Tricarboxylic Acid Cycle Activity in Postischemic Rat Hearts

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Background. Although myocardial oxidative tricarboxylic acid (TCA) cycle activity and contractile function are closely linked in normal cardiac muscle, their relation during postischemic reperfusion, when contractility often is reduced, is not well defined.

Methods and Results. To test the hypothesis that oxidative TCA cycle flux is reduced in reperfused myocardium with persistent contractile dysfunction, TCA cycle flux was measured by analyzing the time course of sequential myocardial glutamate labeling during \(^{13}\)C-labeled substrate infusion with \(^{13}\)C nuclear magnetic resonance spectroscopy in beating isolated rat hearts at 37°C. Total TCA cycle flux, indexed by both empirical and mathematical modeling analyses of the \(^{13}\)C data, was not reduced but rather increased in hearts reperfused after 17–20 minutes of ischemia (left ventricular pressure, 73±5% of preischemic values) compared with flux in developed pressure–matched controls (e.g., total flux, 2.5±0.4 versus 1.6±0.1 \(\mu\)mol·min\(^{-1}\)·g wet wt\(^{-1}\), respectively; \(p<0.01\)). No TCA cycle activity was detectable by \(^{13}\)C nuclear magnetic resonance in hearts reperfused after 40–45 minutes of ischemia, which lacked contractile recovery and had ultrastructural evidence of irreversible injury.

Conclusions. These results suggest that TCA cycle activity is not persistently decreased in dysfunctional reperfused myocardium after a brief ischemic episode and therefore cannot account for the reduced contractile function at that time. (Circulation 1993;87:270–282)

Key Words • tricarboxylic acid • contractility • reperfusion • metabolism, intermediary • nuclear magnetic resonance • spectroscopy • myocardium, stunned • ultrastructure • Krebs cycle

Normal myocardial contractile function is critically dependent on high levels of oxidative tricarboxylic acid (TCA) cycle activity,\(^1\),\(^2\) and inhibition or impairment of TCA cycle flux can significantly reduce myocardial contractile function.\(^3\) During myocardial ischemia, oxidative metabolism rapidly halts, high-energy phosphate levels decline, and contractile failure ensues. Contractile failure during postischemic reperfusion has been attributed by some to low levels of ATP,\(^4\) whereas others\(^5\) have observed that ultimate contractile recovery is more closely related to recovery of oxidative metabolism supported by the TCA cycle than to ATP levels alone. It remains unclear whether incomplete contractile recovery after brief episodes of ischemia is related to delayed or impaired recovery of oxidative TCA cycle metabolism. Evidence supporting the hypothesis that postischemic metabolic recovery is incomplete includes observations that critical enzymes in intermediary metabolism are inhibited during reperfusion,\(^6\) that certain metabolic substrates can improve reperfusion contractile performance,\(^7\)–\(^9\) and that irreversible ischemic injury is associated with severe functional and structural mitochondrial abnormalities.\(^10\),\(^11\) Other studies, however, have reported that high-energy phosphate (ATP and creatine phosphate) levels\(^12\),\(^13\) and myocardial oxygen consumption\(^14\) recover earlier than contractility in reperfused viable myocardium. The inability to directly quantify oxidative TCA cycle metabolism in intact hearts has, in part, limited efforts to directly test the hypothesis, which requires simultaneous measurements of TCA activity and contractile function during reperfusion in intact hearts.

\(^{13}\)C nuclear magnetic resonance (NMR) spectroscopy can be used to nondestructively probe intermediary metabolism in intact tissues. By obtaining sequential or steady-state spectra after the introduction of \(^{13}\)C-enriched substrates, these unique properties have been exploited to quantify several aspects of metabolism in intact hearts,\(^15\)–\(^20\) including TCA cycle flux.\(^21\),\(^22\) The latter has been described with mathematical modeling\(^21\),\(^22\) and empirical\(^22\) analysis of the time course of \(^{13}\)C enrichment of the isotopomers of glutamate, an amino acid that is in rapid equilibration with the TCA cycle intermediate 2-oxoglutarate and present in high concentrations.

The present study uses \(^{13}\)C NMR spectroscopy to test the hypothesis that oxidative TCA cycle activity is reduced in reperfused viable myocardium with persistent contractile dysfunction (e.g., “stunned” myocardium). TCA cycle flux was studied under reperfusion conditions of unchanging intermediate pools and a
stable cardiac work load. To separate the effects of ischemia and reperfusion from those of altered work load per se, flux was also measured under conditions of reduced cardiac work achieved by lowering perfusate calcium without a prior ischemic period. Contractile and citric acid cycle activities, as well as histology and ultrastructure, were assessed after both short and long ischemic insults.

**Methods**

**Isolated Rat Heart Preparation**

Nonfasting, ex-breeder, male Wistar rats (approximate weight, 500–700 g) were anesthetized with 100–150 mg pentobarbital i.p. After rapid excision of the heart, the aorta was cannulated for retrograde constant-flow, nonrecirculating perfusion at 15 ml/min via a peristaltic pump with oxygenated solution at 37°C (pH 7.4). The perfusate contained (in mM) sodium 144, potassium 5, calcium 1.5, HEPES 6, magnesium 0.9, inorganic phosphate (P) 1, chloride 152, and acetate 5. Acetate was chosen as the exogenous substrate since its metabolism occurs via the TCA cycle and is not altered by changes in glycolytic or pyruvate dehydrogenase activity. HEPES was used because bicarbonate buffer solutions contained within the long perfusion lines inside the magnet cannot be bubbled during prolonged, total ischemia. If bicarbonate buffer was used in the NMR protocols, the reperfusion solution may transiently have a different pH and calcium concentration. Lidocaine (5 µg/ml) was added to all perfusates to reduce the incidence of ventricular fibrillation during reperfusion. Control hearts (n=4) perfused in separate experiments with bicarbonate buffer and without lidocaine had developed pressures, creatine phosphate–to–ATP ratios, and 13C NMR TCA flux indexes equal to those observed in control hearts perfused with HEPES buffer and lidocaine. Hearts were paced at a rate of 140 min⁻¹ using a Grass SD-9 stimulator via a KCl wick electrode placed into the right ventricle. A polyvinyl chloride balloon attached to PE 190 tubing was inserted into the left ventricle through the mitral valve and filled in increments to obtain the maximum isovolumic developed pressure (DP). The balloon solution contained [6-¹³C]hexanoate and phenylphosphonic acid (pH 7), which served as ¹³C and ¹⁵P NMR standards. The other end of the PE tubing was connected to a Gould P23Db transducer for continuous measurement of left ventricular pressure. After the calibration period described below, the volume of the balloon was not changed throughout the remainder of the experiment. [2-¹³C]Acetate (99% enriched) was obtained from Isotec, Miami, Ohio; [6-¹³C]hexanoate (99% enriched) was obtained from Merck Frosst Isotopes, Montreal; and other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

