Quantitative assessment of anaplerosis from propionate in pig heart in vivo

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Martini, Wenjun Z., William C. Stanley, Hazel Huang, Christine Des Rosiers, Charles L. Hoppel, and Henri Brunengraber. Quantitative assessment of anaplerosis from propionate in pig heart in vivo. Am J Physiol Endocrinol Metab 284: E351–E356, 2003.—Normal cardiac metabolism requires continuous replenishment (anaplerosis) of catalytic intermediates of the citric acid cycle. Little is known about the quantitative aspects of propionate as a substrate of in vivo anaplerosis; therefore, we measured the rate of propionate entry into the citric acid cycle in hearts of anesthetized pigs. [U-13C3]propionate (0.25 mM) was infused in a coronary artery branch for 1 h via an extracorporeal perfusion circuit, and cardiac biopsies were analyzed for the mass isotopomer distribution of citric acid cycle intermediates. Infusion of propionate did not affect myocardial oxygen consumption, heart rate, or contractile function. In the infused territory, propionate infusion did not affect uptake of glucose and lactate but decreased free fatty acid uptake by one-half (P < 0.05). Propionate extraction and uptake were 57.4 ± 3.3% and 0.078 ± 0.009 μmol·min⁻¹·g⁻¹. Anaplerosis from propionate, calculated from the mass isotopomer distribution of succinate, accounted for 8.9 ± 1.3% of the citric acid cycle flux. Propionylcarnitine extraction accounted for only 0.033 ± 0.002% of propionate uptake. Methylcitrate did not accumulate. Thus administration of a low concentration of propionate appears to be a convenient and safe way to boost anaplerosis in the heart.

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study, we measured the rate of propionate entry into the CAC in hearts of anesthetized pigs using an infusion of [U-\(^{13}\text{C}_3\)]propionate (0.25 mM) in the left anterior descending (LAD) artery. The contribution of anaplerosis from propionate was quantified by mass isotopomer distribution analysis of heart succinate using gas chromatography/mass spectrometry (GC-MS). To relate the anaplerotic effects to in vivo cardiac function, we also measured functional parameters, such as left ventricular pressure (LVP), myocardial oxygen consumption (MV\(\text{O}_2\)), and regional segmental shortening.

**METHODS**

**Animal model.** An in vivo pig model was used to administer [U-\(^{13}\text{C}_3\)]propionate (sodium salt) in the LAD (19, 20). Six overnight fasted pigs (35.4 ± 1.1 kg) were sedated with 6 mg/kg im Telazol, anesthetized by mask with isoflurane (5%), intubated via a tracheotomy, and ventilated with pure O\(_2\) to maintain blood gases in the normal range (P\(_{\text{O}_2}\) > 100 mmHg, P\(_{\text{CO}_2}\) 35–45 mmHg, and pH 7.35–7.45). Anesthesia was maintained with isoflurane (0.75–1.5%). The right femoral artery and vein were cannulated for extracorporeal bypass and venous infusion of heparin, respectively. Heparin was infused to prevent clotting (200 U/kg bolus, followed by 100 U·kg\(^{-1}\)·h\(^{-1}\) iv). The heart was exposed via a midline sternotomy with left-side rib resection. An extracorporeal perfusion circuit was set up between the femoral artery and the LAD coronary, and its flow rate was controlled via a roller pump (19, 20). LAD arterial blood samples were obtained from a constant-flow (10 ml/min) withdrawal loop from the perfusion circuit so that blood sampling would not disturb coronary artery blood flow. A 7-Fr Millar Mikrotip dual-transducer catheter was used to assess LVP. A polyethylene cannula was placed in the anterior interventricular vein and was used to collect venous blood samples from the perfused territory. Regional segment length was measured in the anterior free wall (LAD bed) using sonomicroscopy, as previously described (Triton Technologies, San Diego, CA; see Ref. 4). The crystal pair was positioned at approximately midwall depth. The LAD flow was adjusted to give an interventricular venous Hb saturation of 35–40%. Heart rate, LVP, and contractility were continuously monitored and recorded through the experiment using an online data acquisition system (BioFac).

