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A ^{13}C isotopomer kinetic analysis of cardiac metabolism: influence of altered cytosolic redox and $[Ca^{2+}]_o$

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Carvalho, Rui A., Tiago B. Rodrigues, Piyu Zhao, F. Mark H. Jeffrey, Craig R. Malloy, and A. Dean Sherry. A ^{13}C isotopomer kinetic analysis of cardiac metabolism: influence of altered cytosolic redox and $[Ca^{2+}]_o$. *Am J Physiol Heart Circ Physiol* 287: H889–H895, 2004. First published March 25, 2004; 10.1152/ajpheart.00976.2003.—Rat hearts were perfused with mixtures of [3-^{13}C]pyruvate and [3-^{13}C]lactate (to alter cytosolic redox) at low (0.5 mM) or high (2.5 mM) Ca^{2+} concentrations to alter contractility. Hearts were frozen at various times after exposure to these substrates, were extracted, and were then analyzed by ^{13}C NMR spectroscopy. The time-dependent multiplets observed in the ^{13}C NMR resonances of glutamate in all hearts and in malate and aspartate in hearts perfused with high-pyruvate/low-lactate concentrations were analyzed using a kinetic model of the tricarboxylic acid (TCA) cycle. The analysis showed that TCA cycle flux (V_{TCA}) and exchange flux (V_X) that involved cycle intermediates were both sensitive to cell redox and altered Ca^{2+} concentration, and the ratio of these fluxes (V_X/V_{TCA}) varied >10-fold.

^{13}C nuclear magnetic resonance; tricarboxylic acid cycle; intermediary metabolism; malate-aspartate shuttle; modeling; extracellular calcium concentration

A COMMON ASSUMPTION in early dynamic NMR studies was that exchange between α-ketoglutarate (α-KG) and glutamate was rapid compared with tricarboxylic acid flux (V_{TCA}), and hence temporal enrichment of glutamate C4 provided a direct readout of cycle activity. Later kinetic studies of isolated hearts demonstrated that two fluxes, V_{TCA} and exchange flux (V_X), are required to adequately describe the rates of ^{13}C enrichment at glutamate C4 and C3 (3, 4, 8, 18, 19, 22–26). V_X has been ascribed to an exchange process associated with the malate-aspartate shuttle in addition to the transaminase exchange (15). The malate-aspartate shuttle is highly active in cardiac tissue and thought to be the primary process for transporting reducing equivalents (NADH) from cytosol to mitochondria (16). The shuttle consists of the cytosolic and mitochondrial glutamate-oxaloacetate (OAA) transaminase (GOT) and two transporter proteins in the inner mitochondrial membrane: the reversible α-KG-malate exchanger and the unidirectional glutamate-aspartate exchanger (10). It has been shown (15) in isolated mitochondria that increased Ca^{2+} (6, 9, 20, 21) or H^+ (decreased pH) concentration increases the affinity of α-KG dehydrogenase for α-KG and alters mitochondrial levels of α-KG. This in turn has been reported to stimulate aspartate formation due to less inhibition of aspartate aminotransferase by α-KG and, consequently, overall stimulation of the malate-aspartate shuttle (21). Thus if V_X reflects compartmental exchange of glutamate-α-KG, this working model would predict that increased Ca^{2+} levels should not only increase contractility and O_2 consumption (and hence V_{TCA}) but increase V_X as well. A positive correlation between V_{TCA} and V_X was indeed observed recently (3) in mouse hearts perfused with various single substrates, although in this case, the variations in V_{TCA} and V_X were due to differences in the oxidation state of the substrates presented to the hearts rather than to changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). V_X also increased significantly in hearts perfused with butyrate upon addition of lactate (25) purportedly due to increased malate-aspartate shuttle activity. More recently, O'Donnell et al. (15) have shown in isolated rabbit heart mitochondria that lowering the pH results in a decrease in the K_m of α-KG dehydrogenase for α-KG and hence a reduced efflux of α-KG from mitochondria (reflecting lower malate-aspartate shuttle activity). This was also reflected by a lower V_X (F_1) value measured by NMR in intact hearts perfused at pH 6.6. These results were consistent with the hypothesis that direct competition between α-KG dehydrogenase (K_m, ~0.10 mM at pH 6.8) and the α-KG-malate transporter (K_m, ~1.5 mM) for mitochondrial α-KG is responsible for the observed decrease in malate-aspartate shuttle activity. Hence, either increased $[Ca^{2+}]_o$ or H^+ concentration results in a decrease of the K_m of α-KG dehydrogenase, yet Ca^{2+} purportedly stimulates malate-aspartate shuttle activity, whereas H^+ moderates it. This suggests that the effects of Ca^{2+} and H^+ differ in mechanistic origin.