**Nuclear Magnetic Resonance Spectroscopy**

The hearts were positioned in a remotely switched triple-tuned (¹³C, ¹H, and ¹⁵P) 20-mm commercial probe of a Bruker AM 360-WB NMR spectrometer (field strength, 8.5 T), as described previously. Magnetic field homogeneity was optimized while the water proton signal was observed using the decoupler coil. Proton-decoupled, minimally saturated ¹⁵P spectra were obtained using 60º pulses with a 2.1-second interpulse delay and a 2K data table, and proton-decoupled ¹³C spectra were collected using 60º pulses with a 1.1-second interpulse delay and 2K points and zero-filled to 8K before Fourier transformation. Free induction decays were multiplied by an exponential function that introduced a line broadening of 15–20 Hz. Intracellular pH was measured by the chemical shift of the P, peak relative to the creatine phosphate peak. ²³

Absolute ¹³C metabolite concentrations were calculated in each heart by comparison of metabolite and standard peak areas after correction for partial saturation and nuclear Overhauser enhancement (NOE) effects. Specifically, a calibration graph of peak area versus amount for each heart was obtained at the beginning of each experiment by acquiring separate ¹³C NMR spectra as the volume within the intraventricular balloon, containing known concentrations of [6-¹³C]hexanoate as a standard, was incremented by precise amounts. Each increment in balloon volume placed known amounts of ¹³C compound within the sensitive volume of the NMR coil containing the heart. Such spectra acquired before administration of [2-¹³C]acetate also indicated the amount of naturally abundant (1.1%) ¹³C present in the hearts and perfusate. Difference spectra obtained by subtracting natural abundance spectra from those obtained during [2-¹³C]acetate perfusion indicated the appearance of ¹³C in intracellular metabolites. The areas under metabolite and reference peaks were determined by hand digitization (SigmaScan, Jandel Scientific, Corte Madera, Calif.), as described previously, with a mean intraobserver variability of 2% and a mean interobserver variability of 4–5% in this laboratory. Of importance, the areas of all peaks were corrected for partial saturation and NOE by comparison of fully relaxed spectra (interpulse delay, ≥6–8 seconds) without NOE with partially relaxed spectra with NOE (described above), as measured at isotopic steady state in parallel experiments. The absolute amount of a metabolite was therefore calculated from the peak area of the metabolite peak, which was then corrected for saturation and NOE effects and compared with corrected standard [6-¹³C]hexanoate peak areas representing known amounts of ¹³C compound. Hearts were weighed at the beginning of each experiment, and results are expressed in micromoles of metabolite per gram wet weight. Wet-to-dry weight ratios measured under comparable conditions in glucose-perfused hearts range from 6.5 to 7.0.

The quantity of each ¹³C metabolite was plotted versus time after substitution of [2-¹³C]acetate for unlabeled acetate. The time to half-maximal enrichment (t₅₀) for each metabolite of each heart was interpolated from a least-squares fit to the experimental glutamate ¹³C enrichment data of a sum of exponentials of the form:

\[
[¹³C]\text{Glutamate (time)} = A + [B \exp(-\text{time}/t₁)] + [C \exp(-\text{time}/t₂)]
\]

where [¹³C]glutamate (time) is the ¹³C enrichment of a given glutamate isotope as a function of time, and A, B (where B<0), C, t₁, and t₂ are constants. For enrichment curves that had a monoexponential form, C was taken to be 0.
**Data Analysis: TCA Cycle Flux Calculations**

Estimates of TCA cycle activity from the $^{13}$C NMR data were obtained by two previously described, independent methods. The first, an empirical approach, provides a flux parameter, $K_F$ (µmol • min$^{-1}$ • g$^{-1}$), that is proportional to total TCA cycle flux. A strength of this method is that it is relatively independent of pool size considerations for metabolites not detected by $^{13}$C NMR. The second method is a numerical model of the $^{13}$C-labeling kinetics of the TCA cycle intermediates. Although this model requires input for each cycle intermediate pool size, it can be used to calculate absolute TCA cycle flux.

**Indexing TCA cycle flux empirically.** The rationale underlying this approach is exemplified by a schematic representing the TCA cycle (Figure 1) that has been described in detail. After substitution of enriched acetate for unenriched acetate, $^{13}$C nuclei from [2-13C]acetate enter the TCA cycle as acetyl-CoA (acetyl-CoA) and appear in the C4 position of citrate and, subsequently, of 2-oxoglutarate and glutamate. At subsequent steps in the TCA cycle, the C4 of 2-oxoglutarate becomes the C2 or C3 of succinate and, eventually, the C2 or C3 of 2-oxoglutarate and glutamate.

Most citric acid cycle intermediates occur in concentrations too low to permit $^{13}$C NMR detection. Glutamate is an amino acid that is in rapid equilibrium with 2-oxoglutarate and is present in sufficiently high concentrations to permit $^{13}$C NMR quantification. Because [4-13C]glutamate and [2-13C]glutamate are both derived from the same intermediate in the TCA cycle but are enriched from subsequent “turns” of the TCA cycle, we proposed that the time difference ($\Delta t$) between the $^{13}$C appearance in glutamate C4 and glutamate C2 (or C3) after the administration of $^{13}$C-labeled substrate indexes $^{13}$C flux through reactions [2]-[10], or one turn of the TCA cycle. The time to $t_0$ of glutamate C4 and C2 can be calculated, and then the difference in that time (glutamate $\Delta t_0$) can be calculated, which is inversely related to TCA cycle activity. The $t_0$ values of glutamate C4 and C2, as well as the glutamate $\Delta t_0$, are also directly dependent on TCA cycle-derived intermediate pool sizes since the larger the pools, the longer it will take for label to move through the cycle.

The total TCA cycle flux is the sum of the $^{13}$C-labeled flux and the unlabeled $^{13}$C flux. The latter arises from endogenous stores of unenriched triglycerides and glycogen. The relative contribution of these to total cycle flux can be calculated with the knowledge of the fractional enrichment of total acetyl-CoA ($F_{Ea}$) entering the TCA cycle. The parameter $K_F$, which relates to total TCA cycle flux, can therefore be defined as the following:

$$K_F = \frac{\Sigma \text{[13C-NMR–detected TCA metabolites (µmol/g)]}}{F_{Ea} [\Delta t_0 \text{ (min)}]}$$

This parameter correlates closely with contractile function and with myocardial oxygen consumption over a range of cardiac work loads, as expected for an index of TCA cycle flux. The assumptions in this method are that $^{13}$C entry into the TCA cycle from [2-13C]acetate occurs via acetyl-CoA, the metabolism of $^{13}$C substrates is the same as that of $^{12}$C substrates, $^{13}$C NMR accurately quantifies the $^{13}$C enrichment of the noncarboxyl carbons of the glutamate isotopomers, intermediate pool sizes are not changing during $^{13}$C infusion under steady-state conditions ("Results"), and the substitution of [2-13C]acetate for unenriched acetate is instantaneous.