**Study protocol.** After the surgical procedure, a physiologically steady state was achieved within 20–30 min, as indicated by constant blood gas measurements, heart rate, and LVP. Next, basal blood samples were taken from the artery and the anterior interventricular vein. The study started with a constant infusion of unlabeled sodium propionate for 20 min at a rate that achieved an increase of 0.25 mM over the baseline concentration. At 20 min, the infusion of unlabeled propionate was switched to sodium [U-\(^{13}\text{C}_3\)]propionate for 60 min. The total dose of sodium administered over the 80-min infusion was 0.51 ± 0.06 meq. This amount of sodium infused in the extracellular fluid of the pig (20% of 35 kg = 7.0 liters) would increase the basal sodium concentration by a negligible and undetectable 0.07 meq/l. Blood samples were taken simultaneously from the coronary artery perfusion line and the coronary vein at 20, 25, 40, 60, and 80 min. At the end of [U-\(^{13}\text{C}_3\)]propionate infusion, large punch biopsies (~3 g) were taken from the LAD bed and from the untreated posterior left ventricular free wall. The biopsies were immediately freeze-clamped using aluminum blocks precooled in liquid nitrogen. The heart was excised, and black ink was injected down the right and left main coronary arteries to identify the LAD-infused tissue bed (25.3 ± 3.1 g; see Ref. 20).

**Analytical methods.** Arterial and venous pH, P\(_{\text{CO}_2}\), and P\(_{\text{O}_2}\) were determined on a blood gas analyzer, and Hb concentration and saturation were determined on a hemoximeter. The concentrations of plasma glucose, lactate, and free fatty acids were determined using standard spectrophotometric enzymatic assays. Plasma propionylcarnitine concentrations and M\(_3\) enrichments were determined by a modification of the HPLC acylcarnitine method (17) coupled to mass spectrometry (P. E. Minkler and C. L. Hoppel, unpublished observation).

Plasma samples were spiked with \(^{2}\text{H}_3\)propionate internal standard and treated to prepare the pentfluorobenzyl esters before ammonia negative chemical ionization GC-MS (8). The concentration and M\(_3\) enrichment of propionate were calculated from the signals at mass-to-charge ratio (m/z) 73–78. Heart tissue (250 mg) was powdered and extracted by 2 ml 8% sulfosalicylic acid and 0.1 ml of 5 M hydroxylamine hydrochloride. After centrifugation, the supernatant was brought to pH 8 with 5% KOH and incubated at 65°C for 1 h to convert ketoacids to hydroxamates. The solution was then acidified to pH 1–2 with 6 N HCl, saturated with NaCl, and extracted three times with 9 ml ethyl acetate. The pooled extract was dried under N\(_2\). The residues were derivatized with N-methyl-N-(\(t\)-butyldimethylsilyl)trifluoroacetamide at 85°C for 1 h. The mass isotopomer distributions of CAC intermediates were measured by GC-MS (7). The ion signals were monitored at m/z 289–293 for succinate, 287–291 for fumarate, 419–423 for malate, 432–436 for oxaloacetate, 459–465 for citrate, and 446–451 for \(\alpha\)-ketoglutarate. In separate samples, the concentrations of succinate, fumarate, and citrate in heart tissue from the infused tissue and non-infused tissue were measured by GC-MS using \(^{2}\text{H}_3\)succinate, \(^{3}\text{H}\)fumarate, and \(^{2,2,4,4-}\text{H}_4\)citrate as internal standards.

**Calculations.** MV\(_2\)O and uptakes of glucose, lactate, and free fatty acids were calculated as the products of the arterial-venous concentration difference and LAD blood flow. The propionate uptake by the perfused territory was calculated as the arterial-venous concentration difference times the LAD plasma flow, with correction of the venous concentration for contamination with venous effluent from outside the LAD bed using a dilution factor of 0.91, as previously described (20). The LAD blood flow was taken as the perfusion pump rate divided by the mass of LAD tissue. The product (LVP; segment length loop) was calculated off-line from 30 consecutive beats using Matlab software and was used as an index of external wall work of the anterior free wall. Anterior wall external power was calculated as the product of heart rate and the area of the LVP vs. segmental length loop area.

The mass isotopomer distribution of metabolites, M\(_i\), is defined as

\[
M_i = A_i \sum_i A_i
\]

where \(A_i\) is the peak area of ion \(M_i\) from computer integration and adjusted for the natural abundance obtained from baseline plasma samples, and \(\Sigma A_i\) (\(i = 0\)–\(n\)) is the sum of the peak areas from ion \(M_0\) to ion \(M_n\).

The flux ratio of anaplerosis from propionate/CAC was calculated as the enrichment ratio of M\(_3\) succinate in tissue/\(M_3\) propionate in coronary vein. It was also calculated from the ratio propionate uptake/myocardial CAC flux. The CAC flux was calculated from the MV\(_2\)O assuming that one-third of the energy was derived from glucose plus lactate and...
two-thirds from fatty acids (20). It was also assumed that 1 μmol O\textsubscript{2} consumed results in the formation of 0.6 and 0.351 μmol citrate from glucose and palmitate, respectively. Thus the CAC flux was calculated as:

\[
\text{CAC flux} = \text{MVO}_{2} \left[ (0.6 \times 0.33) + (0.351 \times 0.67) \right]
\]

**Statistical analysis.** Data are presented as means ± SE. All comparisons were made between the averaged data during [U-1\textsuperscript{3}C\textsubscript{3}]propionate infusion and the data at baseline (time 0) using the paired Student’s t-test. Statistical significance was set at the 0.05 level.

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**RESULTS**

Physiological measurements were made on the pig heart before the propionate infusion started (time 0, referred to as baseline), during the infusion of unlabeled propionate (20 min), and during the infusion of [U-1\textsuperscript{3}C\textsubscript{3}]propionate (60 min). There were no significant changes in MVO\textsubscript{2}, heart rate, peak LVP, and peak negative and positive dP/dt over the course of the study period (Fig. 1). Anterior wall external power did not change over the course of the study (351 ± 41, 343 ± 34, 391 ± 81, 299 ± 70, and 330 ± 47 mmHg·mm·s\textsuperscript{-1} at 0, 20, 40, 60, and 80 min, respectively). Thus increasing arterial propionate concentration to 0.25 mM did not alter global or regional cardiac function.

The arterial concentrations of glucose (4.08 ± 0.23 mM), lactate (1.52 ± 0.20 mM), and free fatty acids (0.46 ± 0.06 mM) were stable during the 80-min study period. There were no significant changes in the uptake of glucose (0.44 ± 0.09 μmol·min\textsuperscript{-1}·g\textsuperscript{-1} at baseline and 0.40 ± 0.10 μmol·min\textsuperscript{-1}·g\textsuperscript{-1} at 80 min) and lactate (0.19 ± 0.06 μmol·min\textsuperscript{-1}·g\textsuperscript{-1} at baseline and 0.15 ± 0.06 μmol·min\textsuperscript{-1}·g\textsuperscript{-1} at 80 min) across the LAD bed. However, the uptake of plasma total free fatty acids was decreased from 0.091 ± 0.018 (baseline) to 0.049 ± 0.010 μmol·min\textsuperscript{-1}·g\textsuperscript{-1} (at 80 min, P < 0.05).

Plasma propionate concentrations were low (0.04 ± 0.01 mM in the artery and 0.03 ± 0.01 mM in the coronary vein) before the infusion started (Fig. 2). After 20 min of propionate infusion, propionate concentrations in the artery and vein were increased to 0.28 ± 0.02 and 0.15 ± 0.02 mM, respectively. The propionate concentrations were stable at these levels afterward (Fig. 2). Propionate uptake across the perfused LAD territory was 0.078 ± 0.02 and 0.15 ± 0.02 μmol·min\textsuperscript{-1}·g\textsuperscript{-1} at baseline and 0.9% in the artery and 81.3 ± 1.6% in the coronary vein 20 min into the infusion of [U-1\textsuperscript{3}C\textsubscript{3}]propionate. Thus there was some dilution of propionate enrichment during a single passage of blood through the myocardium. This dilution derives presumably from the hydrolysis of unlabeled propionyl-CoA formed from the degradation of heart proteins.

