Reverse flux through cardiac NADP⁺-isocitrate dehydrogenase under normoxia and ischemia

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Comte, Blandine, Geneviève Vincent, Bertrand Bouchard, Mohamed Benderdour, and Christine Des Rosiers. Reverse flux through cardiac NADP⁺-isocitrate dehydrogenase under normoxia and ischemia. Am J Physiol Heart Circ Physiol 283: H1505–H1514, 2002. First published June 6, 2002; 10.1152/ajpheart.00287.2002.—Little is known about the role of mitochondrial NADP⁺-isocitrate dehydrogenase (NADP⁺-ICDH) in the heart, where this enzyme shows its highest expression and activity. We tested the hypothesis that in the heart, NADP⁺-ICDH operates in the reverse direction of the citric acid cycle (CAC) and thereby may contribute to the fine regulation of CAC activity (Sazanov and Jackson, FEBS Lett 344: 109–116, 1994). We documented a reverse flux through this enzyme in rat hearts perfused with the medium-chain fatty acid octanoate using [U-¹³C₅]glutamate and mass isotopomer analysis of tissue citrate (Comte et al., J Biol Chem 272: 26117–26124, 1997). In this study, we assessed the significance of our previous findings by perfusing hearts with long-chain fatty acids and tested the effects of changes in O₂ supply. We showed that under all of these conditions citrate was enriched in an isotopomer containing five ¹³C atoms. This isotopomer can only be explained by substrate flux through reversal of the NADP⁺-ICDH reaction, which is evaluated at 3–7% of flux through citrate synthase. Small variations in reversal fluxes induced by low-flow ischemia that mimicked hibernation occurred despite major changes in contractile function and O₂ consumption of the heart as well as citrate and succinate release rates and tissue levels. Our data show a reverse flux through NADP⁺-ICDH and support its hypothesized role in the fine regulation of CAC activity in the normoxic and O₂-deprived heart.

citric acid cycle; citrate release; isotopomer analysis; ¹³C substrate; anaplerosis

CARDIAC ISCHEMIC DISEASES have been associated with chronic alterations of energy metabolism such as increased myocardial citrate release (24, 42). The cause of this deregulated cardiac citrate metabolism is unclear. Myocardial citrate release reflects its efflux from mitochondria (43) where it is synthesized by citrate synthase during normal operation of the citric acid cycle (CAC; Fig. 1). Citrate release, which is modulated by substrates and/or O₂ supply (24, 28, 29, 43), represents at most 1% of CAC flux. Mitochondrial citrate efflux appears normally to be compensated largely by flux through anaplerotic reactions such as pyruvate carboxylation, which represents between 2 and 8% of CAC flux (5, 28, 29).

Citrate synthesis could also occur through a reductive process, which involves the participation of the CAC enzymes aconitase and NADP⁺-linked isocitrate dehydrogenase (NADP⁺-ICDH). Aconitase catalyzes the reversible interconversion between citrate and isocitrate. Its activity is modulated by oxidative stress (3, 25). NADP⁺-ICDH catalyzes the reversible interconversion between isocitrate and α-ketoglutarate (α-KG). It has no known allosteric effector. This is in contrast with NAD⁺-ICDH, which has kinetic properties that are consistent with its unidirectional operation in vivo toward α-KG formation. The latter enzyme is highly regulated by a variety of positive (Ca²⁺, ADP, and citrate) and negative (ATP, NADH, and NADPH) effectors (10).

Little is known about mitochondrial NADP⁺-ICDH in the heart. It is unclear whether this enzyme operates in the forward direction of the CAC cycle, generating α-KG and NADPH, or in the reverse direction, generating isocitrate and NADP⁺. For livers perfused under normoxia, we estimated (7) that 45% of total citrate formation occurs through the reversal of NAD⁺-ICDH and aconitase reactions. Investigating the reverse flux of NADP⁺-ICDH in the heart appears to be even more relevant than in the liver for many reasons. First, NAD⁺-ICDH shows its highest activity and mRNA expression in the heart (19, 20). Second, according to Thomassen et al. (42), reverse flux through NAD⁺-ICDH may participate in the formation of citrate from glutamate and hence possibly explains the higher myocardial citrate release in cardiac patients. Third, according to Sazanov and Jackson (35), reverse flux through mitochondrial NADP⁺-ICDH could be part of a substrate cycle that contributes to fine regulation of CAC activity, thereby providing en-
hanced sensitivity to changes in energy demand. This cycle also includes the participation of NADPH/ICDH and H+/transhydrogenases (H+/Thase) to regenerate α-KG and NADPH, respectively (Fig. 1). That NADP+/ICDH could operate in vivo in the reverse direction is supported by its kinetic properties in terms of the Michaelis-Menten constant (Km) and available information on its substrate concentrations in the matrix of isolated mitochondria as well as its thermodynamic parameters (33, 35, 44). Finally, Jo et al. (14) recently presented evidence from a NIH3T3 cell line that NADP+/ICDH could function as an antioxidant defense enzyme. This role requires, however, that NADP+/ICDH operates in the forward direction of the CAC to form NADPH for regenerating reduced glutathione by glutathione reductase.