**Calculation of absolute TCA cycle flux with numerical modeling.** The dynamics of $^{13}$C labeling of citric acid cycle intermediates were also computed using a mathematical model originally derived from the analysis of Chance et al and described previously. The description, formulation, assumptions, and implementation of the model are described in detail in the "Appendix." In the present study, glutamate and aspartate pool sizes were calculated from the $^{13}$C NMR data for each heart, and citric acid cycle intermediate pool sizes too small to be quantified by $^{13}$C NMR (e.g., oxaloacetate, 2-oxoglutarate, succinate, malate, and oxaloacetate) were assumed to be comparable to those previously reported in acetate-perfused hearts and constant under the control and reperfusion conditions. The validity of these
assumptions was also assessed using the numerical model (“Results”). The citric acid cycle flux and the aspartate aminotransferase flux were determined for each heart by fitting the model, comprised of the solution to 17 simultaneous differential equations, to the glutamate C4 and C2 data, using the modeling language MLAB (Civilized Software, Bethesda, Md.).

**Tissue Extracts**

Hearts that underwent tissue extraction were frozen by rapid compression between tongs cooled to the temperature of liquid nitrogen. The frozen tissue was pulverized in a mortar under liquid nitrogen in 6% perchloric acid and centrifuged for 10 minutes at 20,000g. The supernatant was reneutralized with 2 M potassium hydroxide and centrifuged again. Hearts undergoing amino acid analysis by high-performance liquid chromatography (HPLC) had 0.73 mM tyramine added as an internal standard before centrifugation. Amino acid concentrations were evaluated by gradient elution on a 150x4.6-mm inner-diameter analytical column packed with 5-µm diameter Excellopak ODS with quantitative fluorometric elute analysis. The peak ratios of glutamate, aspartate, and internal tyramine standard were compared as previously described.

**Pathology**

**Perfusion fixation.** At the end of the reperfusion period, the perfusate was switched to an ice-cold solution with 30 mM KCl and then to one containing 4% formaldehyde/1% glutaraldehyde. Serial transverse slices of left ventricle of 2-mm thickness from the midpapillary muscle level were then processed for light and electron microscopy.

**Light microscopy.** After 24 hours of immersion fixation, the slices were rinsed in distilled water and then dehydrated in a series of ethyl alcohol solutions. They were infiltrated for 3 hours with glycol methacrylate monomer and a peroxide catalyst (Historesin, Cambridge Instruments, Chicago, Ill.) in an Infiltron rotary mixer (Ted Pella Inc., Redding, Calif.) and then placed in a mixture of catalyzed glycol methacrylate and an amine activator and plasticizer (Historesin). The embedded block was sectioned on a Historange 2218 microtome (LKB Inc., Bromma, Sweden) at a thickness of 1.5–2.0 µm, and the sections were mounted on slides and stained with toluidine blue and hematoxylin and eosin.

Interstitial space widening was assessed using hematoxylin and eosin–stained transverse sections with a 10x10 grid reticle eyepiece on x40 power. Ten random fields of longitudinally oriented fibers were chosen within the left ventricle. The relative number of grids falling within interstitial versus myocyte spaces was determined for each field. In addition, the entire circumference of the middle third of the left ventricular wall (longitudinally oriented fibers) was scanned on x40 power for the presence and severity of tissue injury using the following scoring system: 0, normal myocardium; 1+, mild injury (occasional contraction bands confined to a few areas of individual myocytes with minimal intracellular edema); 2+, moderate injury (more extensive hypercontraction and disruption of sarcomeres with intracellular and extracellular edema and sarcolemmal lifting and/or tearing); and 3+, severe injury (severe disruption of cellular architecture caused by hypercontraction and rupture of sarcomeres, numerous breaks in sarcolemma, and dissociation of intercalated discs).

Final injury scores for each specimen were expressed as the number of high-power fields with each grade divided by the total number of fields for each specimen. For example, if 15 of 20 assessed high-power fields showed 2+ ischemic injury, then the specimen is said to have 0.75 of its total area showing 2+ ischemic injury.

**Transmission electron microscopy.** Samples from the endocardial third of the free wall of the left ventricle were placed in phosphate-buffered 4% formaldehyde/1% glutaraldehyde. These were twice washed in buffer and then postfixed in 1% buffered OsO4. Semi-thin (1 µm) sections were cut on a Reichert-Jung Ultracut E Ultramicrotome, mounted on slides, and stained with toluidine blue. The semithin sections were examined for technical suitability with the observer blinded as to its origin. In general, two blocks from each specimen were chosen for further processing so that one block had myocytes in longitudinal orientation and the other had myocytes in cross-sectional orientation. Thin sections (75 nm) from the selected blocks were cut serially and mounted on copper grids. Grids were stained with 2.5% uranyl acetate and lead citrate. The severity of mitochondrial swelling, disorganization of cristae, nuclear chromatin margination, intracellular edema, loss of intracellular glycogen, integrity of sarcolemmal membranes, and severity of amorphous dense bodies within mitochondria were noted.

**Heart Perfusion Protocols**

All hearts were initially perfused at a constant flow of 15 ml/min during a stabilization period. Of the 23 hearts studied with NMR, one group (n=6) underwent 17–20 minutes of zero-flow ischemia and subsequent reperfusion. A second group (n=5) underwent 40–45 minutes of zero-flow ischemia before reperfusion. The coronary flow rate during reperfusion in both groups was 15 ml/min—the same as initial and control conditions. Two control groups were also studied. The first (n=6) was studied under baseline conditions, and the second (n=6) was studied at a reduced work load, induced by lowering perfusate Ca2+ from 1.5 to 0.8–1.0 mM to match the recovery left ventricular DP of the first group of hearts, which were reperfused after the 17–20-minute ischemic period. Data from some of the control hearts, principally the high–work load group, have been reported previously. The data from three additional hearts have been excluded because of ventricular fibrillation during reperfusion.