After 20 min of infusion of unlabeled propionate (0.25 mM), plasma propionylcarnitine concentration in the coronary vein was elevated from a baseline value of 51.8 ± 3.5 to 93.0 ± 7.2 pmol/ml. After 60 min of
[\textsuperscript{U-\textsuperscript{13}C_3}]propionate infusion, the total propionylcarnitine concentration in the vein was increased further to 112.0 ± 14.1 pmol/ml. At the end of 60 min, [\textsuperscript{U-\textsuperscript{13}C_3}]propionate infusion and plasma [\textsuperscript{U-\textsuperscript{13}C_3}]propionylcarnitine concentration in the artery and vein were 11.5 ± 1.8 and 43.8 ± 2.2 pmol/ml, respectively. The \( M_3 \) enrichment of propionylcarnitine in the artery and vein were 14.6 ± 1.6 and 41.2 ± 3.4%, respectively. The net release of [\textsuperscript{U-\textsuperscript{13}C_3}]propionylcarnitine from the infused territory was 21.7 ± 3.6 pmol·min\(^{-1} \)·g\(^{-1} \), accounting for 0.033 ± 0.002% of [\textsuperscript{U-\textsuperscript{13}C_3}]propionate uptake.

The concentrations of CAC intermediates in heart tissue did not change during the infusion (Table 1). However, there was substantial labeling of CAC intermediates in the perfused LAD territory after 1 h of infusion of [\textsuperscript{U-\textsuperscript{13}C_3}]propionate (Table 2). There was a progressive decrease of \( M_3 \) enrichments from succinate to α-ketoglutarate. The contribution of propionate to the CAC flux was calculated by dividing the \( M_3 \) enrichment of succinate by the \( M_3 \) enrichment of propionate in the LAD vein. Anaplerosis from propionate was equivalent to 8.9 ± 1.3% of the total CAC flux. One can also calculate this percentage by dividing the uptake of propionate (0.078 ± 0.009 μmol·min\(^{-1} \)·g\(^{-1} \)) by the rate of CAC flux (0.96 ± 0.07 μmol·min\(^{-1} \)·g\(^{-1} \)) calculated from the MV0₂ (2.22 ± 0.16 μmol·min\(^{-1} \)·g\(^{-1} \), see Methods). The value thus calculated, i.e., 8.0 ± 0.5% of the CAC flux, is very close to that calculated from the labeling data.

There was no detectable \( M_1 \), \( M_2 \), and \( M_3 \) enrichment of pyruvate or lactate in the perfused tissue. This supports our previous observation that there is no detectable flux from malate to pyruvate via malic enzyme in the in vivo pig heart (20).

**DISCUSSION**

This study demonstrates that a modest elevation in arterial propionate concentration (0.25 mM) results in substantial uptake of propionate and anaplerosis from propionate in the heart. We observed substantial enrichments in CAC intermediates from [\textsuperscript{U-\textsuperscript{13}C_3}]propionate in the infused heart tissue and found that 8% of the CAC flux derived from propionate. Anaplerosis from propionate did not increase the pool of CAC intermediates nor did it adversely affect myocardial contractile function.

Current calculations of anaplerosis using \[^{13}\text{C}\]NMR spectroscopy yield the ratio total anaplerosis flux/CAC flux, which includes all possible entries of intermediates into the cycle (11, 26, 29). Our technique, based on GC-MS and mass isotopomer analysis, quantifies relative anaplerosis from propionate. When coupled to a metabolic measurement of CAC activity (based on oxygen uptake), it yields an absolute anaplerotic flux from propionate. This rate was confirmed by measuring the myocardial uptake of propionate.