In the present study, the temporal evolution of ^{13}C isotopomers in malate, aspartate, and glutamate in perfused hearts exposed to two different levels of Ca^{2+} and with either high-lactate/low-pyruvate or high-pyruvate/low-lactate concentrations to alter cytosolic redox was measured in freeze-clamped tissues by ^{13}C NMR with an aim toward better defining the metabolic factors that contribute to V_{TCA} and V_X. A kinetic analysis of the data shows that V_{TCA} is influenced more by changes in $[Ca^{2+}]_o$, whereas V_X is more sensitive to changes in cytosolic redox. Interestingly, the V_X/V_{TCA} ratio was found to

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be especially sensitive to these physiological variables and could prove useful as an indicator of tissue physiology.

MATERIALS AND METHODS

Materials. The [3-^{13}C]pyruvate and [3-^{13}C]lactate (99% BC enrichment) were purchased from Cambridge Isotope Laboratories (Andover, MA). All other materials were of the highest quality available from commercial sources. Male Sprague-Dawley rats (250–300 g) were obtained from Sasco (Houston, TX).

Heart perfusion. This study was performed under a protocol approved by the University of Texas Southwestern Animal Care and Use Committee. After administration of general anesthesia, hearts were rapidly excised and immersed in cold perfusate. The aorta was immediately cannulated for retrograde Langendorff perfusion at 100 cm of hydrostatic pressure (14) with a modified Krebs-Henseleit bicarbonate buffer maintained at 37°C that contained (in mM) 119.2 NaCl, 4.7 KCl, 0.5 (or 2.5) $CaCl_2$, 1.2 $MgSO_4$, and 25 $NaHCO_3$. The buffer was saturated with 95% O_2-5% CO_2 and a pH of 7.4 was maintained. A small latex balloon, which was sutured to one end of a polyethylene (PE) tube and attached at the other end to a pressure transducer, was inserted into the left ventricle through the mitral valve. This balloon enabled the continuous measurement of left ventricular developed pressure (LVDP) and heart rate (HR) throughout the experiment. Myocardial O_2 consumption ($M\dot{V}_{O_2}$) was calculated from the difference in O_2 tension (measured with a blood gas analyzer) between the perfusion medium in the arterial supply line and the coronary effluent, which was collected via a cannula placed in the pulmonary artery (13). Coronary flow was measured using a stopwatch and a graduated cylinder.

Hearts were initially perfused with substrates that contained natural-abundance levels of ^{13}C to ensure a metabolic steady state (constant pool sizes) before switching to perfusates that contained ^{13}C-enriched substrates at the same concentrations. Four experimental perfusate conditions were chosen: 4 mM [3-^{13}C]pyruvate, 0.4 mM [3-^{13}C]lactate, plus either 0.5 or 2.5 mM Ca^{2+} (*groups 1* and *2*) and 0.4 mM [3-^{13}C]pyruvate, 4 mM [3-^{13}C]lactate, plus either 0.5 or 2.5 mM Ca^{2+} (*groups 3* and *4*). After perfusion with ^{13}C-enriched substrates for varying periods of time, hearts were freeze-clamped using aluminum tongs precooled in liquid N_2. A small portion of frozen tissue was used for determination of the wet-to-dry ratio by weighing the tissue before and after slow oven drying. The remainder of the frozen tissue was pulverized into a fine powder under liquid N_2, extracted in 8% perchloric acid, centrifuged at 15,000 *g* for 15 min at 4°C, neutralized with KOH, and centrifuged a second time. The supernatant was freeze-dried, and the lyophilized heart extract was dissolved in 0.55 ml of ^2H_2O plus 0.05 ml of a concentrated solution of EDTA in ^2H_2O. EDTA was added to remove Ca^{2+} from its citrate complex so that the C2-C4 resonance of citrate was narrow enough to allow analysis of isotopomer populations.

^{13}C NMR. Proton-decoupled ^{13}C NMR spectra of heart extracts (pH measured in ^2H_2O solutions, 7.2; 25°C) were acquired at 125.7 MHz on a Varian INOVA spectrometer (Varian Instruments; Palo Alto, CA) using a 5-mm broadband NMR probe, a 45° observed pulse, and a 3-s delay between pulses. Broadband proton decoupling was achieved using Waltz decoupling at two power levels: low during the preacquisition delay and high during acquisition. Relative peak areas for each multiplet component were determined by deconvolution using the personal computer-based NMR program NUTS (Acorn; Freemont, CA).

Tissue measurements. After acquisition of NMR spectra, samples were freeze-dried, redissolved in H_2O, and assayed for total tissue aspartate, glutamate, citrate, and malate using standard enzymatic assays measured spectrophotometrically (1). Appropriate calibration curves prepared from standards were used to derive metabolite tissue contents.