Because the metabolism of fatty acids, glucose, and pyruvate could be influenced by ischemia/reperfusion–induced changes in β-oxidation, glycolysis, or pyruvate dehydrogenase activity, respectively, independent of TCA cycle activity, acetate was chosen as the sole exogenous substrate since it directly enters the TCA cycle. When [2,13C]acetate was introduced, it replaced the nonenriched form in equimolar concentrations (5 mM). In the reperfused hearts, [2,13C]acetate was administered at 7 minutes of reperfusion, when preliminary research with this model demonstrated that contractile function and high-energy phosphate recovery reach their eventual levels. To confirm that the gluta-
mate and aspartate pools were not changing during the time of glutamate $^{13}$C enrichment and since glutamate and aspartate pool sizes decrease during ischemia, $^{28}$ these pools were quantified in 20 separate hearts that were rapidly frozen under nonischemic conditions at the end of 20 minutes of ischemia and at 6, 15, and 30 minutes of posts ischemic reperfusion ($n=4$ for each condition) for later extraction and analysis by HPLC techniques.

**Statistical Analysis**

All results are expressed as mean±SEM values. The data are compared using the unpaired $t$ test for two groups, such as comparison of histological data between reperfusion after short or long periods of ischemia, or ANOVA for more than two groups, such as comparison of contractile and metabolic data among the four groups.$^{29}$ Where ANOVA suggested a significant or borderline difference, a Bonferroni procedure was performed to determine whether significant differences existed between groups. Least-squares fitting of sequential $^{13}$C data to monoeponential or higher-order exponential functions was performed with SYSTAT software (SYSTAT Inc., Evanston, Ill.).

**Results**

**Contractile Data**

During the initial baseline period, mean left ventricular DP (peak systolic minus end-diastolic pressure [EDP]) was 136±6 mm Hg, and EDP was 14±2 mm Hg in all hearts ($n=23$) studied in the NMR spectrometer. The contractile response to the various experimental interventions as measured by DP is shown in Figure 2. Control hearts maintained a stable DP throughout the period of $[2^{-13}$C]acetate infusion with a mean DP of 132±15 mm Hg in the high-work load ($n=6$) hearts and 89±5 mm Hg in the low-work load ($n=6$) hearts. Global, zero-flow ischemia abolished contractions rapidly in all hearts. Mean EDP was 68±19 mm Hg at the end of the 17–20-minute ischemic period ($n=6$) and 41±6 mm Hg at the end of the 40–45-minute ischemic period ($n=5$). During reperfusion, mean DP in the hearts exposed to the shorter ischemic period recovered to 94±9 mm Hg, or 73±5% of the initial value, and was stable for 5–45 minutes of reperfusion (see Figure 2). Note that the mean DP of the reduced-work load hearts not exposed to prior ischemia was matched and not different from that of the stunned hearts, which were reperfused after 17–20 minutes of ischemia. Contrast, four of five hearts exposed to 40–45 minutes of ischemia exhibited no measurable posts ischemic contractile recovery; in one heart, DP eventually reached 22 mm Hg, or only 16% of its initial value. Mean EDP in these five severely damaged hearts was markedly elevated at 106±10 mm Hg during the reperfusion period.

**Histological Findings**

Six additional hearts underwent morphological examination after reperfusion following either 20 minutes ($n=3$) or 45 minutes ($n=3$) of global, total ischemia. The contractile activity of these hearts was similar before, during, and after ischemia to that of the respective hearts studied with NMR and reported above. Hearts reperfused after the shorter 20-minute ischemic period had less histological widening of the interstitial spaces than did those reperfused after the longer 45-minute ischemic period. This was quantitatively manifested by a larger fraction of high-power grid intersections falling on myocytes in the former group ($32.9±5.6$ versus $17.2±4.0$, $p<0.001$). Light microscopic morphological changes in the short ischemia group revealed some heterogeneity but predominantly normal or mildly injured morphology and only occasional zones of contraction bands with minimal intracellular edema. Of all high-power views, $13±8$% were grade 0, $72±4$% were grade 1, and only $15±11$% were grade 2. In striking contrast, the light microscopic morphological changes after the longer ischemic period revealed a uniform appearance consisting of extensive, multifocal zones of contraction band injury associated with granular degeneration of poorly delineated contractile elements. In these cases, $92±10$% of all high-power views were graded 2 or higher, and no microscopic field was graded 0 for normal myocardium.

Electron microscopy revealed that after the short ischemic period, the majority of myocytes showed reversible myocyte injury. The vast majority of myocytes evidenced reversible mitochondrial changes (see Figure 3, top panel), whereas dense bodies within mitochondria were seen rarely and only in severely hypercontracted cells. Electron microscopy revealed morphological evidence of irreversible injury in hearts after the longer ischemic period. Many of these myocytes contained large numbers of degenerated mitochondria with numerous dense bodies and disrupted sarcoplasmic membranes (see Figure 3, bottom panel). These changes were seen in densely hypercontracted cells as well as in adjacent noncontracted cells. Moderate-to-severe intracellular edema; numerous mitochondria
FIGURE 3. Top panel: Electron micrograph of two adjacent myocytes from a heart subjected to 20 minutes of ischemic reperfusion. The upper myocyte has increased intracellular edema and increased matrix between mitochondrial cristae. Mitochondria do not contain dense bodies. Note the intact intercalated disc (magnification, ×7,500). Bottom panel: Low-power transmission electron micrograph from a heart subjected to 45 minutes of total ischemia and subsequent reperfusion. Note the dense contraction bands with no discernible normal contractile elements. Numerous mitochondria appear pale with increased matrix between disorganized cristae. There is marked intracellular edema (magnification, ×9,000).
with increased separation of widened, disorganized cristae; and nuclear changes with marked peripheral aggregation of myocytes were present in the majority of myocytes. In summary, hearts reperfused after 20 minutes of zero-flow ischemia had minimal ultrastructural evidence of irreversible injury, whereas those reperfused after 45 minutes of zero-flow ischemia had extensive evidence of irreversible injury.\textsuperscript{11,30,31}

\textbf{Metabolic Data}

Under baseline, preischemic conditions, the mean ratio of creatine phosphate to ATP determined by \textsuperscript{31}P NMR after correction for partial saturation and NOE effects was $2.6\pm0.1$, and mean intracellular pH was $7.20\pm0.01$. These values agree with observations reported from other laboratories.\textsuperscript{32-34} Intracellular P, was not quantified because of the overlying resonance of P, contained in the perfusate. Levels of both creatine phosphate and ATP decreased during ischemia. The extent of their recovery during subsequent reperfusion differed dramatically in the two groups, as shown in the representative \textsuperscript{31}P NMR spectra in Figure 4. Hearts reperfused after 17–20 minutes of ischemia demonstrated recovery of creatine phosphate and ATP to mean levels of $89\pm6\%$ and $54\pm6\%$ of preischemic values, respectively, whereas those reperfused after 40–45 minutes of ischemia had a mean creatine phosphate level of only $6\pm4\%$ of preischemic values and no detectable ATP.