The objective of this study was to investigate substrate flux through the reversal of NADP+/ICDH in the intact heart. We expanded on a previous study (4) where we documented this reverse flux in rat hearts perfused under normoxia with the medium-chain fatty acid (MCFA) octanoate and physiological concentrations of glucose, lactate, pyruvate, and glutamate. This was achieved using the 13C protocol developed for perfused rat livers (7). In the current study, we assessed the physiological significance of our previous finding by perfusing hearts in the presence of the physiological long-chain fatty acid (LCFA) oleate. In addition, we tested the effects of O2 deprivation, a condition for which Sazanov and Jackson's hypothesis (35) predicts increased reverse flux through NADP+/ICDH. Flux values were extrapolated from the 13C mass isotope distribution (MID) of tissue citrate and α-KG levels, which were assessed by gas chromatography-mass spectrometry (GC-MS). The mechanical and metabolic responses of the heart were documented under all conditions through various measurements: 1) indices of the heart's contractile activity, 2) release rates and tissue concentrations of CAC intermediates, and 3) activity of the tissue CAC enzymes citrate synthase, aconitase, NAD+/ICDH, and NADP+/ICDH.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

The sources of chemicals, biological products, and 13C substrates have been identified previously (5, 43). The dia-
lyzed 13.4% albumin solution (BSA fraction V, fatty acid poor; Bayer) and the stock solution of 20 mM sodium oleate...
complexed to albumin were prepared and stored as described previously (43).

Heart Perfusions

Animal experiments were approved by the local animal ethics committee in compliance with the guidelines of the Canadian Council on Animal Care. Procedures for the isolation and perfusion of rat hearts in the Langendorff mode have been described elsewhere (5, 16, 43). Briefly, the hearts of fed male Sprague-Dawley rats (body wt 160–200 g; Charles River Breeding Laboratories) were perfused for 15–20 min at a constant pressure of 70 mmHg with nonrecirculating modified Krebs-Henseleit buffer, pH 7.4, that contained 119 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 5.5 mM glucose, 8 nM insulin, 1 mM lactate, 0.2 mM pyruvate, 0.5 mM glutamate, and 50 μM carnitine. After this equilibration period, which allowed for balloon insertion into the left-ventricular cavity, the hearts were switched to another buffer reservoir that contained equilibration buffer supplemented with a fatty acid without or with albumin as indicated below. The setup for heart perfusions with albumin-containing buffer and for continuous monitoring of functional parameters using instruments linked to a microcomputer has been described earlier (45). Samples of effluent and influent perfusate, which were collected on ice, were processed immediately for determinations of PO₂, P CO₂, pH, and free Ca²⁺ or stored at −20°C until analysis for citrate and succinate release rates. At the end of the experiments, the hearts were freeze-clamped and stored in liquid nitrogen.

Perfusion Protocols

Hearts were perfused under normoxia or low-flow ischemia (LFI) with nonrecirculating equilibration buffer supplemented with a fatty acid: either 0.2 mM octanoate (a MCFA) or 0.4 mM oleate (a LCFA complexed to 4% albumin). Perfusion experiments were conducted sequentially. First, we tested our 13C protocol with a MCFA because this avoids the use of albumin. We then used a LCFA complexed to albumin to assess the physiological significance of our findings with the MCFA.

After an equilibration period of 15–25 min, two groups of hearts were perfused under normoxia for 30 min before freeze-clamping. Four other groups of hearts underwent an additional 90 min of perfusion at 1 ml/min in the absence or presence of 1 μM norepinephrine (LFI/NE). The addition of NE simulates the increase of catecholamines that occur under ischemia and in cardiac patients (21, 37, 38). Unlabeled glutamate was replaced by [U-13C₅,15N]glutamate (99%) for I) the last 30 min of normoxic perfusion, and 2) 1 min after the beginning of LFI. Note that the concentrations of ionized calcium and endogenous free fatty acids for the 4% albumin-containing buffer were determined to be 1.2 and 0.3 mM, respectively. Thus total free fatty acid concentration for the LCFA group was 0.7 mM.