Modeling. Metabolite pool sizes and ^{13}C NMR multiplet data were used as input to a kinetic model of the TCA cycle. The program tcaFLUX (8), which is an extension of the model reported by Chance et al. (5), provides a best fit of V_{TCA}, V_X, anaplerosis (y), and fractional enrichment of acetyl-CoA (F_{C2}) to time-dependent ^{13}C multiplet data (12). The temporal changes in the glutamate C1 doublet (1D), C2 doublets (D12 and D23), C2 quartet (2Q), C3 doublet and triplet (3D and 3T), and C4 doublet (D34) were used in the fitting procedure for hearts perfused with low-pyruvate/high-lactate concentrations (other metabolites were not detected with these perfusion conditions). In addition to these same glutamate multiplets, temporal data for malate C2 (D23 and Q), malate C3 (D23 and Q), aspartate C2 (D23 and Q), and aspartate C3 (D23 and Q) were also included in the fitting procedure for hearts perfused with high-pyruvate/low-lactate concentrations. Dilution of [3-^{13}C]pyruvate/[3-^{13}C]lactate by other endogenous substrates, expressed as the fraction of acetyl-CoA that is unenriched ($F_{C0} = 1 - F_{C2}$), was also a variable fitted by the kinetic analysis. All results are presented as means ± SD. Differences in mean values were considered statistically significant at a probability level of <5% ($P < 0.05$). Student's *t*-test was used to compare means.

RESULTS

Cardiac performance. HR, LVDP, and $M\dot{V}_{O_2}$ were measured for each heart. The rate pressure product (RPP) was not sensitive to the pyruvate-lactate ratio but was significantly higher ($P < 0.0001$) at increased $[Ca^{2+}]_o$. At 0.5 mM Ca^{2+}, the RPP values were (12 ± 4) × 10^3 and (10 ± 4) × 10^3 mmHg/min at low- and high-pyruvate concentrations, respectively, and at 2.5 mM Ca^{2+}, RPP values were (42 ± 3) × 10^3 and (43 ± 5) × 10^3 mmHg/min at low- and high-pyruvate concentrations, respectively. $M\dot{V}_{O_2}$ values also increased significantly between the low- and high-$[Ca^{2+}]_o$ groups as expected. Interestingly, $M\dot{V}_{O_2}$ was also higher between the low- vs. high-pyruvate groups with either 0.5 or 2.5 mM Ca^{2+} in the perfusate.

Tissue levels of TCA cycle intermediates. The most abundant metabolite in hearts perfused with a mixture of 0.4 mM [3-^{13}C]pyruvate and 4.0 mM [3-^{13}C]lactate was glutamate (Table 1). The tissue contents of other cycle intermediates and aspartate were small and could therefore be neglected in the kinetic analysis (22). Tissue glutamate content tended to decrease at higher $[Ca^{2+}]_o$ (20.4 ± 5.3 vs. 17.5 ± 4.2 μmol/g dry wt at 0.5 vs. 2.5 mM Ca^{2+}, respectively) but did not reach statistical significance. The metabolite content of hearts perfused with 4.0 mM [3-^{13}C]pyruvate and 0.4 mM [3-^{13}C]lactate was, however, quite different; glutamate con-

Table 1. *Tissue contents of major heart metabolites*

Pyruvate-to-Lactate Ratio	$[Ca^{2+}]_o$, mM	Metabolite, μmol/g dry wt			
		Glutamate	Aspartate	Malate	Citrate
0.1	0.5	20.4±5.3			
0.1	2.5	17.5±4.2			
10	0.5	4.8±1.6*	3.6±0.5*	2.7±0.7*	3.6±0.3
10	2.5	6.6±0.9*	8.1±1.3*	4.6±0.9*	3.6±0.3

Values are means ± SD; n = 15 hearts in each extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) group. Metabolite contents were measured by enzymatic assay. A [3-^{13}C]pyruvate-to-[3-^{13}C]lactate ratio of 0.1 refers to hearts perfused with 0.4 mM [3-^{13}C]pyruvate and 4.0 mM [3-^{13}C]lactate; ratio of 10 refers to hearts perfused with 4.0 mM [3-^{13}C]pyruvate and 0.4 mM [3-^{13}C]lactate. *$P < 0.05$ for respective 0.5 vs. 2.5 mM $[Ca^{2+}]_o$ groups.

tent was lower, whereas citrate, malate, and aspartate were all present in significant amounts (Table 1). With this substrate mix, a increase in $[Ca^{2+}]_o$ from 0.5 to 2.5 mM did produce significant increases in glutamate content (4.8 ± 1.6 vs. 6.6 ± 0.9 μmol/g dry wt), aspartate (3.6 ± 0.5 vs. 8.1 ± 1.3 μmol/g dry wt), and malate (2.7 ± 0.7 vs. 4.6 ± 0.9 μmol/g dry wt). Thus an increase in the pyruvate-lactate ratio from 0.1 (typical of physiological conditions) to 10 resulted in an approximately fourfold reduction in tissue glutamate level at low $[Ca^{2+}]_o$ (from 20.4 ± 5.3 to 4.8 ± 1.6 μmol/g dry wt) and an approximately threefold reduction at high $[Ca^{2+}]_o$ (from 17.5 ± 4.2 to 6.6 ± 0.9 μmol/g dry wt) and concomitant increases in aspartate, malate, and citrate (Table 1).

