Profiling substrate fluxes in the isolated working mouse heart using $^{13}$C-labeled substrates: focusing on the origin and fate of pyruvate and citrate carbons

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Profiling substrate fluxes in the isolated working mouse heart using $^{13}$C-labeled substrates: focusing on the origin and fate of pyruvate and citrate carbons. Am J Physiol Heart Circ Physiol 286: H1461–H1470, 2004. First published December 11, 2003; 10.1152/ajpheart.00942.2003. —The availability of genetically modified mice requires the development of methods to assess heart function and metabolism in the intact beating organ. With the use of radioactive substrates and ex vivo perfusion of the mouse heart in the working mode, previous studies have documented glucose and fatty acid oxidation pathways. This study was aimed at characterizing the metabolism of other potentially important exogenous carbohydrate sources, namely, lactate and pyruvate. This was achieved by using $^{13}$C-labeling methods. The mouse heart perfusion setup and buffer composition were optimized to reproduce conditions close to the in vivo milieu in terms of workload, cardiac functions, and substrate-hormone supply to the heart (11 mM glucose, 0.8 nM insulin, 50 µM carnitine, 1.5 mM lactate, 0.2 mM pyruvate, 5 nM epinephrine, 0.7 mM oleate, and 3% albumin). The use of three differentially $^{13}$C-labeled carbohydrates and a $^{13}$C-labeled long-chain fatty acid allowed the quantitative assessment of the metabolic origin and fate of tissue pyruvate as well as the relative contribution of substrates feeding acetyl-CoA (pyruvate and fatty acids) and oxaloacetate (pyruvate) for mitochondrial citrate synthesis. Beyond concuring with the notion that the mouse heart preferentially uses fatty acids for energy production (63.5 ± 3.9%) and regulates its fuel selection according to the Randle cycle, our study reports for the first time in the mouse heart the following findings. First, exogenous lactate is the major carbohydrate contributing to pyruvate formation (42.0 ± 2.3%). Second, lactate and pyruvate are constantly being taken up and released by the heart, supporting the concept of compartmentation of lactate and glucose metabolism. Finally, mitochondrial anaplerotic pyruvate carboxylation and citrate efflux represent 4.9 ± 1.8 and 0.8 ± 0.1%, respectively, of the citric acid cycle flux and are modulated by substrate supply. The described $^{13}$C-labeling strategy combined with an experimental setup that enables continuous monitoring of physiological parameters offers a unique model to clarify the link between metabolic alterations, cardiac dysfunction, and disease development.

Substantial evidence indicates that a defect in energy substrate metabolism is an independent determining factor of cardiac dysfunction and disease development (5, 14, 33, 57, 60, 62, 66). However, much remains to be learned about the impact of manipulating substrate metabolism on the heart’s contractile function and pathophysiological status. Genetically modified mice, especially if they carry inducible cardiomyocyte-specific changes in the expression of a single metabolic gene, have proven to be extremely valuable models for addressing this issue (10, 32, 37). The dynamic nature of cardiac metabolism and contractile functions requires, however, that investigations be conducted in the intact beating organ. Hence, methodologies developed for larger animals have to be applied to the mouse. Despite technical limitations associated with the small size of the organ, Grupp and colleagues (32) successfully characterized the mechanical function of the working mouse heart perfused ex vivo. The great advantage of this model is the ability to precisely manipulate and control experimental conditions such as the afterload, the preload, and the buffer composition. Subsequently, this ex vivo study model was refined by Belke et al. (8) for cardiac energy metabolism investigations. By using radioactive substrates and by providing the heart with a source of fatty acids, in addition to glucose, Belke et al. reported rates of glycolysis and glucose and fatty acid oxidation and showed a regulation of substrate selection according to the Randle cycle.

Although the prevailing view in the field of cardiac metabolism remains that long-chain fatty acids and glucose are the primary energy source, an increasing number of studies conducted both in vivo and ex vivo emphasize the importance of 1) the contribution of other energy substrates, such as lactate and pyruvate (13, 16, 17, 39, 45, 67); and 2) pyruvate metabolism through anaplerosis (19, 20, 30, 46, 52, 58, 67–69) for optimal cardiac energy metabolism. Lactate and pyruvate metabolism have been shown to be altered under pathophysiological conditions (13, 16, 30, 40, 51, 56, 58, 63, 69). Hence, it appears essential that the isotopic method used for the metabolic profiling of the mouse heart includes measurements of substrate fluxes through the pyruvate branch point. Compared with radioisotope methods, stable isotope techniques can provide, with less sample treatment, more information on the isotopic enrichment of intermediary precursor metabolites (e.g., acetyl-CoA or citrate for CO$_2$ production) and on the metabolic fate of labeled molecules. In fact, the use of stable isotopes combined with mass isotoper analysis has proven...
to be a very powerful approach for metabolic investigations in the ex vivo and in vivo perfused heart (15–20, 43, 45, 47, 51, 52, 67–69).

Thus this study was aimed at characterizing the metabolism of exogenous pyruvate and lactate in the working mouse heart perfused ex vivo. In addition, we considered it important to assess the metabolism of exogenous glucose and fatty acids to integrate our findings with those of others (1, 8). For this purpose, we expanded on a previously described 13C-labeling strategy using GCMS that was developed in the ex vivo working perfused rat heart (67, 69). Specifically, three differentially 13C-labeled carbohydrates and a 13C-labeled long-chain fatty acid were used to assess the metabolic origin and fate of tissue pyruvate as well as the relative contribution of substrates feeding acetyl-CoA (pyruvate and fatty acids) and oxaloacetate (OAA; pyruvate via anaplerosis), which are necessary for mitochondrial citrate synthesis. Perfusion conditions and buffer composition were optimized to mimic in vivo conditions in terms of cardiac functions and substrate-hormone concentrations, respectively. In addition, our experimental setup includes continuous monitoring of indexes of cardiac performance, myocardial oxygen consumption (MV\(\hat{O}_2\)), and tissue integrity. Beyond providing data on substrate selection and oxidation rates, this study reports for the first time in the working mouse heart quantitative flux data on several metabolic processes that could potentially be altered by disease, such as lactate and citrate release (69), and highlights the substantial contribution of carbohydrates, other than glucose, in myocardial energy production.