Analytical Procedures

GC-MS. Citrate and succinate releases and 13C MID of tissue CAC intermediates were determined by methods that have been described previously (4, 5, 16, 43). All metabolites were analyzed as tert-butylimidethylsilyl derivatives on a Hewlett-Packard 5890 series II plus GC coupled to a 5972 mass-selective detector equipped with an HP-5 fused silica capillary column (50 m, 0.2-mm inner diameter, 0.33-μm film thickness). Concentrations of CAC intermediates were determined in tissue samples spiked with [1,5-13C₂]citrate, [1,4-13C₅]succinate, and [U-13C₄]fumarate. Quantification was achieved using standard curves for isocitrate, α-KG, and malate.

Other assays. PO₂, P CO₂, free Ca²⁺, and pH were determined in influent and effluent perfusates collected under normoxia (18 min) or LFI (88 min) using a pH, blood gas, and electrolyte analyzer (ABL 70 series, Radiometer; Copenhagen). For the determination of enzyme activities (citrate synthase, aconitate, and NAD⁺- and NADP⁺-ICDH), frozen powdered tissues were homogenized in solution that contained (in mM) 180 KCl, 5 MOPS, and 2 EDTA, pH 7.4. After sequential centrifugation at 800 and 6,000 g for 10 min at 4°C, the final supernatant was collected and stored at −80°C pending the assays. Enzyme activities were assayed by monitoring the kinetics of optical density of NADH or NADPH (340 nm) measured on a Hewlett-Packard 8452A spectrophotometer. Citrate synthase and NAD⁺-ICDH were assayed by standard procedures (22). The procedure of Nulton-Persson and Sweda (25) was slightly modified for aconitase. Heart tissue samples were added to the incubation mixture that contained 5 mM citrate, 0.5 mM MgCl₂, 1 mM NADP⁺, and 1 U/ml ICDH, pH 7.4 (2 ml total volume at 30°C). NAD⁺-ICDH activity was measured with a commercial kit (Sigma Diagnostics). Protein contents were determined with a Bio-Rad kit, and BSA served as a standard. Enzyme activities are expressed as units per milligram of total protein, where 1 unit of enzyme activity is defined as the amount catalyzing the conversion of 1 μmol of substrate per minute at 30°C.

Calculations

Myocardial O₂ consumption (MV O₂, μmol/min) was calculated from the product of O₂ concentration (mM) differences between influent and effluent perfusates and coronary flow rates (ml/min). A value of 1.06 mM was taken as the concentration of dissolved O₂ at 100% saturation (40). Intracellular pH (pHᵢ) was estimated using venous CO₂ pressure (P CO₂, mmHg) by the method of Bunger et al. (2) as follows: pHᵢ = 7.524e⁻^(-0.00087286 × P CO₂). The rate pressure product (RPP, mmHg × beats/min) was calculated from the product of left ventricular developed pressure (LVPD, mmHg) and heart rate (HR, beats/min). As an estimate of cardiac efficiency, RPP was divided by MV O₂.

Areas under the GC fragmentograms were determined by computer integration and corrected for naturally occurring heavy isotopes (8). GC-MS data are expressed as molar percent enrichment (MPE) as defined previously (5, 16, 43). Briefly, the absolute MPE of individual 13C-labeled mass isotopomers (Mᵢ) of a given metabolite was calculated as follows

\[
\text{MPE (Mᵢ)} = \frac{\%A_M}{[A_M + \Sigma A_M]} \tag{1}
\]

where Aₘ and Aₘᵢ represent the peak area of GC ion fragmentograms corrected for natural abundance that correspond to the unlabeled (M) and 13C-labeled (Mᵢ) where i ranges from 1 to n, n being the number of carbon atoms.

Relative substrate flux through the reversal ICDH and aconitase reactions was calculated from the MPE in M₅ of citrate and α-KG as described elsewhere (7)

\[
\text{FC}_{α-KG→CIT} = \text{CIT}_{M₅}/\text{α-KG}_{M₅} \tag{2}
\]

where FCₐ-KG→CIT is the fractional contribution (FC) of α-KG to citrate via the reversal of NADP⁺-ICDH and aconitase reactions, and α-KGₜ₅ and CITₜ₅ are the molar fractions of M₅ α-KG and M₅ citrate, respectively. The molar fraction is the MPE divided by 100. The term (1 − FCₐ-KG→CIT) repre-
sents the fraction of citrate molecules coming from the CAC through the citrate synthase reaction.

