(^+\) production.

**Lactic Acidosis**

A similar slow onset and time course of accumulation for glycolytic H\(^+\) (Fig. 7) and for lactate (Fig. 6) are apparent in ischemic mouse hindlimb muscles. Figure 8 shows that these similar time courses reflect a close correspondence between H\(^+\) generation and lactate production (solid regression line) that is quantitatively linked, as evident by a regression slope near 1 that parallels the identity line (dashed line). Thus H\(^+\) generation matches the elevation in [lactate] up to some of the highest values reported under physiological conditions [i.e., ~25 mM (7)]. This quantitative correspondence and the stability in [ATP] during ischemia provide strong support for the link between lactate generation and acidosis over a wide range of pH in muscle in vivo.

Our laboratory has previously found good agreement between MR determinations of glycolytic H\(^+\) production with lactate generation in vivo (14). In rattlesnake muscle, the MR determination of glycolysis during sustained rattling was confirmed by direct measurement of lactate released by the tailshaker muscles into the blood circulation. The disadvantage of the rattlesnake experiment was that the MR and lactate determinations were done under ischemic and aerobic rattling conditions, respectively. The hindlimb ischemia experiment reported here eliminated this limitation by making lactate and
H⁺ determinations on the same muscle in the same ischemic bout. Despite these experimental differences, the same result was found in the two studies: MR determination of H⁺ production agreed with biochemical measurements of lactate generation. This agreement between glycolytic H⁺ production and elevation of lactate, as well as the stability of [ATP], supports the notion of lactate acidosis under conditions that substantially elevate lactate and drop pH. Thus over a wide physiological range, the generation of H⁺ is quantitatively linked to the accumulation of lactate under ischemia, in accordance with the classical unitary H⁺/lactate stoichiometry of glycolysis.

Stoichiometry of Glycolysis

This quantitative agreement between H⁺ and lactate generation has a mechanistic basis, as demonstrated in numerous analyses (4, 9, 11, 16, 28). Two factors that account for this quantitative agreement are the links among cell energetic reactions (Eqs. 1–6) and the tradeoff of pH sensitivity of the underlying stoichiometries (Fig. 1). Hochachka and Mommsen (9) summarized these two points in their paper entitled “Protons and anaerobiosis”: “Because of opposite pH dependencies of proton production by fermentation and by hydrolysis of the adenosine triphosphate formed in the fermentation, the total number of moles of protons generated is always two per mole of the fermentable substrate.”

A constant 2 H⁺ per 2 lactate (derived from one glucose or glucosyl unit) is the key finding of this and other experiments (14), as well as mechanistic analyses (9, 11, 17, 28). An example of how this constant stoichiometry results with distinct pH dependencies of proton production of the underlying reactions is shown in Fig. 1. Opposite pH dependencies are apparent in the rise in the coefficient involved in PCr splitting to meet ATP demand (γ, Lohmann reaction) vs. the decline in the coefficient involved in glycolytic PCr synthesis that reverses the Lohmann reaction (δ). The net result is a constant glycolytic synthesis of 1 H⁺ per 0.67 PCr [ΔH⁺ = (γ + δ)ΔPCr or more conventionally 1.5 PCr/H⁺], independent of pH (19).

An example of the second factor—the mechanistic links among the ATPase, CK, and glycolytic reactions—that underlies the quantitative agreement between H⁺ and lactate generation in glycolysis in skeletal muscle is shown in Table 1. This table reveals that ATP hydrolysis in skeletal muscle yields a net H⁺ uptake rather than H⁺ generation as the result of coupling the CK reaction (H⁺ consumption) to maintain ATP stability in the face of ATP hydrolysis (H⁺ generation) (Lohmann reaction, Eqs. 1–3). Glycolysis reverses these reactions (reverse Lohmann reaction, Eqs. 4–6) to generate 2 H⁺ (−0.6 + 2.6 = 2.0) per 2 lactate. Thus, in skeletal muscle, the mechanistic link between H⁺ production and lactate generation results from glycolysis reversing the Lohmann reaction that maintains ATP stable in the face of ATP hydrolysis. This reversal restores the H⁺ produced by ATP hydrolysis and simultaneously generates 2 lactate in the process. Thus there is no mismatch in H⁺ production and lactate generation in the balance of reactions involving glycolysis in vivo under physiological conditions in which ATP level is stable. These results demonstrate that the net H⁺ generation by the reactions associated with glycolysis are stoichiometrically linked to the production of lactate, indicating that lactate generation is the cause of cellular acidosis in vivo under physiological conditions.