MATERIALS AND METHODS

Materials

Sources of chemicals, biological products, and 13C-labeled substrates, as well as the procedure for the dialysis of fatty acid-free BSA (BSA fraction V, Intergen), have been reported previously (19, 20, 67–69).

Heart Perfusions in the Semirecirculating Mode

Animal experiments were approved by the local ethics committee in agreement with the guidelines of the Canadian Council on Animal Care. Male C57BL/10 mice (10–12 wk old; average body weight: 29.4 ± 0.2 g; Jackson Laboratories) were anesthetized (1 μg/g ip) with a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) and were heparinized (5,000 U/kg ip) 15 min before surgery. After rapid insertion of an 18-gauge steel cannula into the aorta, hearts were excised and placed on the perfusion system, where they first underwent Langendorff perfusion (55 mmHg perfusion pressure) for ~5 min. The modified Krebs-Henseleit buffer (containing 119 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 25 mM NaHCO\(_3\), 0.1 mM EDTA, 11 mM glucose, 0.8 mM insulin, 50 μM l-carnitine, 1.5 mM lactate, and 0.2 mM pyruvate) was gassed with 95% O\(_2\)-5% CO\(_2\) (pH 7.4) and maintained at 37.5°C. During this retrograde perfusion period, a polyethylene catheter (PE-50) was inserted through the pulmonary vein into the left ventricle, anchored at the apex of the heart by a fluted end, and connected to a pressure transducer for the continuous monitoring of intraventricular functions (Digi-Med Heart Performance Analyzer, Micro-Med). Furthermore, the left atrium was connected to the preload reservoir (oxygenator) by cannulating the pulmonary vein with a 16-gauge steel cannula. Simultaneously beating hearts were then switched to the anterograde working mode and perfused via the left atria cannula with a semirecircular modified Krebs-Henseleit buffer containing oleate complexed to 3% albumin (see Perfusion Protocols for details). The perfusion setup for the working mouse heart in the semirecircular mode, depicted in Fig. 1, was adapted from that previously described for the working rat heart (67, 69). Briefly, in contrast to a recirculating setup, the coronary perfusate, which contains various metabolites released by the heart, is not recirculated but is continuously collected. Thus only the aortic outflow is recirculated into the buffer reservoir. The preload and afterload pressures were maintained at 15 and 50 mmHg, respectively, through continuous monitoring by pressure transducers (Digi-Med Blood Pressure Analyzer and Low Pressure Analyzer, Micro-Med). Other parameters that were continuously monitored (10-s duration for each recording) are 1) the atrial and aortic flow rates (monitored with calibrated electromagnetic flow probes; Carolina Medical Electronics); 2) left ventricular functions, namely, heart rate (HR), maximum left ventricular systolic pressure, left ventricular end-diastolic pressure, and maximum value for the first derivative of maximum left ventricular systolic pressure (measured by using the intraventricular cannula linked to a pressure transducer; Digi-Med Heart Performance Analyzer, Micro-Med); and 3) heart temperature (measured with a thermocouple; Yellow Springs Instruments). In addition, biochemical parameters, among which PO\(_2\), PCO\(_2\), pH, Ca\(^{2+}\), and other ion concentrations, were evaluated by using a blood gas, electrolytes, and pH analyzer (ABL 77 series, Radiometer Copenhagen) in the atrial influent and the coronary effluent perfusates collected every 10 min. Finally, effluent samples were collected every 10 min to assess lactate dehydrogenase (LDH) release, an index of tissue necrosis, by using a standard enzyme activity assay on a Roche Cobas Fara apparatus.

Perfusion Protocol

Working mouse hearts were perfused for 30 min with a semirecirculating modified Krebs-Henseleit buffer, whose composition was identical to that described above (see Heart Perfusions in the Semirecirculating Mode) except that it also contained oleate bound to 3% albumin and 5 nM epinephrine. In addition, for any given perfusion, one of the unlabeled substrates was replaced by its corresponding labeled substrate. Substrate and hormone concentrations are within the range of reported values of mouse plasma concentrations in the non-fasting state (Jackson Laboratories; “Phenome Database”; and refs. 1, 3, 8, 25, 64, 72, and 73). We chose a lactate concentration of 1.5 mM, which is below values reported for the normal rested mouse (between 2.7 and 4.7 mM) (44, 54, 72). However, in blood drawn by cardiac puncture in a syringe containing sulfosalicylic acid to precipitate proteins and prevent anaerobic glycolysis, we evaluated lactate concentration to be 1.8 ± 0.3 mM (n = 4) by using an enzymatic assay (69). Perfusion protocols can be subdivided into two groups that
differed with respect to their oleate concentration. In a first group of experiments, oleate concentration was 0.7 mM. Four different 13C-labeled substrates were used: [U-13C]lactate (initial molar percent enrichment (MPE): 99%; n = 5), [U-13C]pyruvate (initial MPE: 99%; n = 4), [U-13C]glucose (initial MPE: 50%; n = 6), or [U-13C]oleate (initial MPE: 25%; n = 4). In a second set of experiments, we tested whether perfused working mouse hearts responded to a change in substrate supply resulting from a lowering of oleate concentration from 0.7 to 0.4 mM. Two different 13C-labeled substrates were used: [U-13C]pyruvate (initial MPE: 99%; n = 4) or [U-13C]oleate (initial MPE: 25%; n = 4).

Throughout the perfusion, influent and effluent perfusates were collected at the following times: 1) 20–25 min to evaluate the citrate and succinate release rates; and 2) 25–30 min to document the lactate and pyruvate uptake and release rates, of which 1 ml was immediately treated with sodium borodeuteride. These samples were stored at –80°C until further analysis. Subsequent to each perfusion period, hearts were freeze-clamped with metal tongs chilled in liquid nitrogen, weighed, and stored at –80°C.

Analytical Procedures

Perfusate and tissue processing. Procedures for the determination of 1) citrate and succinate release rates and 2) 13C enrichment and concentration of the citric acid cycle (CAC) intermediates (citrate, α-ketoglutarate, succinate, fumarate, malate, and OAA) were adapted from those previously described for the working rat heart (67–69). Changes made concerned the sample size. In addition, the previously described procedure for citrate cleavage was optimized to the smaller tissue sample (30 mg). Briefly, after tissue homogenization and centrifugation, the supernatants were reacted with sodium borodeuteride, acidified, and neutralized before incubation with 2.24 mL of 500 mM triethanolamine buffer (pH 7.6) containing 10 mM MgSO4, 20 mM EDTA, 20 mM methoxylamine, and 1.5 g/l of citrate lyase.

Conditions for the operation and analysis of all metabolites as their t-butyldimethylsilyl derivatives by GCMS (Hewlett-Packard 6890N gas chromatograph coupled to a 5973N mass spectrometer) were previously described (67). Areas under each fragmentogram were determined by computer integration and corrected for naturally occurring stable isotopes.

Calculations

Biochemical and functional status. MVO2 (in μmol/min), intracellular pH, rate-pressure product (in mmHg·beats·min⁻¹·10⁻⁴), cardiac power (in mW), and cardiac efficiency (in mW·μmol⁻¹·min⁻¹) were calculated from previously reported equations (67–69).

Flux parameters. GCMS data are expressed as MPE, as defined previously (19, 20, 68). Briefly, mass isotopomers of metabolites containing 1 to 3 13C-labeled atoms were identified as Mi with i = 1, 2, 3, ..., n, and the absolute MPE of individual 13C-labeled mass isotopomers (Mi) of a given metabolite was calculated as follows:

\[
MPE(Mi) = \%A_Mi/(A_Mi + \Sigma A_{Mi})
\]

where \(A_Mi\) and \(A_{Mi}\) represent the peak areas from ion chromatograms corrected for natural abundance, corresponding to unlabeled (M) and 13C-labeled (Mi) mass isotopomers, respectively. The equations to calculate flux ratios relevant to citrate synthesis in hearts perfused with [U-13C]lactate, [U-13C]pyruvate, or [U-13C]oleate were adapted from those previously described (19, 20, 68) for the use of individual labeling. Equations used for [U-13C] pyruvate or lactate also apply to [U-13C]glucose. In brief, flux ratios were calculated from the measured mass isotopomer distribution (MID) of the following tissue metabolites: 1) citrate and its OAA moiety (OAA\(\text{O}_{\text{O}}\)), from which we extrapolated the acetyl moiety of citrate (AC\(\text{Cit}_{\text{Cit}}\)); 2) pyruvate; and 3) succinate. In this study, we reported the following flux rates, expressed relative to that of citrate synthase (CS): 1) oleate oxidation (OLE): OLE/CS = M2 AC\(\text{Cit}_{\text{Cit}}\)/M18 oleate; 2) pyruvate decarboxylation (PDC): PDC/CS = M2 AC\(\text{Cit}_{\text{Cit}}\)/M3 pyruvate (Eq. 5 of Ref. 19); 3) pyruvate carboxylation (PC): PC/CS = M3 OAA\(\text{O}_{\text{O}}\) corrected/ M3 pyruvate (Eq. 4 of Ref. 19); and 4) the contribution of other substrates (OS), such as endogenous fatty acids and/or amino acids, to the formation of acetyl-CoA: OS/CS = 1 – [(PDC/CS) + (OLE/CS)]. The measured M3 OAA\(\text{O}_{\text{O}}\) was corrected for the fraction of M3 OAA molecules coming from citrate isomoters metabolized in the CAC, as described in Ref. 19 (Eqs. 8–10). To extrapolate the MPE M2 of AC\(\text{Cit}_{\text{Cit}}\) from the measured MID of citrate and of its OAA moiety, we used a mathematical approach, previously described by Vincent et al. (67, 69) (Eqs. 2 and 2a)

\[
\text{MPE M2 AC}_{\text{Cit}} = 0.5|\Sigma \text{MPE M2}|(\text{citrate} - \text{OAA})
\]

The fractional contribution (FC) of individual carbohydrate precursors to pyruvate formation was calculated from the MPE M3 of tissue pyruvate measured in hearts perfused with [U-13C]lactate, [U-13C]glucose, or [U-13C]pyruvate by using the following general equation

\[\text{FC carbohydrate} \rightarrow \text{pyruvate} = M3 \text{pyruvate/Mi carbohydrate precursor} \]

where the term Mi refers to the 13C enrichment of the exogenous carbohydrate precursor. Finally, the FC of other carbohydrates (FC/other carbohydrates \(\rightarrow\) pyruvate), such as glycogen and amino acids, was calculated as follows

\[\text{FC other carbohydrates} \rightarrow \text{pyruvate} = 1 - (\text{FC glucose} \rightarrow \text{pyruvate} + \text{FC exogenous pyruvate} \rightarrow \text{pyruvate} + \text{FC lactate} \rightarrow \text{pyruvate})\]

Lactate and pyruvate uptake and efflux. Lactate and pyruvate uptake and efflux rates were determined by a modification of the procedure described in detail by Vincent et al. (69) for the working rat heart. In brief, rates of uptake and efflux of unlabeled (M) and [U-13C]labeled (M3) lactate and pyruvate are quantified from 1) the difference between their influent and effluent perfusate concentrations, determined by GCMS and enzymatic assays; and 2) the coronary flow rate. However, the use of three different 13C-labeled carbohydrate substrates in this study enables the determination of additional flux parameters. First, in hearts perfused with unlabeled lactate and pyruvate and [U-13C]glucose, the efflux rate of [U-13C]labeled lactate and pyruvate (M3) reflects that of glycolysis from exogenous glucose. Second, in hearts perfused with unlabeled glucose and lactate and [U-13C]pyruvate, the rate of efflux of [U-13C]lactate reflects the rate of conversion of pyruvate into lactate by the LDH reaction. Finally, in hearts perfused with unlabeled glucose and pyruvate and [U-13C]lactate, the rate of efflux of [U-13C]pyruvate reflects the rate of conversion of lactate into pyruvate by the LDH reaction. It is noteworthy that with all three 13C-labeled carbohydrates, the rate of efflux of unlabeled lactate and pyruvate reflects more than one metabolic process, namely glycolysis, lactate-pyruvate interconversion by LDH and/or alanine transamination.

Absolute CAC flux rate. The CAC flux rate was calculated from MVO2, and the stoichiometric relationships between oxygen consumption and citrate formation, from carbohydrates and fats. The equation of Vincent et al. (Eq. 3 of Ref. 67) was modified to take into account 1) the specific contribution of exogenous oleate to citrate formation, as assessed from the flux ratios OLE/CS; 2) the contribution of other sources, as assessed from the flux ratio OS/CS, which we assumed to be endogenous triglyceride stores consisting of equal proportions of oleate and palmitate; and 3) the contribution of carbohydrates to citrate synthesis, as assessed from the flux ratio PDC/CS and the FC of each carbohydrate to pyruvate synthesis, assuming that other carbohydrate sources are mainly glycogen. That is, we used Eq. 4 below, which considers that 1 μmol of consumed O2 results in the formation of 0.333 μmol of citrate from glucose and lactate and 0.4, 0.353, and 0.348 μmol of citrate from pyruvate, oleate, and palmitate, respectively.
CAC flux = M$V_{O2}$([FCglucose → pyruvate]$PDC/CS$) + (FClactate → pyruvate)$PDC/CS$) + (FCExogenous pyruvate → pyruvate)$PDC/CS$) + (FCExogenous carbohydrates → pyruvate)$PDC/CS$) + (OLE/CS) + (OS/CS))

ATP production. Total rates of ATP production were calculated from 1) the various absolute flux rates by using theoretical yields of ATP per mole of substrate oxidized (50), and 2) rates of anaerobic glycolysis determined from the efflux rate of [U-13 C3]lactate measured in hearts perfused with [U-13 C6]glucose.

Statistical Analysis

Data are expressed as means ± SE of n = 4–15 heart perfusions. Statistical significance was reached at P < 0.05 by using an unpaired t-test or a one-way analysis of variance followed by a Bonferroni selected comparison test.

RESULTS

Functional and Physiological Parameters

During the 30-min perfusion period, isolated working hearts perfused with 11 mM glucose, 0.8 nM insulin, 50 μM carnitine, 1.5 mM lactate, 0.2 mM pyruvate, 5 nM epinephrine, 0.7 mM oleate, and 3% albumin maintained stable values for the various absolute and physiological parameters reported in Table 1. HR was stable without the need for external pacing. The integrity of our preparation is indicated by the low values for LDH and succinate release rates, which are used as indexes of necrosis and ischemia, respectively (41, 49, 70). Reducing the oleate concentrations from 0.7 to 0.4 mM did not affect any parameter (data not reported).

Table 1. Functional and physiological parameters of ex vivo perfused working mouse hearts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>360±9</td>
</tr>
<tr>
<td>LVSP max, mmHg</td>
<td>96±2</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>6±1</td>
</tr>
<tr>
<td>RPP, mmHg/beat-min$^{-1}$ × 10$^{-3}$</td>
<td>32.1±1.3</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>110±0.4</td>
</tr>
<tr>
<td>Aortic flow, ml/min</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>Coronary flow, ml/min</td>
<td>3.1±0.3</td>
</tr>
<tr>
<td>MVO2, μmol/min</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>CP, mW</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>CE, mW/μmol$^{-1}$</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>pH</td>
<td>7.406±0.003</td>
</tr>
<tr>
<td>LDH release, μM</td>
<td>26.6±3.3</td>
</tr>
<tr>
<td>Succinate release, mmol/min</td>
<td>2.7±0.7</td>
</tr>
</tbody>
</table>

Data are means ± SE of 4–21 heart perfusion experiments with 11 mM glucose, 0.8 nM insulin, 50 μM carnitine, 1.5 mM lactate, 0.2 mM pyruvate, 5 nM epinephrine, 0.7 mM oleate, and 3% albumin. Values of heart rate (HR), maximum left ventricular systolic pressure (LVSP max), left ventricular end-diastolic pressure (LVEDP), rate-pressure product (RPP), and cardiac flows are those measured between 25 and 30 min of perfusion. Myocardial oxygen consumption (MVO2) and intracellular pH (pH) were calculated from PO2 and PCO2 values determined in influent and effluent perfuse collected at 10 and 20 min. Succinate and lactate dehydrogenase (LDH) release were measured in perfusates collected between 20 and 25 min and at 30 min, respectively. CP, cardiac power; CE, cardiac efficiency.

Table 2. Fractional contribution of exogenous carbohydrates to tissue pyruvate and its normalization to pyruvate equivalents

<table>
<thead>
<tr>
<th>13C-labeled Substrate</th>
<th>Glucose</th>
<th>Lactate</th>
<th>Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute concentrations, mM</td>
<td>11</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Pyruvate equivalents, mM</td>
<td>22</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>FC carbohydrate → pyruvate, %</td>
<td>21.3±3.0</td>
<td>42.0±2.3</td>
<td>10.7±0.4</td>
</tr>
<tr>
<td>(normalized to pyruvate equivalents)</td>
<td>1.0±0.1</td>
<td>28.0±1.5</td>
<td>53.5±2.0</td>
</tr>
</tbody>
</table>

Data are means ± SE of 4–6 heart perfusion experiments, as described in Table 1. The fractional contribution (FC) of each carbohydrate precursor to tissue pyruvate synthesis in hearts perfused with [U-13 C6]glucose, [U-13 C6]lactate, or [U-13 C3]pyruvate was calculated from the molar percent enrichment (MPE) M3 of tissue pyruvate, determined by GCMS, by using Eq. 3 (FC carbohydrate → pyruvate = M3 tissue pyruvate/mass isotopomer (Mi) carbohydrate precursor). Pyruvate equivalents are defined as the number of pyruvate molecules formed per mole of substrate precursor.

Pyruvate Branch Point

The use of individual uniformly labeled carbohydrate substrates allowed the direct assessment of several metabolic flux parameters relevant to pyruvate metabolism in hearts perfused in the presence of 0.7 mM oleate. From the 13C-labelling of tissue pyruvate (reported in Table 2), we can conclude the following. For all three 13C-labeled substrates, tissue pyruvate was only enriched in M3 isotopomers. The absence of M1 or M2 isotopomers (data not reported) indicates negligible decarboxylation of J malate to pyruvate through the malic enzyme reaction or 2) glucose in the oxidative pentose phosphate pathway. From the values of MPE M3 of tissue pyruvate, we conclude that, among all carbohydrates, exogenous lactate contributes the most to pyruvate synthesis (42.0 ± 2.3%; Table 2). However, when the contribution of exogenous carbohydrate substrates to pyruvate synthesis is normalized to the pyruvate equivalents produced by these substrates at their respective concentration, it becomes apparent that the contribution of exogenous pyruvate exceeds that of exogenous lactate and glucose (53.5 ± 2.0 vs. 28.0 ± 1.5 and 1.0 ± 0.1, respectively; Table 2). It is noteworthy that 26.0 ± 0.5% of tissue pyruvate remained unaccounted for and could arise from unlabeled endogenous sources, such as glycogen and alanine.

Second, from the measurements of 13C enrichment of lactate and pyruvate in the influent and effluent perfusates and of coronary flow rates, we assessed the rates of pyruvate and lactate efflux, uptake, and interconversion by the LDH (Fig. 2). Perfused working mouse hearts took up exogenous pyruvate and lactate as determined by using [U-13 C6]pyruvate or [U-13 C6]lactate, respectively, as the 13C-labeled substrate (0.11 ± 0.02 and 0.09 ± 0.03 μmol/min, respectively; Fig. 2, B and C). Although lactate and pyruvate were supplied at the physiological concentration ratio of 7.5:1, their uptake rates were similar, indicating a preferential pyruvate uptake. Consequently, the fractional extraction of lactate was significantly less than that of pyruvate (2.9 ± 1.0 and 34.6 ± 3.6%, respectively; P < 0.001), consistent with the results of Table 2. Working mouse hearts perfused with [U-13 C3]pyruvate also constantly released unlabeled lactate, represented in Fig. 2B (0.40 ± 0.18 μmol/min). This release of unlabeled lactate reflected glycolysis from exogenous glucose as indicated from...
it is apparent that the extent of $^{13}$C-labeling of the various CAC intermediates varied with the type of $^{13}$C-labeled substrate. This can be explained by differences in the initial $^{13}$C enrichment of these substrates (~50% for $[^{1-13}C]$glucose, ~25% for $[^{1-13}C]$oleate, and >99% for $[^{1-13}C]$lactate and $[^{1-13}C]$pyruvate) and their relative concentration as well as their respective contribution to citrate formation. However, for any given $^{13}$C-labeled substrate, there were only small differences in the labeling patterns as well as the total $^{13}$C-labeling of the various CAC intermediates. In fact, the values of the $^{13}$C dilution factors for the various $^{13}$C-labeled substrates were similar and indicated little, if any, entry of unlabeled carbon through anaplerosis at sites other than OAA ($[^{1-13}C]$lactate): 1.01 ± 0.03; $[^{1-13}C]$pyruvate: 0.95 ± 0.13; $[^{1-13}C]$glucose: 1.00 ± 0.10; and $[^{1-13}C]$oleate: 1.43 ± 0.22; P = not significant; Eq. 10 of Ref. 19.

As shown in Table 4, there was a fairly good agreement in flux values obtained with the three $^{13}$C-labeled carbohydrate substrates; small differences between the flux values of PDC/CS and PC/CS did not reach significance. According to the relative flux ratio PDC/CS, pyruvate contributed between 25 and 30% to acetyl-CoA for citrate synthesis. The contribution of anaplerotic pyruvate carboxylation (PC/CS) to OAA for citrate synthesis was on average 5% and was approximately sixfold lower than its decarboxylation. As indicated in Table 5, the oxidation of exogenous oleate represented the predominant source of acetyl-CoA for citrate synthesis (OLE/CS: 63.5 ± 3.9%). The relative contribution of OS, presumably endogenous triglycerides or amino acids, was evaluated to be <10% (OS/CS: 9.8 ± 5.7%).

Table 3. $^{13}$C-labeling of CAC intermediates from working mouse hearts perfused with $[^{1-13}C]$lactate, $[^{1-13}C]$pyruvate, $[^{1-13}C]$glucose, or $[^{1-13}C]$oleate

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Tracer</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>ΣM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>Lactate</td>
<td>15.5±0.9</td>
<td>15.2±1.2</td>
<td>6.4±0.7</td>
<td>2.2±0.3</td>
<td>40.2±3.1</td>
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<tr>
<td></td>
<td>Pyruvate</td>
<td>6.0±0.9</td>
<td>5.1±0.9</td>
<td>0.7±0.1</td>
<td>0.2±0.0</td>
<td>12.0±2.0</td>
</tr>
<tr>
<td></td>
<td>Oleate</td>
<td>13.9±1.7</td>
<td>16.3±2.1</td>
<td>4.3±0.7</td>
<td>1.6±0.3</td>
<td>36.5±4.8</td>
</tr>
<tr>
<td>α-KG</td>
<td>Lactate</td>
<td>15.3±1.2</td>
<td>15.5±0.9</td>
<td>5.3±0.5</td>
<td>2.8±0.9</td>
<td>39.9±2.9</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>5.9±0.9</td>
<td>4.9±0.8</td>
<td>0.5±0.1</td>
<td>0.1±0.0</td>
<td>11.4±1.9</td>
</tr>
<tr>
<td>Fumarate</td>
<td>Lactate</td>
<td>18.5±1.4</td>
<td>9.8±1.8</td>
<td>5.5±1.0</td>
<td>1.3±0.2</td>
<td>35.3±2.3</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>6.2±0.9</td>
<td>2.5±1.0</td>
<td>0.3±0.0</td>
<td>0.0±0.0</td>
<td>8.9±1.7</td>
</tr>
<tr>
<td></td>
<td>Oleate</td>
<td>16.2±0.7</td>
<td>11.7±0.1</td>
<td>2.3±0.2</td>
<td>0.4±0.2</td>
<td>30.6±1.0</td>
</tr>
<tr>
<td>Malate</td>
<td>Lactate</td>
<td>15.3±1.1</td>
<td>10.0±0.8</td>
<td>4.5±0.5</td>
<td>0.8±0.1</td>
<td>30.7±2.4</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>5.6±0.9</td>
<td>3.1±0.6</td>
<td>0.5±0.1</td>
<td>0.0±0.0</td>
<td>9.2±1.5</td>
</tr>
<tr>
<td></td>
<td>Oleate</td>
<td>13.8±1.6</td>
<td>8.8±1.1</td>
<td>2.3±0.4</td>
<td>0.2±0.0</td>
<td>25.1±3.1</td>
</tr>
</tbody>
</table>

Data, expressed as MPE, are means ± SE of 4-6 heart perfusion experiments, as described in Table 1. The MPE M5 and M6 of citrate and M5 of α-ketoglutarate (α-KG) were <1%. The mass isotopomer distribution (MID) of citric acid cycle (CAC) intermediates was determined in freeze-clamped heart homogenates by GCMS. OAA$,\text{oxaloacetate moeity of citrate.}$

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**Fig. 2.** Lactate (Lac) and pyruvate (Pyr) uptake and efflux rates assessed in isolated working mouse hearts perfused with 3 different $^{13}$C-labeled carbohydrates. Data are means ± SE of 4 heart perfusion experiments, as described in Table 1. Hatched bars are unlabeled substrates, and solid bars are $^{13}$C-labeled substrates. The uptake (negative values) and release (positive values) of lactate and pyruvate by the hearts were quantified in hearts perfused with one of the following three different $^{13}$C-labeled carbohydrates: $[^{1-13}C]$glucose (A), $[^{1-13}C]$pyruvate (B), or $[^{1-13}C]$lactate (C). Lactate and pyruvate uptake and efflux rates were calculated from the product of values of coronary flow rates and concentration differences in the influent and effluent perfusates, determined by GCMS and enzymatic assays.

**Table 3. $^{13}$C-labeling data relevant to the calculation of the relative contribution of substrates to citrate synthesis are shown in Tables 3-5. Table 3 portrays the MID of the CAC intermediates isolated from mouse hearts perfused with physiological concentrations of $[^{1-13}C]$pyruvate, $[^{1-13}C]$lactate, $[^{1-13}C]$glyceraldehyde, or $[^{1-13}C]$glycerol. Table 4 reports the MPE values for ACC, the corrected OAA, and the various flux ratios relevant to pyruvate metabolism. Table 5 reports substrate flux ratios relevant to fatty acid β-oxidation for hearts perfused with the two different oleate concentrations.

**Substrate Selection for Citrate Synthesis**

$^{13}$C-labeling data relevant to the calculation of the relative contribution of substrates to citrate synthesis are shown in Tables 3-5. Table 3 portrays the MID of the CAC intermediates isolated from mouse hearts perfused with physiological concentrations of $[^{1-13}C]$pyruvate, $[^{1-13}C]$lactate, $[^{1-13}C]$glyceraldehyde, or $[^{1-13}C]$glycerol. Table 4 reports the MPE values for ACC, the corrected OAA, and the various flux ratios relevant to pyruvate metabolism. Table 5 reports substrate flux ratios relevant to fatty acid β-oxidation for hearts perfused with the two different oleate concentrations.
Lowering of oleate concentration from 0.7 to 0.4 mM resulted in the expected increase in the flux ratio PDC/CS (1.5-fold; Table 4), although it did not reach significance, at the expense of a decreased OLE/CS flux (1.3-fold; Table 5). Interestingly, at 0.4 mM oleate, the relative rate of anaplerotic pyruvate carboxylation was also decreased significantly compared with hearts perfused with 0.7 mM oleate (Table 4). To expand on our finding of a lower flux ratio PC/CS at 0.4 than 0.7 mM oleate, we documented the rates of citrate release and tissue levels of CAC intermediates in both perfusion conditions (Fig. 3). Compared with hearts perfused with 0.7 mM oleate, those perfused with 0.4 mM oleate showed a significantly lower citrate release rate (Fig. 3A) and tissue concentration (Fig. 3B). Tissue concentrations of other CAC intermediates did not vary with oleate concentration.

**DISCUSSION**

With the use of a 13C-labeling methodology, this study profiles metabolic pathway fluxes linked to energy production in the working mouse heart perfused ex vivo under conditions in which cardiac flows and work as well as the buffer substrate-hormone composition mimic those prevailing in vivo. In addition to measurements of exogenous glucose (glycolysis, oxidation) and fatty acid utilization for energy production, our 13C-labeling strategy provides for the first time quantitative data on 1) the contribution of various exogenous carbohydrate sources to pyruvate synthesis; 2) the rates of pyruvate and lactate efflux, uptake, and interconversion by the LDH; 3) the cytosolic redox state; and 4) the pyruvate anaplerosis and citrate efflux. Our data substantiate the notions of a predominant use of fatty acids over glucose and a regulation of substrate fuel selection according to the Randle cycle (27). However, more importantly, they reveal new information on the metabolism of other exogenous substrates, namely, lactate and pyruvate. As discussed sequentially in greater detail below, we show that these substrates contribute significantly to energy production and participate in anaerobiosis. Furthermore, with the use of MV O2 values, our relative flux values are converted into quantitative flux rates to enable comparisons with previous studies using radioactive substrates (1, 8).

Our results demonstrate a substantial role for lactate and pyruvate as energy fuels and underscore the dynamic aspects of the pyruvate metabolic branch point in the mouse heart. These concepts were supported by the measured 13C enrichments of 1) tissue pyruvate as well as 2) influenza (arterial and effluent) lactate. Data were normalized to pyruvate molar equivalents produced by these substrates and expressed as a percentage, exogenous pyruvate became the predominant contributor (>50%). The importance of exogenous pyruvate as an energy substrate for the heart when compared with lactate is also emphasized by their similar uptake rates despite a 7.5-fold difference in their “arterial” perfusate concentration. A higher percentage of pyruvate extraction relative to that of lactate, which was also observed by Laughlin et al. (42) in vivo in the dog heart and emphasized by Lloyd et al. (45) in perfused rat hearts, can possibly be explained by the capacity of pyruvate to inhibit both lactate transport (34) and its metabolism by LDH (21). It is noteworthy that the contribution of exogenous glucose to tissue pyruvate (21.3 ± 3.0%) is subject to some uncertainties. It may represent a minimal estimate. Consequently, the contribution of endogenous sources, possibly glycogen or amino acids, to tissue pyruvate (26%) would be overestimated. Indeed, the 13C-label of glucose enrichment would be diluted if glucose was incorporated into glycogen, estimated to be ~11.6% (35), before its conversion to tissue pyruvate. To further clarify the relative contributions of exogenous glucose and endogenous glycogen to tissue pyruvate formation, one would need to design a different 13C-labeling protocol. Furthermore, potential com-