**Statistical Analysis**

Individual enrichments are the average of two to five GC-MS injections. Data are expressed as means ± SE of n heart perfusions. Statistical significance at P < 0.05 of differences between mean values was assessed by one-way ANOVA followed by a Bonferroni multiple-comparison post-test as indicated.

**RESULTS**

As a whole, most parameters (functional, physiological, and metabolic) measured in hearts perfused with nonrecirculating buffer that contained 5.5 mM glucose, 8 nM insulin, 50 μM carnitine, 1 mM lactate, 0.2 mM pyruvate, 0.5 mM glutamate, and either 0.2 mM octanoate or 0.4 mM oleate were greatly affected by LFI.

Because the effects of LFI in the absence or presence of NE were similar, we present the results obtained with NE only as it has greater clinical relevance. Although a detailed comparison of the effects of MCFA vs. LCFA was beyond the scope of this paper, the data are presented together, because we observed only small differences between these two groups of heart perfusions.

**Functional and Physiological Parameters**

Values for the various functional and physiological parameters of hearts perfused under normoxia or LFI/NE with octanoate or oleate are shown in Figs. 2 and 3. All hearts perfused under normoxia beat spontaneously at a rate of 319 ± 6 beats/min (n = 29) during the entire protocol with a coronary flow rate of 9.4 ± 0.6 ml/min and systolic and diastolic pressures of 106 ± 1.9 and 6.4 ± 0.6 mmHg, respectively. Upon reduction of coronary flow to 1 ml/min, there was a progressive decrease in HR, LVDP, and dP/dt max, which stabilized after 10 min (Fig. 2). MV̇O₂ showed a concomitant decrease (Fig. 3A), and consequently cardiac efficiency was increased (Fig. 3B), although the difference did not reach significance for the LCFA group (P = 0.07). Finally, pHᵢ was lower under LFI/NE (MCFA, 7.210 ± 0.012; LCFA, 7.289 ± 0.030) than under normoxia (MCFA, 7.293 ± 0.006; LCFA, 7.359 ± 0.008). This difference in pHᵢ reached significance only for the LCFA group (P < 0.05).

**Release Rates and Tissue Levels of CAC Intermediates**

The release rates of citrate and succinate, which are, respectively, indices of mitochondrial fuel abundance (43) and O₂ deprivation (16), were differentially affected by LFI/NE (Fig. 4). A similar trend was observed for the release rates of hearts perfused with octanoate or oleate. However, as a whole, these rates were greater with octanoate than with oleate under both normoxia and LFI/NE. Citrate release rates were decreased three- to fourfold by LFI. However, when expressed relative to MV̇O₂ (Fig. 4B), which reflects CAC activity, the rates were increased two- to threefold to reach 1–2.5% of MV̇O₂ values. The succinate release rates under normoxia were three- to fivefold lower than those of citrate. They were increased two- to fivefold by

![Fig. 2. Cardiac function. Data are means ± SE for 3–5 experiments. Hearts were perfused with 5.5 mM glucose, 8 nM insulin, 50 μM carnitine, 1 mM lactate, 0.2 mM pyruvate, 0.5 mM [U-13C₅]glutamate, and 0.2 mM octanoate (for MCFA) or 0.4 mM oleate (for LCFA) for 30 min under normoxia (N, open bars) or for 90 min under low-flow ischemia (LFI, 1 ml/min) in the presence of 1 μM norepinephrine (NE, solid bars). Values for heart rate (A), left ventricular developed pressure (LVDP, B), and dP/dt max (C) were averaged for 30 min during normoxia and for 30–90 min during LFI. LVDP was calculated from the difference in systolic and diastolic pressures. #P < 0.001, LFI vs. normoxia.](http://ajpheart.physiology.org/)
LFI/NE (Fig. 4C) and reached values similar to those observed for citrate under normoxia. This effect of LFI/NE was amplified by the expression of succinate formation from the ratio in M5 enrichment between tissue citrate and α-KG (Eq. 2). Under our perfusion conditions, M5 citrate can only be formed through the reversal of NADP⁺:ICDH and aconitase reactions. The formation of M5 citrate through other pathways involves the following sequence of reactions: M4 malate→M3 pyruvate→M2 acetyl-CoA, or M4 malate→M3 pyruvate→M3 oxaloacetate followed by recombination of M3 oxaloacetate with M2 acetyl-CoA. The contribution of these pathways was considered negligible because of the low values of MPE M4 for malate and fumarate than for succinate reflecting the entrance of unlabeled oxaloacetate coming from pyruvate carbon. As a whole, LFI hearts showed higher MPE values for most CAC intermediates. A similar trend was observed under LFI/NE for the MCFA and LCFA groups. Note that the higher value of MPE M5 in tissue α-KG is consistent with the reported increase in glutamate uptake by the O2-deprived heart (42) and hence probably reflects increased anaplerosis rather than simple exchange.