^{13}C NMR spectra. The [3-^{13}C]pyruvate and [3-^{13}C]lactate (after conversion to pyruvate) are avidly oxidized by heart tissue first via conversion to [2-^{13}C]acetyl-CoA at the level of pyruvate dehydrogenase and subsequently via the TCA cycle. Condensation of [2-^{13}C]acetyl-CoA with OAA on each turn of the cycle results in the entry of ^{13}C into all cycle intermediates and metabolites derived from those intermediates. After multiple cycle turns, each metabolite resonance appears as multiplets largely due to one-bond ^{13}C-^{13}C spin-spin couplings. Figure 1 shows expanded aliphatic regions (10–80 ppm) of ^{13}C spectra of heart extracts after perfusion to steady state with either 4.0 mM [3-^{13}C]pyruvate and 0.4 mM [3-^{13}C]lactate or 0.4 mM [3-^{13}C]pyruvate and 4.0 mM [3-^{13}C]lactate, both at low $[Ca^{2+}]_o$. In Fig. 1*A* (high-pyruvate/high-lactate concentrations), the resonances of glutamate, aspartate, malate, and citrate all appear as multiplets, whereas in Fig. 1*B* (low-pyruvate/low-lactate concentrations), only glutamate was detected. A significant amount of [3-^{13}C]alanine (a singlet) was also evident in spectra of hearts perfused with high-pyruvate/high-lactate concentrations, which reflects the high intracellular content of [3-^{13}C]pyruvate.

Figure 2 illustrates the temporal evolution of several multiplet components (glutamate C4D34, aspartate C3D23 and C3Q, and malate C3D23 and C3Q) for hearts perfused with

