Mitochondrial transporter responsiveness and metabolic flux homeostasis in postischemic hearts

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O’Donnell, J. Michael, Lawrence T. White, and E. Douglas Lewandowski. Mitochondrial transporter responsiveness and metabolic flux homeostasis in postischemic hearts. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H866–H873, 1999.—The transport of metabolites between mitochondria and cytosol via the α-ketoglutarate-malate carrier serves to balance flux between the two spans of the tricarboxylic acid (TCA) cycle but is reduced in stunned myocardium. To examine the mechanism for reduced transporter activity, we followed the postischemic response of metabolite influx/efflux from mitochondria to stimulation of the malate-aspartate (MA) shuttle. Isolated rabbit hearts were either perfused with 2.5 mM [2-13C]acetate (n = 7) or similarly reperfused (n = 5) after 10-min ischemia. In other hearts, the MA shuttle was stimulated with a high cytosolic redox state (NADH) induced by 2.5 mM lactate in normal (n = 6) or reperfused hearts (n = 7). In normal hearts, the MA shuttle response accelerated transport from 8.3 ± 3.4 to 16.2 ± 5.0 µmol·min⁻¹·g dry wt⁻¹. Although transport was reduced in stunned hearts, the MA shuttle was responsive to cytosolic NADH load, increasing transport from 3.4 ± 1.0 to 9.8 ± 3.7 µmol·min⁻¹·g dry wt⁻¹. Therefore, metabolite exchange remains intact in stunned myocardium but responds to changes in TCA cycle flux regulation.

reperfusion; redox potential; malate-aspartate shuttle; tricarboxylic acid cycle

Respiratory inefficiency in stunned myocardium is characterized by normal levels of oxygen consumption despite reduced contractile function (14). Consistent with normal oxygen use and apparent inefficiency in oxygen consumption (5, 27), tricarboxylic acid (TCA) cycle flux (V TCA) has also been found to be normal in postischemic hearts (16, 17, 33). Despite normal rates, isolated hearts oxidizing 13C-enriched substrates indicate that carbon turnover within the NMR-detectable glutamate pool is slower in postischemic hearts relative to normal hearts because of reduced metabolite transport between the mitochondria and cytosol across the α-ketoglutarate (α-KG)-malate transporter (16, 33). Although clearly representing a change in the balance between oxidation in the TCA cycle and metabolite exchange between mitochondria and cytosol, the mechanism for this reduced exchange has yet to be elucidated as either a fundamental defect in transporter protein function or a consequence of competition between the oxidative rate within the TCA cycle and mitochondrial influx/efflux of carbon units. This study examines these possibilities as the reason for reduced metabolite exchange via the α-KG-malate exchanger transporter and in the process examines a mechanism for maintenance of oxidative flux in response to altered TCA cycle enzyme activity in stunned myocardium.

The balance between V TCA and the exchange of mitochondrial and cytosolic metabolites is regulated by the coordinated activity of the mitochondrial matrix enzyme, α-KG dehydrogenase, and the α-KG-malate transporter of the mitochondrial membrane. The oxidative reaction catalyzed by α-KG dehydrogenase represents a rate-limiting step within the TCA cycle by balancing flux through two spans of the TCA cycle (23). The dehydrogenase also competes with the reversible α-KG-malate transporter for exchange of carbon units between subcellular compartments (12, 13, 22). The reversible α-KG-malate transporter functions independently or, working in tandem with the unidirectional glutamate-aspartate exchanger, forms the malate-aspartate shuttle (4, 25, 31). Our laboratory has previously (37) demonstrated that the rate of glutamate labeling in normal hearts, via 13C enrichment of TCA cycle intermediates, is responsive to an increase in flux through the α-KG-malate transporter during recruitment of net forward malate-aspartate shuttle activity. Whether reduced flux through the α-KG-malate transporter of stunned myocardium is also responsive to such stimulation has yet to be determined and would aid in elucidating the mechanism for this reduced transport and the altered balance between oxidative flux and metabolite influx/efflux across the mitochondrial membrane.

Therefore, we examined the mechanism for reduced metabolite exchange between the mitochondria and cytosol in postischemic hearts. The findings confirm a shift in the balance between oxidative rate and mitochondrial/cytosolic interactions, suggesting a homeostatic mechanism for preserving V TCA during pathophysiological changes in mitochondrial dehydrogenase activity.

Materials and Methods

Isolated heart model. Hearts were excised from Dutch Belted rabbits (500 g) that were given an intraperitoneal injection of heparin (20 U) and anesthetized with ketamine (500 U) and Telozol (200 U). Before either ischemia or perfusion with labeled substrate, isolated hearts were retrograde-perfused at 100-cm hydrostatic pressure, using a modified Krebs-Henseleit buffer solution containing (in mM) 116 NaCl, 4 KCl, 1.5 CaCl₂, 1.2 MgSO₄, 1.2 NaHPO₄, 25 NaHCO₃, and 5 glucose, and oxygenated with 95% O₂-5% CO₂. The temperature of the buffer entering the heart was maintained at 37°C. A latex balloon containing water was inserted into the left ventricle, and end-diastolic pressure was set at 5–10 mmHg. After 10 min of ischemia, hearts were reperfused with substrate-labeled buffer for 40 min. Hearts were then freeze-clamped and the left ventricle and septum were cut into small pieces, which were subsequently extracted and analyzed.

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mmHg. The balloon was connected to a pressure transducer to monitor left ventricular developed pressure (LVDP) and heart rate (HR). At the end of the experiment, myocardial oxygen consumption (MV\dot{O}_2) was calculated from the difference in O_2 content of the perfusion medium in the supply line and coronary effluent (21). Coronary effluent was collected without exposure to air by inserting a catheter into the pulmonary artery and withdrawing perfusate into a syringe. Coronary flow was established by collecting the fluid dripping from the pulmonary artery and heart in a graduated cylinder for 1 min.

Experimental protocol. All hearts were initially perfused with modified Krebs-Henseleit buffer containing 5 mM unlabelled glucose. Coronary effluent was discarded, and the hearts were given 10 min to stabilize rate-pressure product (RPP = HR \times LVDP). \textsuperscript{31}P NMR spectra were acquired to establish myocardial viability based on high-energy phosphate content (phosphocreatine, ATP). Isolated hearts were subdivided into four experimental groups perfused with 2.5 mM acetate, with or without 2.5 mM lactate: acetate control group (n = 7), acetate + lactate control group (n = 6), acetate reperfusion group (after 10 min ischemia, n = 5), and acetate + lactate reperfusion group (after 10 min ischemia, n = 7). Exogenous lactate was added to augment cytosolic redox state (NADH/NAD\textsuperscript{+}) (24, 26, 37). In reperfused hearts, global ischemia was induced by stopping the perfusion supply and clamping the aortic line. Immediately after glucose perfusion or the ischemic period, the buffer was switched to the unenriched substrates listed for each group. All hearts were perfused for 10 min, and background \textsuperscript{13}C NMR spectra were acquired. The buffer substrate was then switched from unlabeled acetate to a recirculated reservoir of 2.5 mM 2,\textsuperscript{13}C-enriched acetate (Isotec, Miamisburg, OH). Subsequent sequential \textsuperscript{13}C NMR spectra (1.3- or 2.6-min blocks) were acquired for 30–40 min. MV\dot{O}_2 measurements were taken after the heart was removed from the magnet. The heart was then freeze-clamped and prepared for biochemical assays and high-resolution \textsuperscript{13}C NMR analysis.

MV\dot{O}_2 was also measured from additional hearts during the course of 40 min reperfusion with either 2.5 mM acetate (n = 3) or 2.5 mM acetate with 2.5 mM lactate (n = 3). Coronary effluent was collected from a catheter in the pulmonary artery at 5, 10, 20, 30, and 40 min of reperfusion.

NMR measurements. NMR parameters required for acquisition of \textsuperscript{31}P and \textsuperscript{13}C NMR spectra are as previously reported (16, 22, 38). Briefly, perfused hearts were positioned in a 20-mm broadband probe in the 9.4-T/89-mm vertical-bore superconducting NMR magnet. Magnetic field homogeneity was optimized by shimming to a proton linewidth of 15–30 Hz. A \textsuperscript{31}P spectrum of heart was acquired to confirm normal energetic status based on phosphocreatine, α-, β-, and γ-ATP content. Carbon spectra were then acquired at 100 MHz with bievel broadband decoupling and subtraction of endogenous signal from naturally abundant \textsuperscript{13}C (16, 22, 38). In addition, in vitro \textsuperscript{13}C NMR high-resolution spectra were acquired from perchloric acid extracts of myocardium in a 5-mm probe to determine the fraction of 2,\textsuperscript{13}C-labeled acetyl-CoA.

Tissue chemistry. Perchloric acid extracts were obtained from ventricular muscle as previously described. Glutamate, α-KG, citrate, and aspartate concentrations were determined from ultraviolet spectrophotometric and fluorometric techniques (3, 34).

Kinetic model. For purposes of data analysis, a simple kinetic model of nine differential equations, describing known biochemistry and the isotopic enrichment of key metabolic pools, was applied as previously described in great detail (16, 22, 36, 37). The model has been previously used under appropriate experimental conditions to examine both V_{TCA} and metabolite transport in normal and postischemic hearts (16, 22, 37). V_{TCA} and the interconversion rate between cytosolic glutamate and mitochondrial α-KG (F_1) were determined by nonlinear least-squares fitting of the model to \textsuperscript{13}C NMR data of the second and fourth carbons of glutamate (C-2 and C-4) enrichment.

Statistical analysis. Data set comparisons were performed with Student's unpaired two-tailed t-test. Differences in mean values were considered statistically significant at a probability level of <5% (P < 0.05).

RESULTS

Contractile function and MV\dot{O}_2. All hearts were perfused with glucose before the start of each protocol under either control conditions or ischemia-reperfusion. In this manner the ischemic insult was standardized for substrate availability, and we were able to focus on a comparison of metabolic flux during oxidation of acetate in normal or postischemic hearts. All isolated heart preparations were essentially the same before an experimental fate was delineated. Hearts were randomly chosen for each of the experimental groups, and no difference in mechanical work was evident between eventual groups of hearts before the start of any of the protocols. During the initial setup period of perfusion with glucose, before the initiation of any experimental protocol, the mean RPP of hearts entering the control protocol and the mean RPP for hearts before the ischemic protocol were 17,347 ± 2,597 and 17,031 ± 2,313 mmHg·beats·min\textsuperscript{-1}, respectively.

After glucose perfusion, normal hearts perfused with [2-\textsuperscript{13}C]acetate alone displayed an MV\dot{O}_2 of 21 ± 6 µmol O_2·min\textsuperscript{-1}·g dry wt\textsuperscript{-1}. Normal hearts perfused with [2-\textsuperscript{13}C]acetate and unlabeled lactate displayed an MV\dot{O}_2 of 23 ± 7 µmol O_2·min\textsuperscript{-1}·g dry wt\textsuperscript{-1}. MV\dot{O}_2 was not different between normal and postischemic hearts (17 ± 3 µmol O_2·min\textsuperscript{-1}·g dry wt\textsuperscript{-1} for reperfused acetate hearts and 19 ± 11 µmol O_2·min\textsuperscript{-1}·g dry wt\textsuperscript{-1} for reperfused acetate + lactate hearts).

MV\dot{O}_2 measurements taken throughout the 40-min reperfusion period did not differ between groups. Hearts reperfused outside the magnet with acetate (n = 3) reveal an oxygen consumption of 22 ± 6 µmol O_2·min\textsuperscript{-1}·g dry wt\textsuperscript{-1} at 5 min reperfusion vs. 17 ± 7 µmol O_2·min\textsuperscript{-1}·g dry wt\textsuperscript{-1} at 40 min. MV\dot{O}_2 of hearts reperfused with acetate supplemented with lactate (n = 3) was 31 ± 9 µmol O_2·min\textsuperscript{-1}·g dry wt\textsuperscript{-1} at 5 min reperfusion vs. 21 ± 6 µmol O_2·min\textsuperscript{-1}·g dry wt\textsuperscript{-1} at 40 min. The drop in MV\dot{O}_2 observed over time is not statistically significant, and the final MV\dot{O}_2 measurement is similar to hearts perfused in the magnet.

Postischemic hearts showed contractile dysfunction in comparison to the corresponding normal group that was consistent with earlier observations (16). Figure 1 displays RPP over the course of perfusion in normal and postischemic hearts receiving acetate alone or acetate supplemented with lactate. RPP was depressed an average of 45% (P < 0.05) in both groups. Whereas RPP was reduced in postischemic hearts, the major component of contractile dysfunction was depressed pressure development and not changes in HR. LVDP
Metabolite content and isotopic enrichment. Steady state metabolite contents are listed in Table 1 for all experimental groups. Metabolite contents were similar to those of previously published results (16), showing the expected drop in postischemic glutamate content to 40% (P < 0.005) lower than values in normal hearts.

The fractional isotopic enrichment of acetyl-CoA (Fc) and the ratio of anaplerotic flux to citrate synthase activity (y) were similar among all groups. For acetate controls, values were Fc = 90 ± 6% and y = 8 ± 5%; values in the postischemic hearts supplied acetate were Fc = 92 ± 9% and y = 10 ± 6%; values from hearts oxidizing acetate in the presence of lactate were Fc = 91 ± 8% and y = 10 ± 5%; and values from heart reperfusion with acetate and lactate were Fc = 90 ± 9% and y = 14 ± 10.

13C NMR spectroscopy, isotope kinetics, and metabolic flux. A representative sequential 13C NMR spectrum acquired from normal hearts oxidizing [2-13C]acetate is shown in Fig. 2. Similar spectra (not shown) were acquired for normal and reperfused hearts provided acetate supplemented with lactate. Figure 3 graphically displays the time course of 13C enrichment of glutamate (means ± SD) at C-2 and C-4 from spectra of normal and reperfused acetate hearts. The time course of 13C enrichment of glutamate at C-2 and C-4 from spectra of normal and reperfused acetate + lactate hearts is shown in Fig. 4. Note that the flux parameters shown in Fig. 5 are obtained from data combining such isotope enrichment curves with the corresponding metabolite pool sizes. The least-squares fitting of the kinetic model to the data is also shown in Figs. 3 and 4. The correlation coefficient between the data and the fit was 0.98. Output from the model provided V TCA and F 1.

Figure 5 displays V TCA and F 1 for each of the experimental groups. In normally perfused hearts (Fig. 5A) V TCA in the presence of acetate + lactate was slightly lower than that with acetate alone. This is consistent with a slightly lower steady-state RPP and a potential increase in NADH oxidation. F 1 was significantly higher in the acetate + lactate group (P < 0.05) compared with the acetate normals in response to the effects of elevated cytosolic redox state on recruiting malate-aspartate shuttle activity. This is consistent with earlier work in normal hearts (37).

In postischemic hearts (Fig. 5B) V TCA was not significantly different from that in corresponding normals of Fig. 5A, whereas F 1 was significantly decreased (P <
0.05) as expected (16). The postischemic hearts per-
fused with acetate supplemented with lactate showed
raised F1 relative to postischemic hearts without
lactate (P, 0.05). The increase in F1 was comparable to
that of normal hearts oxidizing acetate. This induced
increase in metabolite exchange during myocardial
stunning indicates that the α-KG-malate transporter
remained responsive to increased cytosolic redox state.

DISCUSSION

This study explores the regulation of mitochondrial
oxidative function in intact, stunned hearts with 13C
NMR at an investigative level previously restricted to
isolated mitochondria (11–13, 23, 25, 31). Sequential
13C NMR spectra were obtained from intact hearts
under conditions of normal and postischemic perfusion
with [2-13C]acetate, with or without elevated cytosolic
redox potential. TCA cycle rate and the rate of
α-KG efflux from the mitochondria for interconversion with
cytosolic glutamate (α-KG-malate transport rate) were
determined by fitting a kinetic model to the dynamic
13C enrichment data of glutamate (16, 22, 37, 38). As
previously reported, net V_{TCA} was unchanged between
controls and reperfused hearts perfused with acetate,
whereas α-KG-malate transport rate was significantly
reduced in the postischemic hearts perfused with buffer
containing acetate (16).