We calculated the contribution of α-KG to citrate formation from the ratio in M5 enrichment between tissue citrate and α-KG (Eq. 2). Under our perfusion conditions, M5 citrate can only be formed through the reversal of NADP⁺:ICDH and aconitase reactions. The formation of M5 citrate through other pathways involves the following sequence of reactions: M4 malate→M3 pyruvate→M2 acetyl-CoA, or M4 malate→M3 pyruvate→M3 oxaloacetate followed by recombination of M3 oxaloacetate with M2 acetyl-CoA. The contribution of these pathways was considered negligible because of the low values of MPE M4 for tissue malate (Table 1) and MPE M3 for tissue pyruvate (<0.2%, data not shown).

Under normoxia, 3–7% of citrate molecules were formed through the reversal of NADP⁺:ICDH (Fig. 6). The remaining (1 – Fc⁺⁻ICDH) 93–97% of citrate arose from citrate synthase. Fatty acids differently modulated the effects of LFI on the proportion of citrate formed through the reversal of NADP⁺:ICDH: LFI increased this proportion by 40% in the presence of MCFA, whereas it had no effect in the presence of LCFA.

### Enzyme Activities

Figure 7 shows the activities of aconitase, NAD⁺:ICDH, and NAD⁺:ICDH expressed as a percentage of citrate synthase as measured in extracts of hearts perfused with MCFA or LCFA (2.10 ± 0.12 and 1.53 ±
0.19 U/mg protein, respectively, under all conditions; \( P < 0.05 \). Note the 100-fold difference in the y-axis scale for the three sets of graphs. Tissue aconitase activity (Fig. 7A) was 10-fold lower than that of citrate synthase and was decreased by LFI/NE. As a whole, aconitase activity was lower for the LCFA group than the MCFA group. NAD\(^+\) and NADP\(^+\)-ICDH activities (Fig. 7, B and C) were not significantly modified by LFI/NE or the nature of the fatty acid. However, there was more than a 100-fold difference in activities between NAD\(^+\)-ICDH and NADP\(^+\)-ICDH, which represents 1.2–2% and 200–300% of citrate synthase activity, respectively.

**DISCUSSION**

This study was undertaken to shed some light on the role of mitochondrial NADP\(^+\)-ICDH in the heart. We expanded on a previous investigation, which provided evidence for the reversal of NADP\(^+\)-ICDH in normoxic rat hearts perfused with glucose, lactate, pyruvate, octanoate, and \([U-^{13}C_5]\)glutamate (4). In the current study, we assessed the physiological significance of our previous finding by documenting the reversal of NADP\(^+\)-ICDH in hearts perfused with the LCFA oleate. In addition, we tested in part the hypothesis of Sazanov and Jackson (35) on the role of NADP\(^+\)-ICDH.

In brief, this hypothesis proposes that in mitochondria, a substrate cycle operates between isocitrate and α-KG where NAD\(^+\)-ICDH generates α-KG and NADP\(^+\)-ICDH generates isocitrate. The NADPH used in the reverse reaction of NADP\(^+\)-ICDH is supplied by the H\(^+\)-transhydrogenases driven by the proton electrochemical gradient (see Fig. 1). The isocitrate ↔ α-KG cycle provides a mechanism by which CAC activity could be tightly controlled by modifiers of NAD\(^+\)-ICDH and the energy state of the inner mitochondrial membrane. This hypothesis was formulated on the basis of the known properties of the isolated enzymes and the available information on substrate concentrations in the matrix of isolated mitochondria as well as thermodynamic parameters. One prediction of this hypothesis is that compared with the CAC, flux through the reversal of NADP\(^+\)-ICDH should increase under conditions of O\(_2\) deprivation.

As a model of O\(_2\) deprivation, we chose LFI that was achieved by reduction of flow to 1 ml/min in the absence or presence of NE. As a whole, measured changes in the various indices reflecting the functional, physiological, and metabolic status of perfused hearts after reduction of flow were independent of NE and indicated the heart’s adaptation to this state of O\(_2\) deprivation. First, in LFI hearts, the indices of contractile function and MV\(_{O_2}\) were decreased, whereas cardiac efficiency was unchanged or increased (see Figs. 2 and 3). This probably results from a switch from fatty acid to carbohydrate utilization (23, 27, 32). Second, LFI hearts perfused with MCFA or LCFA maintained values similar to normoxic hearts for the total pool size of CAC intermediates and the tissue citrate-to-isocitrate concentration ratios (30–33 with MCFA and 70–90

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**Fig. 4.** Release rates of citrate (A and B) and succinate (C and D). Data are means ± SE for 3–5 experiments. Hearts were perfused as for Fig. 2. Release rates were determined from samples of effluent perfusates collected between 10 and 15 min under normoxia and between 80 and 90 min under LFI. Release rates are expressed in nanomoles per minute (A and C) relative to MV\(_{O_2}\) (nmol/μmol O\(_2\) consumed; B and D). \# \( P < 0.001 \), LFI vs. normoxia; \$ \( P < 0.001 \), \^ \( P < 0.05 \), LCFA vs. MCFA.
with LCFA). This indicates tight regulation of CAC enzymes under both normoxia and LFI. The site of this regulation differed between normoxia and LFI as indicated by substantial differences in the release and tissue levels of citrate and succinate (see Figs. 4 and 5). Expressed relative to MVO₂, the combined release of citrate and succinate was raised from 0.5% under normoxia to 10% under LFI (see Fig. 4). Because CAC pool size is unchanged or increased under LFI, CAC efflux must have been compensated by the entry of anaplerotic carbons at the level of pyruvate (5, 28, 29, 43) and/or α-KG (42). Observed differences in tissue levels of citrate and succinate under normoxia and LFI, which are in agreement with other studies (28–30), are consistent with the CAC operating in two spans: one from acetyl-CoA to α-KG, and the other from α-KG to oxaloacetate (31).

We observed a similar trend for the effects of LFI on the release and tissue levels of CAC intermediates for hearts perfused with octanoate and oleate (see Figs. 4 and 5). However, the magnitude of changes observed under both normoxia and LFI was greater for the MCFA group. These results are consistent with the fact that in contrast to LCFA, MCFA α-KG-oxidation is not regulated at the level of carnitine palmitoyl transferase 1 (39). The addition of NE under LFI only marginally affected most contractile, physiological,

![Fig. 5. Tissue levels of citric acid cycle (CAC) intermediates. Data are means ± SE for 3–5 experiments. Hearts were perfused as for Fig. 2. Magnifications of the scale (insets) present the concentrations of isocitrate and α-KG. #P < 0.001, **P < 0.01, *P < 0.05, LFI vs. normoxia; #P < 0.001, **P < 0.01, *P < 0.05, LCFA vs. MCFA. CIT, citrate; ICIT, isocitrate; SUC, succinate; FUM, fumarate; MAL, malate.](http://ajpheart.physiology.org/)

![Fig. 6. Fractional contribution (FC) of α-KG to citrate formation through the reversal of NADP⁺-ICDH. Data are means ± SE for 3–5 experiments. Hearts were perfused as for Fig. 2. Mass isotopomer distributions of α-KG and citrate (see Table 1) were introduced into Eq. 2. *P < 0.05, LFI vs. normoxia; **P < 0.05, LCFA vs. MCFA.](http://ajpheart.physiology.org/)

### Table 1. Molar percent enrichment in M₄ and M₅ isotopomers of tissue CAC intermediates

<table>
<thead>
<tr>
<th></th>
<th>Citrate</th>
<th>α-KG</th>
<th>Succinate</th>
<th>Fumarate</th>
<th>Malate</th>
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<tr>
<td></td>
<td>M₄</td>
<td>M₅</td>
<td>M₄</td>
<td>M₅</td>
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<tr>
<td>MCFA</td>
<td></td>
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<tr>
<td>Normoxia</td>
<td>0.20 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.31 ± 0.04</td>
<td>4.26 ± 0.41</td>
<td>1.54 ± 0.08</td>
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<tr>
<td>LFI/NE</td>
<td>0.63 ± 0.03*</td>
<td>0.50 ± 0.03*</td>
<td>0.66 ± 0.03*</td>
<td>7.08 ± 0.18</td>
<td>1.46 ± 0.04</td>
</tr>
<tr>
<td>LCFA</td>
<td>0.24 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.30 ± 0.07</td>
<td>3.21 ± 0.18</td>
<td>2.04 ± 0.31</td>
</tr>
<tr>
<td>Normoxia</td>
<td>0.96 ± 0.12§</td>
<td>0.41 ± 0.06</td>
<td>0.76 ± 0.12§</td>
<td>9.59 ± 1.48*</td>
<td>1.69 ± 0.28</td>
</tr>
<tr>
<td>LFI/NE</td>
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Values are means ± SE for 3 or 4 experiments. Hearts were perfused with 5.5 mM glucose, 8 nM insulin, 50 μM carnitine, 1 mM lactate, 0.2 mM pyruvate, 0.5 mM [U-¹⁵C]glutamate, and 0.2 mM octanoate (medium-chain fatty acids, MCFA) or 0.4 mM oleate (long-chain fatty acids, LCFA) for 30 min under normoxia or for 90 min under low-flow ischemia (LFI, 1 ml/min) in the presence of 1 μM norepinephrine (NE). Note that under all conditions tested, molar percent enrichment (MPE), values for M₁–M₃ for all citric acid cycle CAC intermediates were between 0.06 and 0.50%, and MPE values for M₆ for citrate were negligible. *P < 0.001, †P < 0.01, ‡P < 0.05, LFI vs. normoxia; §P < 0.01, LCFA vs. MCFA.
and metabolic parameters. Possibly, under this specific condition, the increase in calcium levels resulting from NE addition is counteracted by adaptive mechanisms such as the opening of sarcolemmal ATP-sensitive K⁺ channels, which decreases intracellular calcium levels and hence downregulates contractile activity (9). Thus based on the aforementioned results, we conclude that similar to the pig heart perfused in vivo (28), LFI in the rat heart perfused ex vivo mimics to some extent a state of hibernation (34), which is independent of the nature of the fatty acid added.

The results of this study demonstrate a small flux through the reversal of NADP⁺-ICDH in both normoxic and LFI rat hearts perfused in the presence of MCFA or LCFA, representing 3–7% of flux through citrate synthase (see Fig. 6). This small reverse flux in perfused rat hearts contrasts with that measured in perfused rat livers (∼45%; see Ref. 7). This may be explained by the fact that in the heart, NADP⁺-ICDH is almost exclusively located in mitochondria, whereas in the liver, the cytosolic isoform represents 85% of total NADP⁺-ICDH activity (19) and participates in fatty acid synthesis from glutamate (13). Nevertheless, based on the following reasoning, we concluded low reverse-flux values measured in perfused hearts reflected net flux through reversal of NADP⁺-ICDH rather than simple isotopic equilibration. The values of MPE M5 for tissue α-KG were more than 10-fold greater than those of citrate (Table 1). Rapid isotopic equilibration between these two metabolites would result in similar MPE values. Such a situation is observed for malate and fumarate where rapid interconversion is catalyzed by fumarase (Table 1). Unfortunately, we were unable to determine with precision the MPE M5 value of tissue isocitrate in all tissue samples because of its low concentration. Furthermore, the small peak of isocitrate elutes from the GC column very shortly after the much larger peak of citrate. However, the analysis of some tissue samples revealed that the MPE of citrate reflected that of isocitrate (data not shown), which supports a reversible interconversion by aconitase.

In hearts perfused in the presence of octanoate with physiological concentrations of glucose, insulin, lactate, pyruvate, and glutamate, the magnitude of the reverse NADP⁺-ICDH flux, expressed relative to the flux through citrate synthase, was increased by LFI as predicted by Sazanov and Jackson (35) (see Fig. 6, left). The reverse NADP⁺-ICDH flux was, however, not affected by LFI when octanoate was replaced with the physiological LCFA oleate complexed to fatty acid-poor albumin (see Fig. 6, right). One possible explanation for the differential effects of MCFA and LCFA on the reverse NADP⁺-ICDH flux could be the inhibition of H⁺-transhydrogenases by palmitoyl-CoA (12). In the proposed regulatory substrate cycle between isocitrate and α-KG, H⁺-transhydrogenases, whose activity occurs in the heart (36), generates NADPH for NADP⁺-ICDH (see Fig. 1). Although palmitate was not supplied exogenously to the heart, it could be present as endogenous free fatty acids in our albumin preparation. Another possible explanation is that the metabolism of MCFA and LCFA differently affects the concentrations of effectors of enzymes involved in the metabolism of citrate, isocitrate, and α-KG (18). In rat hearts perfused under normoxia, state 4 respiration (high NADH/NAD⁺ and ATP/Pi limited) prevails with octanoate, whereas state 3 respiration (NADH limited) prevails with the LCFA palmitate (17). A high rate of NADH production (especially under conditions of limited O₂ supply) could shift the redox state of the NAD⁺ pool via H⁺-transhydrogenases, and this in turn would lead to a change in flux through the reversal of NADP⁺-ICDH. Additional investigations are, however, needed to verify and substantiate these explanations. The effects of MCFA could be of clinical relevance because substitution of LCFA with MCFA in the diet prevents the development of cardiac hypertrophy in spontaneously hypertensive rats (11).