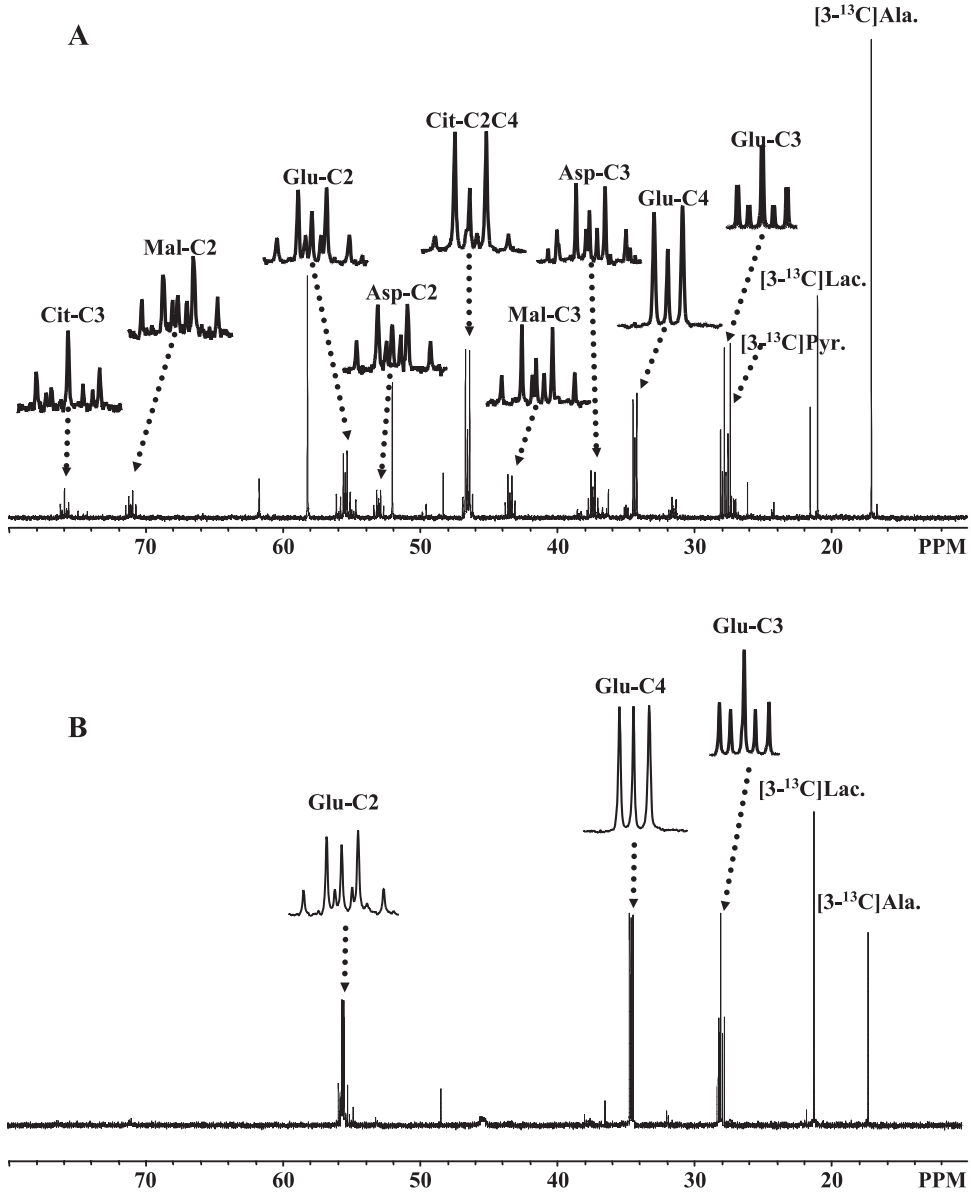

Fig. 1. 125.7-MHz ^{13}C NMR spectra of extracts from rat hearts perfused with 4.0 mM [3-^{13}C]pyruvate plus 0.4 mM [3-^{13}C]lactate (*A*) or 0.4 mM [3-^{13}C]pyruvate plus 4.0 mM [3-^{13}C]lactate (*B*), both in the presence of low (0.5 mM) extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Aside from glutamate carbons (Glu-C2, Glu-C3, and Glu-C4), expansions in *A* also show (*left to right*) citrate C3 (Cit-C3), malate C2 (Mal-C2), aspartate C2 (Asp-C2), citrate C2 and C4 (Cit-C2C4), malate C3 (Mal-C3), and aspartate C3 (Asp-C3). Expansions in *B* refer to glutamate carbons C2 (Glu-C2), C3 (Glu-C3), and C4 (Glu-C4).

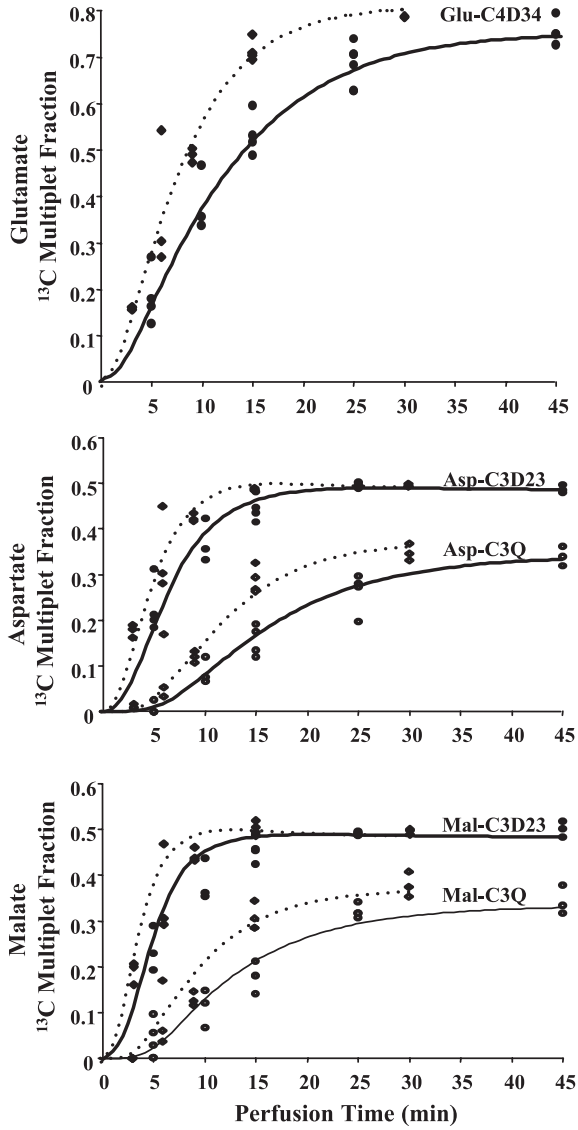

Fig. 2. Temporal evolution of ^{13}C NMR multiplet components for glutamate, aspartate, and malate in hearts perfused with 4.0 mM [3-^{13}C]pyruvate plus 0.4 mM [3-^{13}C]lactate in the presence of either 0.5 mM (solid line) or 2.5 mM (dotted line) $[Ca^{2+}]_o$. Curves for aspartate and malate approach one another for both C3D23 and C3Q, reflecting similar kinetics of ^{13}C appearance in these two metabolites. Evolution of the C4D34 of glutamate is slower, reflecting either a slow exchange with tricarboxylic acid cycle intermediates or a dilution of the ^{13}C labeling by the large glutamate cytosolic pool. An increase in $[Ca^{2+}]_o$ caused an increase in the rate of ^{13}C incorporation for all metabolites.

high-pyruvate/low-lactate concentrations at 0.5 and 2.5 mM $[Ca^{2+}]_o$. Similar graphs were obtained for hearts perfused with low-pyruvate/high-lactate concentrations, but in this case, only glutamate multiplets were detected. As indicated above, changes in the pyruvate-lactate ratio result in considerable redistribution of metabolite contents, and this makes a dramatic difference in the temporal evolution of the ^{13}C multiplets (evolution of isotopomer populations). Hearts perfused with high vs. low $[Ca^{2+}]_o$ were freeze-clamped at different time intervals (as noted in Fig. 2) to account for differences in TCA cycle kinetics in these two groups. Metabolic steady state was reached after ~30 min in hearts perfused with 2.5 mM $[Ca^{2+}]_o$ and after ~45 min in hearts perfused with 0.5 mM $[Ca^{2+}]_o$.