In this study, increasing cytosolic redox state with
addition of lactate caused α-KG-malate exchange to
increase in both normal and reperfused hearts. Despite
countering of the reduced rate of α-KG-malate ex-
change in stunned hearts to demonstrate that the
transporters remained responsive to cytosolic redox
state in the stunned heart, contractility remained
depressed during reperfusion. Consequently, we were
able to demonstrate that despite reduced α-KG-malate
transporter activity in stunned myocardium, the trans-
porter, as part of the malate-aspartate shuttle, remains
responsive to redox state changes. Thus the results
show that the reduced exchange of metabolites across
the mitochondrial membrane is not caused by dysfunc-
tion of the α-KG-malate exchange protein but rather
indicates a change in the balance between α-KG trans-
port and α-KG oxidation, as described above. Because
transporter function appears to be intact, the cause for
the reduced metabolite exchange rates across the mito-
chondrial membrane of the stunned myocardium must
be a response to altered rates of oxidation at the α-KG
dehydrogenase reaction. This finding indicates a meta-
bolic component of stunning at the level of mitochon-
drial dehydrogenase activity, affecting metabolite ex-
change across the mitochondrial membrane. The
mechanisms of metabolic flux homeostasis that account
for these findings are discussed below.

The regulatory enzymes that control the rate of V_{TCA}
in hearts oxidizing acetate have been discussed previ-
ously by Randle et al. (23). They described two separate
spans of the TCA cycle that are regulated by different
rate-limiting enzymes: acetyl-CoA to α-KG, which is
controlled by citrate synthase; and α-KG to oxaloac-
etate, which is controlled by α-KG dehydrogenase. At
steady state, metabolite concentrations attain stability
as the rates of the two cycle spans become equal. One
mechanism for attaining equilibrium among the TCA
cycle intermediate pools and maintaining coordinated
flux through the two spans is the competition between
the oxidation of $\alpha$-KG and the transport of $\alpha$-KG between the mitochondria and cytosol by the reversible $\alpha$-KG-malate exchanger (12, 13, 22). Thus this competition for $\alpha$-KG between the $\alpha$-KG dehydrogenase and the reversible $\alpha$-KG-malate transporter serves as a balance point for VTCA homeostasis.

The balance between $V_{TCA}$ and the exchange of metabolite between the mitochondria and cytosol is regulated by the mitochondrial redox state (4, 35), intramitochondrial calcium and hydrogen ion levels (6, 9), and substrate availability (23). Although the $\alpha$-KG dehydrogenase is sensitive to all these factors, the pH-independent $\alpha$-KG-malate transporter is sensitive to substrate availability and, indirectly, to cytosolic redox state (NADH/NAD$^+$) as part of the malate-aspartate shuttle. Whereas it is clear from our earlier work that this $\alpha$-KG-malate transport rate is reduced in postischemic heart (16), it is not clear whether the transporter is dysfunctional or simply responds to regulation. The results of the present study demonstrate that the transporter remains responsive in stunned myocardium and does not limit the availability of metabolite to the mitochondria for the oxidative processes of the TCA cycle and respiratory chain.

The $\alpha$-KG-malate transporter also functions as part of the malate-aspartate shuttle. The activity of the shuttle is key to coordinating exchange of metabolites between the mitochondria and cytosol and transferring reducing equivalents from the cytosol into the mitochondria. Net forward flux through the malate-aspartate shuttle involves the coordinated activity of the reversible $\alpha$-KG-malate exchanger and the unidirectional glutamate-aspartate exchanger.