### Table 4. Effects of differential labeling and oleate concentration on relative carbohydrate contribution to acetyl-CoA and OAA for citrate synthesis in ex vivo perfused working mouse hearts

<table>
<thead>
<tr>
<th>Flux Ratio</th>
<th>0.7 mM Oleate</th>
<th>0.4 mM Oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPE M2 AC</td>
<td>6.2 ± 0.8*</td>
<td>10.1 ± 0.9†</td>
</tr>
<tr>
<td>MPE M2 OAA</td>
<td>0.2 ± 0.05</td>
<td>0.2 ± 0.05</td>
</tr>
<tr>
<td>PC/CS</td>
<td>0.046 ± 0.011</td>
<td>0.061 ± 0.005</td>
</tr>
<tr>
<td>PDC/CS</td>
<td>0.26 ± 0.02</td>
<td>0.17 ± 0.02</td>
</tr>
</tbody>
</table>

Data are means ± SE of 4–6 heart perfusion experiments. Hearts were perfused with 0.7 mM oleate and either [U-13C6]glucose, [U-13C3]lactate, or [U-13C3]pyruvate as the 13C-substrate, or 2) with 0.4 mM oleate and [U-13C3]pyruvate. Flux ratios were calculated from the measured MID of pyruvate, succinate, citrate and its OAA moiety (OAA Cit;C 4) from which we extrapolated the acetyl moiety of citrate (AC Cit; C 4). MPE of the OAACit was corrected for the formation of M3 OAA from CAC metabolism of citrate isotopeomers (see Analytical procedures). Flux ratios are expressed relative to citrate synthase (CS): 1) pyruvate decarboxylation (PDC/CS) = M2 AC M3 pyruvate and 2) pyruvate carboxylation (PC/CS) = OAA corrected M3 pyruvate. *P < 0.01 and †P < 0.001 vs. pyruvate at 0.7 mM oleate.

### Table 5. Effects of oleate concentration on relative contribution of fatty acids to acetyl-CoA for citrate synthesis

<table>
<thead>
<tr>
<th>Flux Ratio</th>
<th>0.7</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPE M2 AC</td>
<td>14.3±0.9</td>
<td>10.2±0.2</td>
</tr>
<tr>
<td>OLE/CS</td>
<td>0.635±0.039</td>
<td>0.496±0.020*</td>
</tr>
</tbody>
</table>

Data are means ± SE of 4 heart perfusion experiments, as described in Table 4. Hearts were perfused with 0.4 or 0.7 mM [U-13C3]oleate as the 13C substrate. Oleate oxidation (OLE/CS = M2 AC M8 oleate), expressed relative to CS, was calculated from the measured MID of OAA Cit (C 2) from which we extrapolated the AC Cit (C 4). *P < 0.05 vs. 0.7 mM oleate.

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*Note: The table and text are based on the extracted content, with the natural text interpretation and the addition of some context for clarity.*
partmentment of glycogen metabolism should be taken into account in the data interpretation (35).

The aforementioned data on the contribution of exogenous glucose, lactate, and pyruvate to tissue pyruvate provided, however, only a partial picture of the complexity of the metabolic trafficking at the pyruvate branch point. Indeed, as revealed by measurements of the $^{13}$C enrichments and concentrations of lactate and pyruvate in the influent and effluent perfusates, the ex vivo perfused mouse heart simultaneously releases and takes up lactate and pyruvate. These results in the mouse heart are consistent with those reported by others both in vivo and ex vivo in humans, lambs, and rats (2, 6, 16, 29, 67). However, by labeling three different carbohydrates in this study, we demonstrate with precision that the metabolic origin of the lactate released by the heart is predominantly exogenous glucose. In fact, when hearts were perfused with $[U-{ }^{13}$C$_6]$-glucose, rates of $[U-{ }^{13}$C$_3]$-lactate production were similar to rates of unlabeled lactate obtained when $[U-{ }^{13}$C$_3]$-pyruvate was the labeled substrate. These findings are in agreement with the proposed compartmentation of lactate metabolism in the heart (11, 16, 35).

Let us now consider the relative contribution of exogenous $^{13}$C-labeled pyruvate, lactate, and glucose to citrate synthesis. With these three $^{13}$C-labeled substrates, the $^{13}$C-labeling of the tissue ACC$_r$ and OAA$_c$ (Table 4) showed a pattern similar to that observed for tissue pyruvate (Table 2). The contribution of carbohydrate oxidation to acetyl-CoA formation for citrate synthesis, as reflected by the flux ratio PDC/CS (on average 27%), is approximately twofold lower than that of fatty acids (63.5 $\pm$ 3.9%; Table 5). These patterns of substrate selection for energy production concur with those observed by Belke et al. (8) using radioisotopes. To enable a direct comparison of our flux data obtained with $^{13}$C-labeling methods and expressed as relative flux rates (i.e., flux ratios) with those of Belke et al. (8), which were obtained with $^{13}$C-labeling methods and expressed as absolute oxidation rates, our relative flux rates were converted to absolute values by using values of MV$_{O_2}$, expressed in a heart weight-specific manner [6.25 $\pm$ 0.40 $\mu$mol-min$^{-1}$g wet wt$^{-1}$]. The calculation is based on theoretical stoichiometric equations for complete substrate oxidation. The following values (in $\mu$mol-min$^{-1}$g dry wt$^{-1}$) were calculated by assuming a conversion factor of 0.2 for dry-to-wet weight conversion (8) and an average wet weight for the mouse heart of 0.24 $\pm$ 0.01 g: $\text{J})$ CAC flux rate: 9.4 $\pm$ 1.4; $\text{K})$ oleate $\beta$-oxidation: 0.7 $\pm$ 0.3; and $\text{L})$ pyruvate decarboxylation: 2.5 $\pm$ 0.5. Converting these absolute flux rates to rates of ATP production revealed a pattern of substrate contributions, which was similar to that observed with data expressed as flux ratios (Fig. 4). It is noteworthy that the total mitochondrial ATP production rate calculated from the extrapolated absolute substrate oxidation rates was similar to that calculated from the theoretical ATP/O ratio of 2.83 (50) and MV$_{O_2}$ (8.1 $\pm$ 1.3 vs. 8.3 $\pm$ 0.6 $\mu$mol/min, respectively).