Figure 4 presents examples of \textsuperscript{13}C NMR spectra approximately 30 minutes after substitution of [\textsuperscript{2-\textsuperscript{13}}C]acetate for nonenriched acetate obtained from hearts under control conditions (upper panel) and during reperfusion after 20 minutes (middle panel) and 45 minutes (lower panel) of ischemia. Note the prominent resonances of the glutamate isotopeomers (C4, 34.5 ppm; C2, 55.5 ppm; C3, 27.7 ppm), which have been identified previously in [\textsuperscript{2-\textsuperscript{13}}C]acetate–perfused hearts.\textsuperscript{22,35,36} Smaller peaks attributable to the aspartate C2 and C3 resonances are also present. Although the same peaks are apparent during reperfusion after 17–20 minutes of ischemia, the glutamate peaks are smaller. The signal-to-noise ratios at isotopic steady state for glutamate C4 and C3 are $28\pm3$ and $23\pm3$, respectively.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Representative \textsuperscript{31}P and \textsuperscript{13}C nuclear magnetic resonance spectra obtained in a control heart (upper panel), a heart reperfused after 17–20 minutes of zero-flow ischemia (middle panel), and a heart reperfused after 40–45 minutes of ischemia (lower panel). Natural abundance \textsuperscript{13}C spectra, acquired before the introduction of [\textsuperscript{2-\textsuperscript{13}}C]acetate, were subtracted from these collections, which were obtained approximately 30 minutes after the introduction of [\textsuperscript{2-\textsuperscript{13}}C]acetate. Each \textsuperscript{13}C spectrum was obtained with 280 pulses (60°) and a 1.1-second interpulse delay, and each \textsuperscript{31}P spectrum was obtained with 160 pulses (60°) and a 2.1-second delay. Peak assignments are Pi, inorganic phosphate (intracellular and buffer); PCr, creatine phosphate; [\textsuperscript{\beta-P}]ATP, \textsuperscript{\beta}-phosphate of ATP; GLU-C2, glutamate C2; GLU-C4, glutamate C4; C3, glutamate C3; Ac, acetate C2; S1, phenylphosphonic acid standard; S2, hexanoate C6 standard. Hearts reperfused after the short ischemic period (middle panel) showed nearly complete recovery of PCr but reduced ATP and, at steady state, lower levels of glutamate. Note that after the prolonged ischemic period (bottom panel), PCr and ATP were barely detectable, and no \textsuperscript{13}C peaks attributable to tricarboxylic acid cycle–derived intermediates were present.

\end{figure}
for control high-work load hearts, 32±4 and 28±4 for control low-work load hearts, and 15±2 and 12±2 for hearts reperfused after 17 minutes of ischemia. In contrast, no 13C enrichment is detectable in the glutamate isotopomers or in any other TCA cycle-derived intermediate after 45 minutes of ischemia.

The time courses of the 13C enrichment of the glutamate C4 and C2 isotopomers are shown in Figure 5. The time to $t_{50}$ for each isotopomer is demarcated by a vertical dotted line, and the time difference between the C4 and C2 isotopomer enrichment, the glutamate $\Delta t_{50}$, is demarcated by the projection onto the x axis of the cross-hatched area between the dotted lines. Because glutamate $\Delta t_{50}$ is inversely proportional to TCA cycle flux, the broadness of the cross-hatched area is directly proportional to the slowness of the TCA cycle flux for a given TCA cycle pool size. Under normal perfusion, high-work load conditions (upper panel), glutamate C4 enrichment (filled circles) precedes that of glutamate C2 (open circles), and maximal enrichment of both isotopomer pools occurs within approximately 10–17 minutes. Under control, lower-work load conditions (second panel), 13C enrichment is slower, the glutamate $\Delta t_{50}$ is longer, and the C4 and C2 plateau levels are similar compared with those of the high-work load hearts. Glutamate enrichment in reperfused stunned hearts (third panel) differs in two main respects from the matched work load nonischemic group (second panel). First, lower plateau levels of both [4-13C]glutamate (2.5±0.2 versus 4.5±0.4 mol/g wet wt) and [2,13C]glutamate (2.2±0.2 versus 4.0±0.4 mol/min·g wet wt−1) are present in reperfused hearts. This could be due to a lower total glutamate pool after ischemia and/or to increased contributions of nonenriched endogenous substrates to TCA cycle oxidation. Second, the time of glutamate 13C enrichment is more rapid, and the glutamate $\Delta t_{50}$ (width of cross-hatched area) is smaller in hearts reperfused after 20 minutes of ischemia (3.0±0.4 versus 7.0±0.4 minutes in the low-work load, nonischemic group; $p<0.005$), suggesting, but alone not demonstrating, that TCA cycle flux is relatively increased in reperfused stunned hearts. Hearts reperfused after 40–45 minutes of ischemia did not have measurable levels of 13C glutamate enrichment (Figure 5, bottom panel) or any other evidence of NMR-detectable TCA cycle activity.

The 13C-13C spin-spin splitting of the glutamate C4 resonance and the steady-state glutamate C2/C4 ratio, after correction for partial saturation and NOE effects, allow calculation of the fractional 13C enrichment of acetyl-CoA entering the TCA cycle. This could not be performed after the longer ischemic period because of the absence of 13C NMR-detectable TCA cycle activity in these hearts. Mean $F_{ac}$ for all other hearts was 0.92±0.03 and did not significantly differ among any of the groups (Table 1). This indicates that [2-13C]acetate was the predominant substrate utilized by these hearts under all of these experimental conditions.

**Citric Acid Cycle Flux Quantification**

TCA cycle flux was calculated from the 13C NMR data for each heart by two independent methods, and the results are presented in Table 1. Mean citric acid cycle flux parameter ($K_f$) differed significantly among the groups ($p<0.001$) and tended to be higher in the nonischemic, high-work load group compared with low-work load control hearts (1.3±0.1 versus 0.9±0.1 μmol·min−1·g wet wt−1). This is expected since TCA cycle flux increases with work load in normal hearts. More important, mean $K_f$ was higher in hearts reperfused after 17–20 minutes of ischemia than in the work load–matched, nonischemic hearts (1.8±0.3 versus 0.9±0.1 μmol·min−1·g wet wt−1, respectively; $p<0.01$).