The exchange of labeled $\alpha$-KG from the mitochondria with the cytosolic glutamate pool does not require both transporters of the malate-aspartate transporter. Instead, the reversible $\alpha$-KG-malate exchanger need only be available to enable the interconversion of labeled $\alpha$-KG with cytosolic glutamate. This was evident in our earlier work in which the observed efflux of labeled $\alpha$-KG from the mitochondria was delayed in the postischemic hearts provided [2-13C]acetate (16). In that study, supplying postischemic hearts with both acetate...
and labeled glucose produced negligible glycolytic activity for cytosolic NADH production, thus indicating very little involvement of the malate-aspartate shuttle. In the present study, increasing cytosolic redox state with lactate induced net forward flux through this shuttle, as demonstrated in a previously published report (37). For the hearts in the current study that were oxidizing acetate, the TCA cycle was the primary source of reducing equivalents that entered the respiratory chain (3). However, this contribution may be less important in hearts oxidizing acetate in the presence of lactate, where the malate-aspartate shuttle may become a major source of reducing equivalents (10). The net forward flux through the shuttle has been shown to be essential for oxidative energy production in postischemic hearts (15). This finding is important because it confirms that the observed F1 value, for α-KG efflux/influx across the mitochondrial membrane under the condition of acetate as sole substrate, represents a basal level of exchange across the α-KG-malate carrier and not malate-aspartate shuttle activity.

In hearts oxidizing acetate in the presence of lactate to increase cytosolic reducing equivalents, the TCA cycle accounted for 62% of the oxygen consumed in both normal and reperfused hearts. Within experimental error, the remainder of oxygen use can be attributed primarily to the recruitment of net forward aspartate-malate shuttle flux. In this case, the shuttle served as a significant source of reducing equivalents entering the mitochondria during the lactate-induced increase in cytosolic redox state.

Results from reperfusion experiments reveal that the α-KG-malate transporter is responsive to stimulation in stunned myocardium. Whereas V TCA is not statistically different from that in respective nonischemic hearts, metabolite exchange is increased nearly threefold in hearts provided acetate + lactate compared with hearts supplied with acetaldehyde alone (9.8 ± 3.7 vs. 3.4 ± 1.0 μmol·min⁻¹·g dry wt⁻¹, respectively). This result suggests that there is not a fundamental defect in transporter protein function. The reduced exchange rate observed in reperfused hearts provided acetate alone is more likely to be a result from substrate competition between the transporter and the α-KG dehydrogenase enzyme of the mitochondria.

This hypothesis is supported by a recent finding from our laboratory (22). In the recent study, we examined the effects of increasing cytosolic and intramitochondrial Ca2⁺ and H⁺ content on α-KG dehydrogenase flux and metabolite exchange across the mitochondrial membrane in both intact hearts and isolated mitochondria. The results indicated that elevated Ca2⁺ and H⁺ content increased α-KG oxidation and reduced α-KG efflux from the mitochondria. This apparent competition for substrate by the α-KG transporter and α-KG dehydrogenase is attributed to their relative Michaelis constant (K m) values (12, 28–30). Earlier work reported that the α-KG-malate transporter of the mitochondrial membrane has a relative K m of 1.5 mM for α-KG on the matrix side of the carrier (28), whereas the K m of α-KG dehydrogenase for α-KG was reported as 0.67 mM (12). In our recent study (22) with isolated mitochondria incubated at low pH, the apparent K m of the α-KG dehydrogenase decreased by 50% relative to mitochondria incubated at normal pH. This makes both oxidation and efflux very sensitive to regulation by the α-KG concentration in the mitochondrial matrix.

Whereas pH is reduced during ischemia, pH recovers immediately after reperfusion (1, 15) and is not likely to stimulate α-KG dehydrogenase activity. However, mitochondrial dehydrogenase activity is also sensitive to Ca2⁺ content (6, 8, 9, 18, 32). If mitochondrial Ca2⁺ overload persists after reperfusion (2, 7, 10, 19, 20), dehydrogenase activity can be stimulated. Thus a decrease in mitochondrial α-KG content (16) paralleled...
with an increase in α-KG dehydrogenase activity, caused by elevated Ca²⁺, shifts the efflux of α-KG from the mitochondria to oxidation by the dehydrogenase in postischemic myocardium. This altered balance between VTCA and metabolite exchange observed in stunned hearts suggests a homeostatic mechanism for preserving VTCA and metabolite exchange between subcellular compartments in intact postischemic rabbit hearts. Circ. Res. 81: 165–179, 1995.