Overall, our range of values for absolute substrate oxidation rates and for substrate contributions to ATP synthesis concurs with data previously reported for perfused working mouse hearts (1, 8). However, there are some slight differences. For example, the pattern of substrate selection for ATP production that we observed at the physiological concentration of 0.7 mM oleate ($\sim$62% fatty acid and $\sim$34% carbohydrates) was closer to that observed by Belke et al. (8) when palmitate was added at 0.4 mM. Moreover, our glycolytic rates are also superior to those of Belke et al., except in one instance when insulin was added to their buffer. To explain this variability, the following considerations should be taken into account. In our perfusion buffer, we routinely added physiological concentrations of the

![Graph A](image1)

![Graph B](image2)

Fig. 3. Effects of oleate concentration on citrate release rates and citric acid cycle (CAC) intermediates tissue concentrations. Data are means $\pm$ SE of 8–13 heart perfusion experiments. Hearts were perfused with either 0.7 (solid bars; $n = 13$) or 0.4 mM (hatched bars; $n = 8$) oleate and other substrates-hormones listed in Table 1. $\text{A})$ citrate release rates were quantified in effluent perfusate samples collected between 25 and 30 min by isotope dilution GCMS and flow rate measurements. $\text{B})$ tissue levels of CAC intermediates were quantitated by GCMS in tissue homogenates spiked with standards. $\alpha$-KG, $\alpha$-ketoglutarate. *$P < 0.05$ and ***$P < 0.001$ vs. 0.7 mM.

![Graph C](image3)

Fig. 4. Relative contribution of various substrates to acetyl-CoA and ATP production. Data are means $\pm$ SE of 4–15 heart perfusion experiments, as described in Table 1. The contribution of carbohydrates (lactate, pyruvate, and glucose; closed bars), exogenous fatty acids (oleate; hatched bars), and other sources (possibly endogenous triglycerides or proteins; open bars) to energy production is depicted as follows. $\text{A})$ contribution to acetyl moiety of citrate, calculated from the flux ratios pyruvate decarboxylation to citrate synthase, oleate oxidation to citrate synthase, and other substrate oxidation to citrate synthase, respectively. $\text{B})$ contribution to ATP production, calculated from $\text{J})$ flux ratios, myocardial oxygen consumption values, and $\text{Eq. 4}$ for mitochondrial oxidative phosphorylation; and $\text{K})$ lactate release rates for anaerobic glycolysis (shaded bar).
following substrates and hormones: lactate, pyruvate, carnitine, insulin, and epinephrine. On the one hand, the last four factors have been reported to individually enhance cardiac function, glycolysis, and glucose oxidation (7, 22, 23, 36, 46, 55). Specifically, at a physiological concentration, carnitine mimics insulin-like effects on glycolysis and carbohydrate oxidation (55). Lactate, on the other hand, can compete effectively with fatty acids for oxidation (65), possibly through its capacity to stimulate cardiac acetyl-CoA carboxylase activity, which gives rise to elevated levels of cytoplasmic malonyl-CoA, a known inhibitor of the mitochondrial fatty acid transporter (carnitine palmitoyltransferase-I) (9). Insulin also inhibits fatty acid oxidation through a similar mechanism (26).

Taken as a whole, our data suggest that substrate selection for energy production in the mouse heart resembles that of other species such as dogs (48), pigs (52), and humans (4). However, it appears to differ somewhat from that of the rat heart (67). When perfused with a similar mixture containing $^{13}$C-labeled substrates as in our study (including lactate, pyruvate, carnitine, insulin, and 0.4 mM olate), working rat hearts show a higher contribution of carbohydrates than fatty acids to ATP synthesis (60 ± 4 and 30 ± 4% compared with 34 ± 4 and 62 ± 10% in mice, respectively) (67). Despite this different pattern of substrate selection, ex vivo perfused rat hearts and mouse hearts have similar CAC flux rates (1.7 ± 0.2 for the rat vs. 1.9 ± 0.3 μmol·min$^{-1}$·g wet wt$^{-1}$) and cardiac efficiency (1.7 ± 0.2 for the rat vs. 1.8 ± 0.1 mW·μmol$^{-1}$·min$^{-1}$ for the mouse).

In addition to providing data on substrate selection for ATP synthesis, we also report for the first time metabolic data on pyruvate anaplerosis and citrate efflux. In fact, using $^{13}$C-labeled carbohydrates, we were able to evaluate the contribution of pyruvate to OAA formation necessary for citrate synthesis through a similar mechanism (26). To that end, we labeled carbohydrates, we were able to evaluate the contribution of pyruvate to OAA formation necessary for citrate synthesis (PC/CS; Table 4). We show a higher contribution of carbohydrates than fatty acids to ATP synthesis (60 ± 4 and 30 ± 4% compared with 34 ± 4 and 62 ± 10% in mice, respectively) (67). Despite this different pattern of substrate selection, ex vivo perfused rat hearts and mouse hearts have similar CAC flux rates (1.7 ± 0.2 for the rat vs. 1.9 ± 0.3 μmol·min$^{-1}$·g wet wt$^{-1}$) and cardiac efficiency (1.7 ± 0.2 for the rat vs. 1.8 ± 0.1 mW·μmol$^{-1}$·min$^{-1}$ for the mouse).

In conclusion, this study, based on measurements of substrate fluxes using $^{13}$C-labeling methods, provides a detailed quantitative profile of the fate and origin of pyruvate and citrate carbons in the working mouse heart perfused ex vivo under conditions in which cardiac flows and work as well as the buffer substrate-hormone composition mimic those prevailing in vivo. We believe that the described strategy of combining $^{13}$C-labeled substrates with an experimental system enabling the continuous monitoring of cardiac mechanics offers a unique and powerful tool for clarifying the link between metabolic alterations and cardiac contractile dysfunction. This should also allow dynamic flux data to be integrated with static data of metabolite concentrations, enzyme activities, protein levels, and gene expression, and provides an integrative view of the regulation of metabolic pathways at all levels, namely, posttranslational (acute) and transcriptional (chronic) levels. Finally, this methodology should provide valuable insights when applied to genetically altered mouse models of disease.

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