Kinetic analysis of isotopomer data. A kinetic analysis of the time-dependent ^{13}C-multiplet areas yielded values for V_{TCA} and V_X as summarized in Table 2 (values in parentheses are 5–95% confidence limits for each flux). The fractional contribution of lactate plus pyruvate to acetyl-CoA (F_{C2}) was identical in all groups: when supplied with low-pyruvate/high-lactate concentrations, their contribution was 0.88 (0.85–0.90) at low $[Ca^{2+}]_o$ and 0.89 (0.87–0.92) at high $[Ca^{2+}]_o$; when perfused with high-pyruvate/low-lactate concentrations, at low $[Ca^{2+}]_o$, the value was 0.90 (0.88–0.92) and at high $[Ca^{2+}]_o$, it was 0.92 (0.90–0.95). An increase in $[Ca^{2+}]_o$ from 0.5 to 2.5 mM resulted in an approximately twofold increase in V_{TCA} for both the low-and high-pyruvate groups. Similarly, an increase in $[Ca^{2+}]_o$ resulted in a V_X increase of approximately threefold for the low-pyruvate group and nearly fourfold for the high-pyruvate group. Changing the substrate presented to hearts from a low- to a high-pyruvate concentration resulted in only small increases in V_{TCA} (~1.4-fold), whereas V_X decreased four to fivefold. Similar trends in V_{TCA} and V_X were obtained by analysis of the temporal changes in glutamate C4D34 alone (Table 3).

It has been suggested that inclusion of additional ^{13}C multiplet data from intermediates such as malate and aspartate in a kinetic analysis may provide more reliable evaluations of V_{TCA} and V_X compared with inclusion of glutamate data alone (4). A separate kinetic fitting of glutamate C4D34 multiplet data alone yielded similar average values for V_{TCA} and V_X (Table 3) as those obtained by analysis of multiplet data from all visible metabolites (see Table 2), although three of the total of eight flux measurements fell outside the 5–95% confidence range established by the more-complete analysis. As anticipated, the confidence levels were about twofold larger on average for the glutamate C4D34 fits alone. The one exception was the value of V_X for the high-pyruvate/low-lactate groups, where the 5–95% confidence spread in V_X was smaller when glutamate

Table 2. *O_2 consumption and fluxes as determined by kinetic analysis of NMR isotopomer data from glutamate, aspartate, and malate*

Pyruvate-to-Lactate Ratio	$[Ca^{2+}]_o$, mM	$M\dot{V}_{O_2}$, μmol·min^{-1}·g dry wt^{-1}	V_{TCA}, μmol·min^{-1}·g dry wt^{-1}	V_X, μmol·min^{-1}·g dry wt^{-1}	V_X/V_{TCA}
0.1	0.5	11.1±2.1*	3.21 (2.77–3.60)	6.68 (4.40–9.46)	2.0
0.1	2.5	21.5±3.7*	6.55 (5.83–7.14)	18.0 (11.9–27.0)	2.7
10	0.5	14.5±2.2*	4.41 (4.23–4.59)	1.27 (0.25–1.71)	0.3
10	2.5	27.3±3.5*	8.48 (8.11–8.87)	4.79 (3.54–6.48)	0.6

Values are means ± SD; 5–95% confidence limits are shown in parentheses. V_{TCA}, tricarboxylic acid cycle flux; V_X, exchange flux. $M\dot{V}_{O_2}$ (myocardial O_2 consumption) was significantly different between the low- (0.05 mM) and high- (2.5 mM) Ca^{2+} groups and between the low-pyruvate/high-lactate (0.1) and the high-pyruvate/low-lactate (10) groups. *$P < 0.001$.

Table 3. *Fluxes as determined by kinetic analysis of glutamate C4D34 alone*

Pyruvate-to-Lactate Ratio	$[Ca^{2+}]_o$, mM	V_{TCA} μmol·min^{-1}·g dry wt^{-1}	V_X μmol·min^{-1}·g dry wt^{-1}	V_X/V_{TCA}
0.1	0.5	3.41 (2.66–3.74)*	7.73 (3.78–12.84)*	2.3
0.1	2.5	6.98 (6.48–8.04)*	31.5 (17.7–100)*	4.5
10	0.5	3.50 (3.30–3.85)*	1.69 (1.67–1.84)*	0.5
10	2.5	6.90 (6.38–7.59)*	5.78 (5.77–6.35)*	0.8

Values are means ± SD; 5–95% confidence limits are shown in parentheses. *$P < 0.05$, significantly different fluxes.