Failure of Lactic Acidosis

The conditions under which nonglycolytic sources of H⁺ production would likely disrupt the quantitative link between H⁺ production and lactate generation in the cell found here can be deduced from the phosphorus metabolite dynamics shown in Fig. 3. The 25 min of ischemia nearly depleted PCr, and longer periods are expected to completely eliminate this energy buffer and necessitate net ATP hydrolysis to meet ATP demands of the cell. Prolonged ischemia (27) and vigorous exercise (7) have been documented to achieve PCr depletion and perturb cell ATP level. Such ATP hydrolysis would generate H⁺ independent of glycolysis and uncouple acidosis from lactate generation. Thus prolonged energy imbalance in the cell would be expected to generate H⁺ by nonglycolytic pathways and disrupt the link between acidosis and lactate generation. A nonstoichiometric appearance of H⁺ vs. lactate in the blood under open-flow conditions would also be expected, if there is a differential transport of these metabolites across the muscle cell wall into the blood.

Summary

This study tested the concept of lactic acidosis under physiological conditions in the muscle cell by generating a large range of H⁺ and lactate levels using timed intervals of ischemia. The H⁺ generation quantitatively agreed with the accumulation of lactate with a slope near identity. The lack of change in ATP level in the cell confirmed the absence of H⁺ generation outside of glycolysis. These results are supported by an analysis of the underlying reactions involved in glycolysis in vivo. This agreement between lactate accumulation and glycolytic H⁺ production without significant ATP hydrolysis supports the link between lactate and acidosis under cellular conditions that substantially elevate lactate and drop pH. They also reveal cell conditions under which net hydrolysis of ATP

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ATP</th>
<th>PCr</th>
<th>H⁺ Production</th>
<th>Lactate</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase</td>
<td>−3</td>
<td>0</td>
<td>3α = 2.2*</td>
<td>0</td>
<td>i</td>
</tr>
<tr>
<td>CK</td>
<td>3</td>
<td>−3</td>
<td>3β = −2.8*</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>PCr splitting: Lohmann reaction</td>
<td>0</td>
<td>−3</td>
<td>3γ = 3(α + β) = −0.6</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Reverse ATPase</td>
<td>3</td>
<td>0</td>
<td>3ε = −0.2*</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Reverse CK</td>
<td>−3</td>
<td>3</td>
<td>−3β = 2.8*</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>PCr synthesis: reverse Lohmann reaction</td>
<td>0</td>
<td>0</td>
<td>3β = 3(ε − β) = 2.6</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Glycolysis in vivo</td>
<td>0</td>
<td>0</td>
<td>2 = (−0.6 + 2.6)</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

PCr, phosphocreatine; CK, creatine kinase. *Coefficient values are from Ref. 16. †ε = −0.08 at pH 7.0, as calculated using the approach described in Ref. 16.
and generation of H⁺ independent of glycolysis are likely and the link between lactate generation and acidosis would be violated. For a physiological range of pH and lactate levels in muscle achieved by moderate ischemia or exercise, lactate acidosis is a robust concept.

ACKNOWLEDGMENTS

Thanks go to Daryl Monear and Sharon Jubrias for comments.

GRANTS

This study was supported by National Institutes of Health R01 Grants AG10853, AR41928, AG028455, and AR45184.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES