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Mass isotopomer study of anaplerosis from propionate in the perfused rat heart

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Abstract

Anaplerosis from propionate was investigated in rat hearts perfused with 0–2 mM [¹³C₃]propionate and physiological concentrations of glucose, lactate and pyruvate. The data show that when the concentration of [¹³C₃]propionate was raised from 0 to 2 mM, total anaplerosis increases from 5 to 16% of the turnover of citric acid cycle intermediates. Then, [¹³C₃]propionate abolished anaplerosis from endogenous substrates, glucose, lactate and pyruvate. Also, while the contents of propionyl-CoA and methylmalonyl-CoA increased with [¹³C₃]propionate concentration, the content of succinyl-CoA decreased, presumably via activation of succinyl-CoA hydrolysis by a decrease in free CoA. Under our conditions, [¹³C₃]propionate was a purely anaplerotic substrate since there was no labeling of mitochondrial acetyl-CoA, reflected by the labeling of the acetyl moiety of citrate.

INTRODUCTION

Anaplerosis, or re-filling of citric acid cycle intermediates, plays a key role in the maintenance of energy transfer in the heart. This is because the physiological leakage of CAC intermediates must be compensated to maintain an adequate rate of acetyl-CoA oxidation and ATP turnover. The main anaplerotic substrates in the heart (Fig 1) are (i) pyruvate via pyruvate carboxylase and/or malic enzyme, (ii) glutamate/glutamine via glutamate dehydrogenase and/or transaminases, and (iii) precursors of propionyl-CoA (odd-chain fatty acids, some amino acids, C₅-ketone bodies (β-ketopentanoate + R-β-hydroxypentanoate)), via propionyl-CoA carboxylase, methylmalonyl-CoA epimerase, and methylmalonyl-CoA mutase [1]. In isolated hearts perfused with acetoacetate, a substrate that generates only acetyl-CoA, the work output of the heart decreases rapidly [2] unless an anaplerotic substrate such as propionate or pyruvate is added to the perfusate [3,4]. Also, after a brief episode of ischemia, the mechanical function of the reperfused isolated heart can be restored by providing mM concentrations of pyruvate [5,6] which, in addition to its anaplerotic effect [7], shows inotropic effects and traps reactive oxygen species [6,8,9]. Anaplerotic therapy can also be beneficial in conditions where one cannot demonstrate a depletion of CAC intermediates. Such depletion may be suspected in patients with inborn errors of long-chain fatty acid oxidation, because of the release of large molecules such as creatine kinase from muscle into plasma (rhabdomyolysis). In support of this inference, a recent small clinical trial has shown that patients with long-chain fatty acid

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¹Mass isotopomers are designated as M, M1, M2,...Mn, where n is the number of heavy ¹³C atoms in the molecule.

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oxidation disorders have their cardiovascular and muscular condition markedly improved by a dietary treatment with triheptanoin, the metabolism of which leads to anaplerotic propionyl-CoA [10]. Unlike treatment with even-medium-chain fatty acids (which generate only acetyl-CoA), treatment with triheptanoin leads to a marked decrease in plasma creatine kinase activity [10]. As a follow up of this clinical study, we sought to characterize anaplerosis from propionyl-CoA in rat heart using propionate as a precursor.

Although the stimulation of anaplerosis can lead to an increase in the total pool of CAC intermediates, this increase is achieved over a short period of time. Then, anaplerosis must be balanced by an equivalent release of excess intermediates, a process often called cataplerosis [11]. To measure rates of anaplerosis, one needs to use labeled substrates and compute the labeling of CAC intermediates to estimate the rate of anaplerosis relative to the turnover of the catalytic intermediates of the cycle which carry acetyl-CoA as it is oxidized. Our strategy for measuring anaplerosis involves administering [$^{13}\text{C}_3$]propionate, and measuring the M3 enrichment ratio (succinyl-CoA)/(propionyl-CoA), since the anaplerotic process starts at propionyl-CoA and ends at succinyl-CoA, an intermediate of the CAC. This strategy does not lead to overestimation of anaplerosis by label recycling, since the only source of M3 succinyl-CoA is anaplerosis from M3 propionyl-CoA. The conversion of labeled α -ketoglutarate (formed in multiple turns of the CAC) would not yield M3 succinyl-CoA.