Our enzyme-activity data substantiate the notion of high NADP⁺-ICDH activity in the heart (Fig. 7). The
measured activities varied in the following order: NADP⁺-ICDH > citrate synthase > aconitase > NAD⁺-ICDH. The decreased activity of tissue aconitase by LFI (Fig. 7) is consistent with inactivation of this enzyme by free radicals (25) whose production is increased in LFI hearts (1). Because of the low activity of tissue NAD⁺-ICDH compared to that of other enzymes, one may conclude that oxidation of isocitrate by NADP⁺-ICDH would be needed to maintain normal CAC flux. However, assuming that 1 g wet weight contains 0.2 g of protein, maximal NAD⁺-ICDH activity is estimated to be 4 μmol/min × g wet weight. This activity is threefold greater than CAC flux values, which are estimated [using the MVO₂ value (29)], to be ~1.5 and 0.1 μmol/min × g wet weight under normoxia and LFI/NE, respectively.

The participation of NADP⁺-ICDH in mitochondrial isocitrate oxidation in the heart is a controversial subject (6, 12, 33). Taken together, our 13C data demonstrate a net substrate flux from α-KG to isocitrate through the reversal of NADP⁺-ICDH as proposed by Sazanov and Jackson’s hypothesis (35). However, our data do not provide evidence for net substrate flux through the following reactions: glutamate → α-KG → isocitrate → citrate → mitochondrial citrate efflux, as proposed by Thomassen et al. (42). Note that mitochondrial citrate efflux occurs with a proton in exchange for malate and hence would affect the electrochemical gradient. The presence of M5 isotoptomers of citrate in heart tissue as well as in the effluent (not shown) is more likely to be explained by isotopic equilibration between citrate and isocitrate catalyzed by aconitase. The concentration ratio of citrate to isocitrate measured in normoxic and LFI hearts perfused with LCFA (70–90) or MCFA (30–33) is greater than that found at equilibrium for the aconitase reaction in vitro (15–20; see Ref. 19). This would be consistent with isocitrate being pulled through the NAD⁺-ICDH reaction. Using modeling data on [14C]bicarbonate incorporation into citrate in perfused rat hearts, Nuutinen et al. (26) concluded that citrate was being labeled through the reversal of NAD⁺-ICDH, but the resulting net substrate flux was in the forward reaction. The 13C protocol that will quantitate the partitioning of isocitrate formed by the reversal of NAD⁺-ICDH between oxidation by NAD⁺-ICDH and citrate synthesis/efflux remains to be identified.

A reverse flux through NADP⁺-ICDH is inconsistent with an antioxidant role, where the enzyme would supply NADPH for regeneration of reduced glutathione by glutathione reductase. Such a role was proposed by Jo et al. (14) based on evidence obtained from NIH3T3 cells. However, the situation differs in the heart, where the mitochondrial NADPH/NADP⁺ ratio is high (>50; Refs. 19, 41) compared with <1 in NIH3T3 cells. Because NADPH potentiates the inhibition of NAD⁺-ICDH by NADH, reverse NADP⁺-ICDH activity could be crucial to prevent a rise in the NADPH/NADP⁺ ratio, especially under conditions where NADH accumulates (for example, under state 4 respiration). It remains to be clarified as to whether cardiac mitochondrial NADP⁺-ICDH could be forced to participate in NADPH generation at least under some conditions. For example, it could occur when the supply of NADH limits the activity of H⁺-transhydrogenases and hence NADPH generation (15). Such a situation may prevail if oxidative stress is increased in normoxic hearts, especially under state 3 respiration when NADH supply also limits the mitochondrial respiratory chain. However, future studies should investigate the possibility of a modulation of the NADP⁺-ICDH by oxidative stress.

In summary, this study shows that 13C substrates and mass isotopomer analysis provide a dynamic picture of substrate fluxes through NADP⁺-ICDH in the rat heart perfused under normoxia or LFI. Our 13C data show that this reaction operates in the reverse direction of the CAC. A reverse NADP⁺-ICDH flux coupled with the H⁺-transhydrogenase activities may be crucial to the fine regulation of CAC activity and hence of energy production for contraction of the normoxic and O₂-deprived heart.

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