The 13C NMR data were also analyzed using a previously described mathematical model of TCA cycle labeling kinetics, which generated findings qualitatively similar to those of the empirical method. Examples of the model fit to the 13C glutamate data are shown in Figure 6 for three of the experimental conditions and demonstrate that the data are well fit by this model. The mean correlation coefficient ($r^2$) for the fit of the model output to the 13C data for all 18 hearts with detectable TCA flux was 0.96±0.016, indicating the excellent modeling fits generally obtained. Total TCA cycle flux also differed significantly among the groups ($p<0.001$) and tended to be higher in control hearts at a high work load than in those at a lower work load (2.2±0.2 versus 1.6±0.1 μmol·min−1·g wet wt−1; $p<0.07$; see Table 1). Of importance, absolute TCA cycle flux in reperfused

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**Figure 5.** Plots of time course of 13C enrichment of glutamate C4 (●) and glutamate C2 (○) for control, high developed pressure hearts (upper panel), control low developed pressure hearts (second panel from top), hearts reperfused after 17–20 minutes of ischemia (third panel from top), and hearts reperfused after 45 minutes of ischemia (bottom panel). The x axis represents time after substitution of [2-13C]acetate for the unenriched form. The time to half-maximal enrichment for each isotopomer is demarcated by a dotted line, and the difference between these times, the glutamate $\Delta t_{50}$, is indicated by the projection of the cross-hatched region onto the time axis for each condition. Note that hearts reperfused after 17–20 minutes of ischemia (third panel from top) have lower glutamate levels and shorter, not longer, glutamate $\Delta t_{50}$ compared with developed pressure–matched control hearts (second panel from top). 13C glutamate enrichment was not observed in any heart reperfused after 40–45 minutes of ischemia (bottom panel).
stunned hearts was again significantly higher than in the load-matched control hearts (2.5±0.4 versus 1.6±0.1 µmol·min⁻¹·g wet wt⁻¹; p<0.01). Mean aspartate aminotransaminase flux, which is faster than TCA cycle flux in normal hearts,21-27 was roughly threefold lower in reperfused stunned hearts than in control hearts, but the difference was of only borderline statistical significance ( p=0.07). In summary, total oxidative TCA cycle activity, as determined by two independent analyses of the 13C NMR data, was not reduced but instead relatively increased in reperfused stunned hearts during minutes 7–45 of reperfusion compared with control hearts exhibiting similar contractile function.

Data analysis by the methods described assumes that cycle intermediate pool sizes are stable during the 13C enrichment period. Because ischemia alters cycle intermediate pool sizes,28 additional experiments were performed to measure and assess the impact of any such alterations. First, HPLC analysis was performed on extracts of hearts rapidly frozen at the end of 20 minutes of ischemia and at 6, 15, and 30 minutes of reperfusion. The results are presented in Table 2. Total glutamate tended to be lower during ischemia compared with control (3.2±0.4 versus 4.2±0.2 µmol/g wet wt). During minutes 6–30 of postischemic reperfusion, however, total glutamate was constant (ranging from 2.5±0.3 to 2.9±0.3 µmol/g wet wt). Aspartate pools were also stable during minutes 6–30 of postischemic reperfusion. Therefore, the sizes of the largest intermediate pools were not changing during the reperfusion period of [2-13C]acetate infusion, a requisite for citric acid cycle flux quantification by both 13C NMR methods used above. These data also demonstrate a close agreement between 13C NMR and HPLC measures of glutamate and aspartate pools in both control and reperfused hearts. Although the measures were obtained in different hearts from parallel experiments, these results suggest that the described methods of quantifying absolute amounts of 13C metabolites using a standard within the intraventricular balloon are valid and that essentially all of the myocardial glutamate and aspartate pools are "NMR visible" under these experimental conditions.

Changes in the sizes of the smaller TCA cycle intermediate pools can occur during ischemia28 but would have less impact on the time course of 13C glutamate enrichment than would changes in the larger pools. These pools were not measured experimentally, but the extent to which potential changes in these pools would alter TCA flux estimates by 13C NMR was assessed by the numerical model. Twenty minutes of total ischemia in glucose-perfused rat hearts causes an 80% reduction in 2-oxoglutarate, does not change citrate and fumarate, and increases the succinate and malate pools by 260% and 150%, respectively.28 If our reperfusion data are analyzed using the mathematical model but with changes in these smaller pools similar to those reported above,28 the estimated total TCA cycle flux would increase in the reperfused stunned hearts by only 3–4%. Thus, the approximate 60% mean increase in total TCA cycle flux in hearts reperfused after 17–20 minutes of ischemia relative to work load-matched control hearts cannot be attributed to differences in these intermediate pool sizes, which were not experimentally determined.

Discussion

Carbon flux through the Krebs cycle was quantified nondestructively during postischemic reperfusion in beating rat hearts. After a relatively short ischemic period, contractile function was depressed, there was little histological or ultrastructural evidence of significant damage, and total TCA cycle flux measured by 13C NMR analyses was higher than in functionally matched nonischemic hearts. The relation between contractile function and TCA cycle activity during postischemic reperfusion has not been previously defined. Because myocardial oxygen consumption is related to the TCA

<table>
<thead>
<tr>
<th>Table 1. 13C Nuclear Magnetic Resonance and Contractile Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>High DP</td>
</tr>
<tr>
<td>DP (mm Hg)</td>
</tr>
<tr>
<td>EDP (mm Hg)</td>
</tr>
<tr>
<td>[4-13C]Glutamate (µmol/g wet wt)</td>
</tr>
<tr>
<td>[3-13C]Aspartate (µmol/g wet wt)</td>
</tr>
<tr>
<td>FEa</td>
</tr>
<tr>
<td>Anaerobic</td>
</tr>
<tr>
<td>Glutamate t0 C4 (minutes)</td>
</tr>
<tr>
<td>Glutamate t0 C2 (minutes)</td>
</tr>
<tr>
<td>Glutamate Δt0 (minutes)</td>
</tr>
<tr>
<td>TCA cycle flux</td>
</tr>
<tr>
<td>K₇ (µmol·min⁻¹·g wet wt⁻¹)</td>
</tr>
<tr>
<td>F (µmol·min⁻¹·g wet wt⁻¹)</td>
</tr>
</tbody>
</table>
cycle flux is reduced only during the earliest moments of reperfusion and then quickly reaches levels above the baseline.

The findings of the present study are not likely an artifact of the methods used to quantify TCA cycle flux since similar results were produced by two independent methods of $^{13}$C NMR analysis and since they qualitatively agree with other studies of myocardial oxygen consumption in reperfused hearts.14,38,40 They also are not due to changes in metabolic intermediate pool sizes since the largest pools were demonstrated to be stable during the time of $^{13}$C administration (Table 2) and the potential impact of the smaller pools was shown by mathematical modeling to be small relative to the observed differences. Finally, the results also are not attributable to changes in other metabolic pathways induced by ischemia/reperfusion because acetate is oxidized solely by the TCA cycle.