In non-ruminant mammals, peripheral blood carries very low concentrations of propionate (0.05 mM, [12]), since 99% of propionate formed by the intestinal flora is taken up in a single passage of portal vein blood through the liver [12]. Thus, the only source of propionyl-CoA available to peripheral tissues derives from the catabolism of proteins. The supply of propionyl-CoA by dietary proteins is low (about 65 mmol propionyl equivalents/100 g mixed protein, [13]), and the fraction of this supply which is formed in the liver is probably converted to glucose. Therefore, in peripheral tissues such as the heart, the rate of physiological anaplerosis *via* propionyl-CoA formed in this organ is probably low. Thus, the goal of the present study was to characterize how the concentration of exogenous propionate affects the content of CAC intermediates in isolated rat hearts perfused with physiological concentrations of glucose, lactate and pyruvate. Since long-chain fatty acids increase the heart content of citrate [14], we perfused hearts without long-chain fatty acids to characterize the effect of propionate on the content of CAC intermediates.

METHODS

Materials

Chemicals and biochemicals were obtained from Sigma-Aldrich. [$^{13}\text{C}_3$]Propionate, [3- ^{13}C]propionate, [$^2\text{H}_5$]propionate, [$^{13}\text{C}_4$]succinic acid, [$^2\text{H}_4$]succinic anhydride, [$^{13}\text{C}_4$]fumaric acid, and [$^{13}\text{C}_6$]citric acid were purchased from Isotec (Miamisburg, OH). [$^2\text{H}_5$]Propionyl-CoA and [2,2,3,3- $^2\text{H}_4$]succinyl-CoA were prepared by reacting [$^2\text{H}_5$]propionic acid and [$^2\text{H}_4$]succinic anhydride with CoA [15]. The two labeled acyl-CoAs were purified by HPLC and their water solutions standardized by UV spectroscopy [15]. ATP citrate lyase was prepared from liver of rats that were fasted for two days, then re-fed with high carbohydrate diet for three days to increase the enzyme activity. The enzyme, isolated from the Bio-Gel column [16] was precipitated with 50% ammonium sulfate, and aliquots of the suspension (2 U/0.1 ml) were kept frozen at -80°C . Before use, the enzyme was dissolved in 1 ml of 250 mM Tris-HCl, pH 8.7 containing 5 mM dithiothreitol.

Heart perfusion experiments

Hearts from overnight-fasted Sprague-Dawley male rats (200–250 g) were perfused in Langendorff mode with non-recirculating bicarbonate buffer (119 mM NaCl, 4.8 mM KCl, 1.3

mM CaCl_2 , 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , 5 mM glucose, 1 mM L-lactate, 0.2 mM pyruvate, 50 μM carnitine, 100 $\mu\text{U/L}$ insulin (Novolin^R, Novo-Nordisk) and 0 to 2 mM $[\text{C}_3^{13}]$ propionate or $[\text{C}_3^{13}]$ propionate. The perfusate was equilibrated with 95% O_2 + 5% CO_2 (pH = 7.4 – 7.42). The coronary flow was kept at 12 ml/min using a roller pump. This set up, which we have used in a number of studies [17–20] insures adequate oxygenation of this non-working heart model. The apparatus was set up in a thermostated cabinet. The inflowing perfusate passed through a water-jacketed Technicon glass coil at the bottom of which the heart was suspended. This insured a constant 38°C temperature of the heart (verified with an ISI thermocouple). At the end of the experiments, the hearts were quick-frozen, powdered under liquid N_2 , and kept at –80 °C until analysis.

Analytical Procedures

The contents and ^{13}C -labeling pattern of propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA were analyzed by the following modification of our previously described method [15]. Powdered frozen heart tissue (300–400 mg), spiked with 10 nmol of $[\text{H}_5^2]$ propionyl-CoA and 12 nmol $[\text{H}_4^2]$ succinyl-CoA, was extracted with 4 vol of ice-cold 6% HClO_4 using a Polytron homogenizer. After centrifugation, the extract was slowly pushed through an Oasis cartridge (Waters) pre-washed with 1 ml methanol and 1 ml water. This cartridge traps water-soluble CoA esters. After rinsing the cartridge with 1 ml of 5% methanol in water, the short-chain acyl-CoAs were eluted with 1ml methanol. After solvent evaporation at 5 °C, the residue was dissolved in 0.5 ml of 75 mM potassium phosphate buffer, pH 4. One-half of this solution was added to a 0.5 ml solution of 1 M potassium sarcosine, pH 9.6, and incubated at 60 °C for 4 h. After adjusting pH to 7.4, N-propionylsarcosine was reacted in sonicator for 1 hr with 0.3 ml of 4 % (v/v) pentafluorobenzylbromide in acetonitrile solution and 0.3 ml of 0.1 M solution of tetrabutylammonium bromide in 0.1 M potassium phosphate buffer, pH 7.4. Use of the interface catalyst tetrabutylammonium bromide allows derivatization with pentafluorobenzylbromide in a heterogeneous system without extraction of N-propionylsarcosine with organic solvent, as we did previously. The pentafluorobenzyl (PFB) derivative of N-propionylsarcosine was extracted in acid with 3 × 4 ml ethyl acetate. The extract was dried with Na_2SO_4 and the solvent evaporated down to about 0.1 ml and was analyzed by ammonia negative chemical ionization gas chromatography-mass spectrometry. Ions at m/z 144 (M - PFB) to 149 (M - PFB + 5) were monitored.