C4D34 was fitted alone. This is more a reflection of the lower concentration of glutamate. A significant difference in flux values was found only for the low-pyruvate/high-lactate, high-$[Ca^{2+}]_o$ group, where V_X was poorly defined using glutamate C4D34 alone. This is also the group that showed the greatest disparity between V_X and V_{TCA} values, which suggests that the glutamate C4D34 describes the kinetics well whenever V_X and V_{TCA} are similar in magnitude and the extra multiplet data add substantially to the fit whenever there is the greatest disparity between these flux values.

DISCUSSION

Tissue metabolites. Exposure of hearts to different ratios of pyruvate and lactate (with total concentrations held constant at 4.4 mM) alters the cytosolic redox state and subsequently the steady-state levels of several tissue metabolites (see Table 1). One should note that these changes do not result from a change in tissue pH, because even the highest level of lactate used here (4 mM) does not exceed the buffering capacity of the tissue. Rather, these changes must originate from changes in the redox state of the tissue (11).

A 10-fold increase in pyruvate from 0.4 to 4 mM would predictably decrease cytosolic NADH and increase cytosolic NAD^+. This in turn should favor formation of OAA from malate (via cytosolic malate dehydrogenase) and subsequent conversion of OAA to aspartate and α-KG to glutamate (via the transaminase GOT). This would result in a net shift in the tissue content of many TCA cycle and related intermediates (aspartate, malate, and citrate). This was observed experimentally at both high and low $[Ca^{2+}]_o$, although it is interesting to note that there was a net decrease in total tissue metabolites in the low-$[Ca^{2+}]_o$ group as pyruvate was increased from 0.4 to 4 mM (20.4 ± 5.3 vs. 14.7 ± 3.0 μmol/g dry wt) and a net increase in total tissue metabolites in the high-$[Ca^{2+}]_o$ group as pyruvate was increased from 0.4 to 4 mM (17.5 ± 4.2 vs. 22.9 ± 3.3 μmol/g dry wt). This demonstrates that $[Ca^{2+}]_o$ does indeed have a significant effect on total tissue metabolite levels, although the effect is more evident when the metabolites are more evenly distributed among glutamate, aspartate, malate, and citrate (high-pyruvate group).

An increase in $[Ca^{2+}]_o$ from 0.5 to 2.5 mM had a more subtle effect on tissue metabolites (see Table 1). With an increase in $[Ca^{2+}]_o$, tissue metabolites (mostly glutamate) tended to be lower in the low-pyruvate group but clearly increased in the high-pyruvate group. Here the observed changes in metabolite levels are not as easily predicted based on shifts in known equilibria, but studies of isolated mitochondria have demonstrated that mitochondrial Ca^{2+} content, which is known to increase with an increase in $[Ca^{2+}]_o$, significantly decreases the K_m of α-KG dehydrogenase and thereby lowers mitochondrial α-KG (21). This in turn should increase mitochondrial aspartate and decrease mitochondrial OAA and glutamate contents.

Influence of altered Ca^{2+} on TCA cycle kinetics. An increase in $[Ca^{2+}]_o$ is known to increase both contractility (2) and $M\dot{V}O_2$ (22). In these Langendorff-perfused hearts, an increase in $[Ca^{2+}]_o$ from 0.5 to 2.5 mM resulted in an approximately twofold increase in $M\dot{V}O_2$. This was also detected in our kinetic analyses as an increase in V_{TCA} (approximately twofold) between the low- vs. high-Ca^{2+} groups for both low-pyruvate and high-pyruvate groups. It was found that V_X was also stimulated at higher Ca^{2+} levels, and, in fact, V_X was about twice as sensitive to the fivefold increase in $[Ca^{2+}]_o$ as was V_{TCA}. The origin of this effect is unknown. It has been reported that increases in $[Ca^{2+}]_o$ stimulate both glycolysis and glucose oxidation by direct stimulation of phosphofructokinase-1 and pyruvate dehydrogenase by Ca^{2+} (17), but glucose was not present in these experiments, so the increase in V_X must have a different origin. Perhaps it is related to the increase in contractility and O_2 demand that is produced by increased $[Ca^{2+}]_o$. It has been proposed that V_X reflects an exchange between α-KG and glutamate (15), which in turn could reflect an increase in the malate-aspartate shuttle activity (21). Hence, higher $[Ca^{2+}]_o$ may stimulate the shuttling of more reducing equivalents from the cytosol into the mitochondria to contribute to the increased O_2 demand. In the present experiments, these reducing equivalents would most likely be contributed by cytosolic lactate, but unfortunately, we cannot differentiate between oxidation of pyruvate vs. oxidation of lactate because both substrates were labeled with ^{13}C equivalently. It is interesting to note, however, that the change in V_X with increased O_2 demand (low- vs. high-$[Ca^{2+}]_o$ groups) was much higher when the cytosol was more reduced (pyruvate-lactate ratio, 0.1).