The relative increase in TCA cycle flux out of proportion to systolic function during reperfusion could be due to increased contractile or noncontractile energy demands or to mitochondrial uncoupling. This study was not designed to distinguish among these possibilities, although evidence from $^{31}$P NMR magnetization-transfer techniques indicates that significant mitochondrial uncoupling does not occur during reperfusion.38 We have previously reported that excess energy-consuming spontaneous diastolic sarcoplasmic reticulum–myofilament calcium oscillations are present during early (7–13 minutes) reperfusion in isolated rat hearts, and this phenomenon could contribute to increased nonsystolic oxidative metabolic demands at that time.13 $^{13}$C NMR can also be used to quantify other aspects of cardiac metabolism. Under these experimental conditions, the fractional contribution of acetate to total acetyl-CoA entering the TCA cycle and the relative contribution of anaplerosis to acetyl-CoA–derived TCA flux were not altered during reperfusion, although the glutamate pools were reduced. Mathematical modeling analysis of the $^{13}$C data suggested a reduction of transaminase activity during reperfusion. Reduced transaminase activity could generate normal or even reduced total tissue label uptake rates, even in the presence of increased TCA cycle activity, because the glutamate and aspartate amino acid pools are orders of magnitude larger than most TCA cycle intermediate pools in cardiac tissues. This exemplifies an important problem that can arise when metabolic rates (e.g., TCA cycle flux) are inferred from methods that measure only total tissue label uptake. Accurate measurement of flux through a specific metabolic pathway often requires

### Table 2. High-Performance Liquid Chromatography Analysis of Myocardial Amino Acid Pool Sizes

<table>
<thead>
<tr>
<th>Condition</th>
<th>Glutamate (μmol/g wet wt)</th>
<th>Aspartate (μmol/g wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>4.2±0.2</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Ischemia (20 minutes)</td>
<td>3.2±0.4</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>Reperfusion (6 minutes)</td>
<td>2.9±0.3</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>Reperfusion (15 minutes)</td>
<td>2.9±0.8</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>Reperfusion (30 minutes)</td>
<td>2.5±0.3</td>
<td>1.0±0.1</td>
</tr>
</tbody>
</table>

$n=4$ for each condition studied. Results are expressed as mean±SEM.
quantification of rates of label uptake within specific carbon positions of particular intracellular metabolites, and the latter can be done only in a noninvasive fashion by $^{13}$C NMR.

Hearts reperfused after a relatively long ischemic period in our study had ultrastructural evidence of irreversible injury, negligible contractile recovery and high-energy phosphate levels, and no $^{13}$C NMR-detectable citric acid cycle flux. Although this study was not designed to assess the use of $^{13}$C NMR detection of TCA cycle activity as a test for postischemic myocardial viability, these observations are consistent with prior research suggesting that irreversibly damaged myocardium has depressed substrate uptake$^{57-60}$ and metabolite levels.$^{51-53}$ Future research in intact animals that permit a longer reperfusion period are suggested and are needed to test for a quantitative relation between different amounts of necrosis and TCA cycle flux. The results may have clinical relevance since recently developed spatially localized NMR spectroscopic techniques now permit studies in humans.$^{54-56}$

A potential limitation of the present study is that histopathological, biochemical, and NMR measures were determined in parallel experiments and not in the same hearts. The similar experimental conditions and the uniformity of the contractile response among hearts studied by the different techniques suggest that the results are representative. It is possible, but not tested here, that TCA cycle flux could limit contractile recovery during reperfusion in hearts at maximal attainable work loads, as some have suggested.$^{57}$ It would also be important in future research to compare hearts with regions of ultrastructural heterogeneity with spatially localized rather than global measures of contractile function, high-energy phosphate levels, and citric acid cycle activity.

In summary, evidence is presented that demonstrates that oxidative TCA cycle activity is not decreased but rather is increased in persistently hypcontractile stunned myocardium relative to comparably functioning hearts not exposed to a prior ischemic period. Hearts demonstrating no contractile recovery after prolonged ischemia exhibit no $^{13}$C NMR-detectable TCA cycle activity, suggesting future studies to determine whether $^{13}$C NMR may also be an important noninvasive means of distinguishing reperfused viable myocardium from nonviable myocardium in the clinical setting.

Acknowledgments

We thank Koenraad Vandegaar and Michelle Leppo for technical assistance.

Appendix

The dynamics of $^{13}$C labeling of citric acid cycle intermediates were computed using a model derived from the analysis initially described by Chance et al.$^{21}$ This is based on a simplified scheme of the cycle, containing citrate, α-ketoglutarate, succinate, malate, and oxaloacetate; connected by effectively irreversible reactions; and coupled to pools of glutamate and aspartate by the alanine aminotransferase and aspartate aminotransferase reactions. Other assumptions are that all reactions are at chemical (as opposed to isotopic) steady state, that the input function instantaneously changes from unenriched to $^{13}$C-enriched substrate, and that pool sizes of metabolic intermediates are uniform in tissues. Because the calculated flux parameters were well determined, the distribution of residuals was random, and the uncertainty of the least squares fitting was within the error of the data, Chance et al.$^{21}$ have concluded that it is not essential to introduce separate extracellular, cytosolic, and mitochondrial spaces.

Because of the symmetry resulting from the isotope independence of reaction rates, we summed the original 176 differential equations as analyzed by Chance et al.$^{21}$ for the various possible labeled species over intermediates labeled at a common position and obtained 17 differential equations that describe the evolution of the fractional labeling of individual carbon positions. This has been previously described,$^{22}$ and the equations are again listed below:

\[
\begin{align*}
\frac{dG_3}{dt} &= \frac{(FAL+FA)(K_3-G_3)}{PG} \\
\frac{dA_3}{dt} &= \frac{FA(O_3-A_3)}{PA} \\
\frac{dA_2}{dt} &= \frac{FA(O_2-A_2)}{PA} \\
\frac{dM_2}{dt} &= \frac{\left[\frac{S_3+S_2}{2}-(FP+1)M_2\right]}{PM} \\
\frac{dO_2}{dt} &= \frac{(FA+F)O_2+F M_2+A_2 FA}{PO} \\
\frac{dC_1}{dt} &= \frac{F(O_2-C_1)}{PCI} \\
\frac{dK_3}{dt} &= \frac{-(FAL+FA+F)K_3+(FAL+FA)G_3+C_13 F}{PK} \\
\frac{dS_2}{dt} &= \frac{F(K_3-S_2)}{PS} \\
\frac{dS_3}{dt} &= \frac{F(K_4-S_3)}{PS} \\
\frac{dM_3}{dt} &= \frac{\left[\frac{S_3+S_2}{2}-(FP+1)M_3\right]}{PM} \\
\frac{dO_3}{dt} &= \frac{(FA+F)O_3+F M_3+A_3 FA}{PO} \\
\frac{dC_2}{dt} &= \frac{F(O_3-C_2)}{PCI} \\
\frac{dK_2}{dt} &= \frac{-(FAL+FA+F)K_2+(FAL+FA)G_2+C_12 F}{PK} \\
\frac{dG_2}{dt} &= \frac{(FAL+FA)(K_2-G_2)}{PG}
\end{align*}
\]
dG4 = (FAL + FA) (K4 - G4) \\