Another goal of this study was to assess the effectiveness of infusing anaplerotic propionate at low arterial concentration in the heart. Stimulation of anaplerosis with propionate has therapeutic potential for the treatment of myocardial ischemia (24, 25) and for the treatment of some inborn defects in the oxidation of long-chain fatty acids (22). A good anaplerotic compound should 1) diffuse easily in cardiomyocytes, 2) be rapidly converted to a CAC intermediate, 3) be effective at a low plasma concentration, 4) be stable in solution, and 5) not result in major sodium overload. Anaplerosis via a precursor of propionyl-CoA is an attractive approach because the conversion of propionyl-CoA to succinyl-CoA is irreversible and, in the case of propionate, substantial at low plasma concentration. In the present study, a flux ratio anaplerosis from propionate/CAC of 8.9% was achieved at a coronary plasma propionate concentration of 0.25 mM. In contrast, in a similar pig study where coronary plasma pyruvate concentration was raised to 1.1 mM, the anaplerosis from the pyruvate-to-CAC ratio was 5.7%.

### Table 1. Concentrations of CAC intermediates in heart tissue

<table>
<thead>
<tr>
<th></th>
<th>Succinate</th>
<th>Fumarate</th>
<th>Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonperfused posterior free wall</td>
<td>0.37 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>Perfused anterior free wall</td>
<td>0.36 ± 0.03</td>
<td>0.07 ± 0.01</td>
<td>0.56 ± 0.08</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Units are μmol/g wet wt. CAC, citric acid cycle. After 80 min of infusion of 0.25 mM propionate into a coronary artery, punch biopsies were taken simultaneously from the perfused anterior wall territory and from the nonperfused posterior wall.

### Table 2. Mass isotopomer distribution of labeled CAC intermediates in cardiac tissue after 1 h of [\textsuperscript{U-\textsuperscript{13}C_3}]propionate infusion into the coronary artery

<table>
<thead>
<tr>
<th>Intermediates</th>
<th>( M_1 )</th>
<th>( M_2 )</th>
<th>( M_3 )</th>
<th>( M_4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>6.0 ± 0.7</td>
<td>4.6 ± 0.4</td>
<td>7.3 ± 1.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Fumarate</td>
<td>7.6 ± 0.7</td>
<td>10.9 ± 2.2</td>
<td>5.8 ± 0.5</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>Malate</td>
<td>7.9 ± 0.6</td>
<td>5.4 ± 0.4</td>
<td>6.2 ± 0.5</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>6.9 ± 0.6</td>
<td>4.1 ± 0.3</td>
<td>4.1 ± 0.4</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Citrate</td>
<td>7.7 ± 0.4</td>
<td>5.5 ± 0.4</td>
<td>4.9 ± 1.3</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>6.8 ± 0.5</td>
<td>4.3 ± 0.5</td>
<td>2.2 ± 0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are means ± SE and are expressed as percent distribution after correction for natural enrichment. Mass isotopomers are denoted as \( M_n \), where \( M \) is the naturally occurring molecule and \( n \) is the number of excess \(^{13}\text{C}\) atoms in the molecule. There is no detectable mass isotopomer heavier than \( M_4 \).
which was not statistically different from the ratio measured at a pyruvate concentration of 0.2 mM (4.7%). It is possible that higher concentrations of pyruvate will increase the rate of anaplerosis via pyruvate carboxylation; however, adequate infusion of sodium pyruvate via a peripheral vein rapidly results in dangerous hypernatremia (18). In any case, the data of the present study suggest that propionate is a more effective anaplerotic agent than pyruvate.

Intracoronary infusion of 0.25 mM propionate did not adversely affect cardiovascular function in normal pigs. Our future work will investigate 1) the time course of anaplerotic flux and its feedback mechanisms, 2) the modulation of anaplerotic flux by the concentration of propionyl-CoA precursor, 3) varying the propionyl-CoA precursor (medium-odd-chain fatty acids, C₅ ketone bodies, or propionylcarnitine), and 4) the effect of propionyl-CoA precursor on performance of hearts damaged by acute anoxia or pharmacological blockage of fatty acid oxidation. Our recent clinical study (23) has shown that the cardiovascular function of patients with defects in long-chain fatty acid oxidation was improved remarkably by a diet containing 30% of the calories as triheptanoin (a precursor of propionyl-CoA) by comparison with a diet containing an equivalent amount of trioctanoin (which is not anaplerotic). The main difference in the metabolisms of heptanoate vs. octanoate is that the former is catabolized to propionyl-CoA plus acetyl-CoA, whereas the latter forms only acetyl-CoA. This supports the hypothesis that the improvement in cardiovascular function of these patients results from the stimulation of anaplerosis via propionyl-CoA.