The second portion the short-chain acyl-CoAs solution was hydrolyzed in alkaline medium. Methylmalonate and succinate (derived from the corresponding CoA esters) were analyzed after conversion to *tert*-butyldimethylsilyl derivatives. Under ammonia- positive chemical ionization, ions monitored were 347.4 (M + H^+) to 352.4 (M + H^+ + 5).

The ^{13}C -labeling of the acetyl moiety of heart tissue citrate (a probe of mitochondrial acetyl-CoA) was assayed by cleaving citrate with ATP-citrate lyase isolated from rat liver, and reacting the acetyl-CoA formed with thiophenol [21]. The labeling pattern of pyruvate, lactate and citric acid cycle (CAC) intermediates were analyzed as described previously [22].

Calculations, Data Presentations and Statistics—Isotopic enrichments are expressed as molar percent enrichments (MPE), i.e., the percentage of a given mass isotopomer over the sum of all mass isotopomers.

Relative anaplerosis from propionate represents the fraction of the turnover of catalytic intermediates of the CAC which carry acetyl-CoA as it is being oxidized. The contribution of exogenous M3 propionate to the pool of CAC intermediates was calculated by dividing the M3 enrichment of succinyl-CoA by 0.97, that is by the M3 enrichment of $[\text{C}_3^{13}]$ propionate in the perfusate. Relative anaplerosis from all sources (endogenous and exogenous) was calculated as the M3 enrichment ratio (succinyl-CoA)/(propionyl-CoA). Relative anaplerosis

from substrates other than exogenous M3 propionate was calculated as the difference (relative anaplerosis from all sources) – (contribution of exogenous M3 propionate to the pool of CAC intermediates).

For each experimental condition, we ran 7–9 perfusions in the presence of [^{13}C]propionate with the concentration or time being allowed to vary. Although $n = 1$ for each time point or concentration, this protocol yields content or time curves which can be subjected to statistical analysis. The enrichments and tissue contents presented are means of duplicate GC-MS injections, which differed by <2%. The statistical significance of differences between selected profiles of ^{13}C -mass isotopomers was tested using a paired t test (Graph Pad Prism Software, version 3).

RESULTS AND DISCUSSION

This study included two groups of experiments. First, we perfused hearts with 0.25 mM [$^{13}\text{C}_3$]propionate for increasing amount of time to follow the time course of metabolite labeling. Second, we perfused hearts with increasing concentrations of [$^{13}\text{C}_3$]propionate.

Experiments with 0.25 mM [U- $^{13}\text{C}_3$]propionate

The two panels of Fig 2 present the time course of M3 labeling of intermediates extracted from hearts perfused with 0.25 mM [$^{13}\text{C}_3$]propionate for increasing times. The labeling of propionyl-CoA and methylmalonyl-CoA reached a plateau by 3 min (Fig 2A), while the M3 labeling of other metabolites reached a plateau by 15 min (Fig 2B). Note that the M3 labeling of fumarate and malate were significantly greater than those of their precursors, succinyl-CoA and succinate ($p < 0.02$). This topic will be discussed below. Based on the data of Fig 2, and on the time course of stabilization of the total content of CAC intermediates after addition of an anaplerotic substrate to the perfusate [23], we concluded that subsequent experiments should be conducted for at least 15 min. Anaplerosis, calculated as the M3 labeling ratio (succinyl-CoA)/(propionyl-CoA), ranged from 6 to 8% of the turnover of CAC intermediates.