\( \Delta G = \frac{(FAL + FA)(K4 - G4)}{PG} \)

\( dCI4 = (AC - CI4) F \) \\
\( \Delta T = \frac{(AC - CI4) F}{PCI} \)

\( dK4 = -(FA + FA + F) K4 + (FAL + FA) G4 + CI4 F \) \\
\( \Delta T = \frac{(FAL + FA + F) K4 + (FAL + FA) G4 + CI4 F}{PK} \)

CI, K, S, M, and O are citrate, \( \alpha \)-ketoglutarate (2-oxoglutarate), succinate, malate, and oxaloacetate, respectively. K4, for example, is the fractional labeling of carbon 4 on \( \alpha \)-ketoglutarate (using the numbering scheme of Chance et al.\(^\text{21}\)), and PCI, PK, PS, PM, and PO are the absolute total pools sizes of the corresponding intermediates. F is the citric acid cycle flux, and FA and FAL are the exchange fluxes of aspartate aminotransferase and alanine aminotransferase, respectively. AC is the fractional labeling of (carbon 2 of) acetylcoenzyme A (acetyl-CoA) (termed FE\(_2\) in the article), and anaplerosis is represented by exchange of malate with an infinite unlabeled pool at a flux rate FF;\(^\text{2F}\), where FF is the dimensionless fractional rate of anaplerosis. Pool sizes and fluxes are measured in micromoles per gram wet weight and micromoles per gram wet weight per minute, respectively; any unit used previously could be used, however, since the calculated fractional labelings are dimensionless. This has the same assumptions as the model described by Chance et al.\(^\text{21}\) and as detailed above.

We previously demonstrated\(^\text{22}\) that this model provides an excellent fit to experimental \( ^{13}\)C-glutamate–enrichment curves observed across a range of tricarboxylic acid cycle activity.

In the current research, the pool sizes of glutamate and aspartate were measured by \( ^{13}\)C nuclear magnetic resonance (NMR) in each heart; other pool sizes were taken from the literature\(^\text{21}\) and were the same for reperfused and control hearts. Specifically, the pool sizes of CI, K, S, M, and O were 0.296, 0.024, 0.047, 0.047, and 0.0015 \( \mu \text{mol/g wet wt} \)\(^\text{21}\), assuming a wet-to-dry weight ratio of 6.75. AC and FP were determined from isotopic steady-state \( ^{13}\)C NMR spectra.\(^\text{20}\) The citric acid cycle flux (F) and aspartate aminotransferase flux (FA) were the only parameters “flattened” during optimization and were determined by fitting the model to the G4 and G2 data measured from the hearts, using a Marquardt-Levenberg iterative least-squares fitting algorithm. All model computations were performed using the modeling language MLAB (Civillized Software, Bethesda, Md.) and implemented on an IBM-compatible personal computer with a 386 or 486 microprocessor. This analysis provided excellent fits for the \( ^{13}\)C NMR data as evidenced by the representative examples, the low average root-mean-square error (0.057±0.016), and high mean correlation coefficient \( (r^2=0.96±0.02) \) for all 18 hearts with detectable tricarboxylic acid cycle activity. Residuals were generally distributed randomly.

In addition, a potential flaw analysis was performed to determine the extent to which uncertainties in experimental measures (pool sizes, AC, FP, and time of spectral acquisition relative to isotope delivery) could influence the results generated by the modeling analysis. Although the mean intraobserver and interobserver variability in \( ^{13}\)C NMR peak digitization in a given data set was less than the variability observed among data sets from different hearts, the flaw analysis was performed using values comparable to the latter and therefore probably indicates maximal, rather than minimal, potential variations. The model was fit to data from control, high-work load hearts with fixed parameters changed as indicated below (Table 1A). This demonstrates that errors of this magnitude in these fixed parameters may individually introduce only an approximate 5% (maximum of 10%) variation in the model calculated tricarboxylic acid cycle activity (F).

The input parameters or data were intentionally altered in the following ways. For pool sizes, the glutamate and aspartate pool sizes were individually altered by 10%, or the citrate, malate, succinate, \( \alpha \)-ketoglutarate, and oxaloacetate pools were all increased by 100%. For acetyl-CoA, the fractional \( ^{13}\)C enrichment of acetyl-CoA entering the tricarboxylic acid cycle was varied from 94%. For anaplerosis, the anaplerotic contribution to citrate synthase activity was varied from 5%. For time, the time registration between isotope delivery and \( ^{13}\)C NMR spectral acquisition was altered by a linear offset of 15 seconds or by a monoeponential input function representing isotope mixing and transport with a very low time constant of 45 seconds.

**References**


**Table 1A. Conditions and Corresponding Total TCA Fluxes**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total TCA flux (( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g wet wt}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>Intermediate pools</td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
</tr>
<tr>
<td>+10%</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>-10%</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>Aspartate</td>
<td></td>
</tr>
<tr>
<td>+10%</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>-10%</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>All others</td>
<td></td>
</tr>
<tr>
<td>+100%</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>Acetyl-CoA (AC)</td>
<td></td>
</tr>
<tr>
<td>92%</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>96%</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>Anaplerosis (FP)</td>
<td></td>
</tr>
<tr>
<td>3%</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>7%</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>Time</td>
<td></td>
</tr>
<tr>
<td>Offset</td>
<td></td>
</tr>
<tr>
<td>+15 seconds</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>-15 seconds</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Input function</td>
<td></td>
</tr>
<tr>
<td>k=45 seconds</td>
<td>2.1±0.1</td>
</tr>
</tbody>
</table>

TCA, tricarboxylic acid; acetyl-CoA, acetoacetylglycerone; AC, fractional labeling of (carbon 2 of) acetyl-CoA; FP, dimensionless fractional rate of anaplerosis.