One potential problem with treating patients with precursors of propionyl-CoA is the possibility of creating a syndrome similar to that of congenital propionic academia. In these patients, the production of large amounts of 2-methylcitrate depletes the oxaloacetate pool and interferes with CAC operation (2). In our pig experiments, the production of 2-methylcitrate was minuscule, since the myocardial 2-methylcitrate concentration was <2% of the citrate concentration. In addition, the concentrations of citrate, succinate, and fumarate were not affected by propionate infusion (Table 1). In a previous study in which conscious dogs were infused with large amounts of C₅ ketone bodies, β-ketopentanoate and β-hydroxypentanoate, which are precursors of propionyl-CoA, we found that the urinary excretion of indexes of propionyl overload was very small (14). Thus, in an animal with a normal propionyl-CoA carboxylase pathway, it is unlikely that anaplerotic doses of propionyl-CoA precursors would have any deleterious effects.

The mass isotopomer distribution of heart CAC intermediates labeled from [U-13C₃]propionate is compatible with current concepts of CAC operation (Table 2). The progressive decrease in M₃ enrichment of CAC intermediates from succinate to α-ketoglutarate reflects the influx of unlabeled substrates into the CAC and/or isotopic exchanges between CAC intermediates and related unlabeled metabolites (aspartate, glutamate, and glutamine). The production of M₂ and M₁ isotopomers results from the loss of label in the cycle under conditions where the incoming acetyl-CoA is unlabeled, as reflected by the absence of labeled tissue pyruvate and lactate. This absence of labeling in pyruvate and lactate must reflect very low flux via phosphoenolpyruvate carboxykinase and malic enzyme in the direction of pyruvate formation. We had reached a similar conclusion in experiments in which [U-13C₃]-pyruvate was infused in the LAD of similarly treated pigs (20).

Our protocol allowed two independent measurements of anaplerosis from propionate as a fraction of CAC flux, i.e., by mass isotopomer distribution of succinate and by propionate uptake (8.9 vs. 8.0%, respectively). There was a close agreement between these two measurements. In addition, we found that the release of propionylcarnitine was very small, accounting for <0.1% of propionate uptake. These data confirm that, in the normal heart, most of the propionate taken up is channeled in the anaplerotic pathway. Thus, secondary pathways of propionate metabolism leading to 2-methylcitrate (2) and 3-hydroxypropionate (1) are quantitatively minor.

Myocardial substrate utilization plays an important role in the recovery of cardiac function after ischemia and during demand-induced ischemia. A shift from fatty acid to glucose utilization proved to be beneficial in animal studies (12, 28) and clinical trials (3, 5, 9). In this study, we found that propionate infusion significantly inhibited myocardial fatty acid uptake, with no changes in glucose and lactate uptake. This decrease was apparently not because of changes in substrate availabilities, since arterial concentrations of glucose, lactate, and fatty acids remained constant. Liedtke et al. (15) also reported a 38% decrease of fatty acid oxidation during propionate infusion (2 mM) in open-chest, extracorporeally perfused pig heart. Although the mechanism of propionate inhibition in fatty acid utilization remains to be investigated, this inhibition may indicate a potential benefit to cardiac functional recovery after ischemic injury.

In conclusion, our study demonstrates that propionate is an effective anaplerotic precursor for the myocardium in vivo. In the context of our first clinical trial of the effect of heptanoate on cardiac function (23), it opens the way to a number of basic science and clinical investigations on the potential of propionyl-CoA precursors for the treatment of cardiac diseases.

We thank Paul Minkler for help in measuring propionylcarnitine concentrations.

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