Influence of changes in cytosolic redox on TCA cycle kinetics. One would anticipate that altering cytosolic redox by changing the pyruvate-lactate ratio would have predictable effects on both V_{TCA} and V_X. As a first approximation, one would anticipate that high-lactate (low-pyruvate) concentration would stimulate the malate-aspartate shuttle (25), and hence, more reducing equivalents would be transported from the cytosol into the mitochondria. Indeed, V_X was about four- to fivefold higher in hearts perfused with low-pyruvate/high-lactate concentrations at both levels of $[Ca^{2+}]_o$. Furthermore, import of more reducing equivalents from the cytosol into the mitochondria should also decrease the requirement of the TCA cycle to produce NADH; hence, one would expect that V_{TCA} would be lower. This was also detected by the kinetic analysis and by a direct measure of $M\dot{V}O_2$. As shown in Table 2, both V_{TCA} and $M\dot{V}O_2$ increased significantly between the low-

pyruvate/high-lactate group compared with the high-pyruvate/low-lactate group (at both low and high $[Ca^{2+}]_o$), although contractile function as measured by RPP did not change at either $[Ca^{2+}]_o$ level. The percent increases in $M\dot{V}_{O_2}$ between the low- and high-pyruvate groups were similar for hearts perfused with 0.5 mM (23% increase) vs. 2.5 mM (21% increase) $[Ca^{2+}]_o$. This indicates that the increase in $M\dot{V}_{O_2}$ is not related to increased demand brought about by the increased contractility promoted by Ca^{2+} but is rather due to the substrates available to the heart. It has been observed in a perfused, working heart preparation that switching the perfusate substrate available to hearts from glucose to pyruvate can result in an increase in $M\dot{V}_{O_2}$ without a concomitant increase in RPP (7). Yu et al. (25) also found that V_X (equivalent to F_1 in Ref. 25) increased by four- to fivefold in hearts perfused with butyrate plus lactate compared with butyrate alone, whereas V_{TCA} was no different in these two groups. They also found no net oxidation of lactate; however, we observed net oxidation of lactate (F_{C2} values, ~0.9 for all groups). These differences likely reflect the expected differences in mitochondrial redox state between hearts perfused with pyruvate vs. butyrate.

Comparisons to previous studies. The primary role of the TCA cycle in heart tissue is to provide reducing equivalents for electron transport and subsequent ATP synthesis. The values reported here for V_{TCA} are significantly smaller than the values reported previously for acetate-perfused hearts but are similar to the values reported for acetate- plus propionate-perfused hearts (4). Although acetate has a single metabolic fate in the heart (to contribute to acetyl-CoA and generate reducing equivalents in the TCA cycle), both lactate and pyruvate are capable of contributing extra reducing equivalents and thus reducing V_{TCA}. Perfusion of hearts with any substrate or substrate mixture that contributes reducing equivalents external to the TCA cycle results in lower cycle flux relative to hearts perfused with acetate alone. This trend was observed by Burgess et al. (3) in mouse hearts, where it was found that V_{TCA} was significantly higher in hearts perfused with acetate alone vs. octanoate alone and vs. lactate alone (27.1 vs. 17.1 vs. 13.1 $\mu mol \cdot min^{-1} \cdot g$ dry wt^{-1}, respectively). Jeffrey et al. (8) also observed a similar trend in rat hearts perfused with glucose plus acetate vs. glucose plus pyruvate, where V_{TCA} decreased from 11.3 to 6.1 $\mu mol \cdot min^{-1} \cdot g$ dry wt^{-1}.

The observations that a more highly oxidized cytosol results in an increase in V_{TCA} and a decrease in V_X whereas an increase in $[Ca^{2+}]_o$ results in an increase in both V_{TCA} and V_X are at least consistent with a parallel between V_X and malate-aspartate shuttle activity. However, it is clear from these and other experiments that the process represented by V_X does not require net transport of reducing equivalents from the cytosol to the mitochondria under all conditions, so V_X may be more accurately described as a metabolite-exchange flux across the inner mitochondrial membrane (15). The V_X/V_{TCA} ratio (see Tables 2 and 3) was found to vary ~10-fold among the four groups of hearts reported here (0.3 for hearts perfused with low-$[Ca^{2+}]_o$ and high-pyruvate concentrations to 2.7 for hearts perfused with high-$[Ca^{2+}]_o$ and low-pyruvate concentrations), so the ability of the heart to alter both V_{TCA} and V_X reflects an intricate coordination of energy demand and exchange kinetics.

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