Experiments with increasing concentrations of [$^{13}\text{C}_3$]propionate

Hearts were perfused for 15 min with increasing concentrations of unlabeled propionate, then for 15 additional min with the same concentrations of [$^{13}\text{C}_3$]propionate. The uptake of propionate by the perfused heart ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) was linear with the mM concentration (slope = $1.33\ \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\cdot\text{mM}^{-1}$; $R^2 = 0.91$). Fig 3A shows that the tissue content of propionyl-CoA increased linearly with the concentration of [$^{13}\text{C}_3$]propionate in the perfusate (slope = $32.2\ \text{nmol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$; intercept = $2\ \text{nmol/g}$; $R^2 = 0.997$). The content of methylmalonyl-CoA (Fig 3B) followed a sigmoid curve and plateaued at about $20\ \text{nmol/g}$ at $0.5\ \text{mM}$ [$^{13}\text{C}_3$]propionate (10-fold increase over baseline). The content of succinyl-CoA (Fig 3C) decreased from 34 to $19\ \text{nmol/g}$ as the concentration of [$^{13}\text{C}_3$]propionate increased from 0 to $1\ \text{mM}$. Thus, although the contents of succinyl-CoA precursors, i.e., propionyl-CoA and methylmalonyl-CoA were increased 18 and 10 fold, respectively by $1\ \text{mM}$ propionate, the content of succinyl-CoA was halved.

To interpret this surprising finding, it is useful to calculate the equilibrium value of the mass action ratio [succinyl-CoA]/[propionyl-CoA] from the combined equilibrium constant of the reactions catalyzed by propionyl-CoA carboxylase, methylmalonyl-CoA racemase, and methylmalonyl-CoA mutase ($K' = 18.6$, [24]). This mass action ratio can be calculated from tissue contents since there is good evidence that, in the heart, (i) propionate, like acetate, is activated in mitochondria by acetyl-CoA synthetase 2 [20], and (ii) propionyl-CoA carboxylase, methylmalonyl-CoA racemase and methylmalonyl-CoA mutase are mitochondrial enzymes [1]. At equilibrium, the [succinyl-CoA]/[propionyl-CoA] should be

about 177. Fig 3D shows that, in fact, this mass action ratio is 15.5 in the absence of [$^{13}\text{C}_3$] propionate, and decreases further to 0.5 as the content of [$^{13}\text{C}_3$]propionate increases from zero to 1 mM. This indicates that the disequilibrium of the three reactions is present even in the absence of exogenous propionate, when the propionyl-CoA pathway is fed only from the catabolism of endogenous cardiac proteins. Also, the [succinyl-CoA]/[propionyl-CoA] ratio decreases when the propionyl-CoA pathway is loaded with substrate. This unexpected finding must reflect an activation of the conversion of succinyl-CoA to succinate when the propionyl-CoA supply is increased.

From panels A to C of Fig 3, one calculates that the total amount of CoA in propionyl-CoA, methylmalonyl-CoA and succinyl-CoA increased from 38 to 70 nmol/g as the concentration of [$^{13}\text{C}_3$]propionate increased from 0 to 1 mM. Thus, although we did not assay the content of free CoA in these hearts (because of the small amount of tissue available), it is likely that it was decreased by the addition of [$^{13}\text{C}_3$]propionate. This is supported by reports that the total content of free CoA decreased four- and five-fold when 0.5 to 2 mM propionate was added to the perfusate of rat hearts [25–27]. This trapping of CoA probably (i) decreased the flux through α -ketoglutarate dehydrogenase, and (ii) increased the activity of mitochondrial succinyl-CoA thiolase, as is the case for the peroxisomal enzyme [28]. Both effects of CoA trapping would accelerate anaplerosis from [$^{13}\text{C}_3$]propionate, in spite of the lower content of succinyl-CoA (Fig 3C).

The contents of propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA discussed above are expressed in nmol per gram tissue (wet weight). It is likely that these CoA esters are located mostly, if not exclusively in mitochondria since they are generated by mitochondrial enzymes. Although we could not assess the intra-mitochondrial concentrations of these CoA esters, we can reasonably assume that their concentration ratios are the same in mitochondria as in total tissue.

Fig 4 shows that the total content of CAC intermediates in the hearts increases seven fold when the concentration of [$^{13}\text{C}_3$]propionate increases from zero to 2 mM. About 80% of this increase is accounted for by the accumulation of malate. Sundqvist *et al* reported similar and even higher increases in the content of CAC intermediates (mostly malate) induced by 2 mM propionate [26,29]. Chance *et al* [30] reported that similar increases in CAC intermediates are induced by 1 mM pyruvate (in the presence of 5 mM glucose). In our study, the only CAC intermediate which decreased in content was succinyl-CoA (Fig 3C). Our measurements of tissue content (i) do not differentiate between cytosolic and mitochondrial concentrations of CAC intermediates, (ii) do not shed light on micro-compartmentation of metabolite concentrations. However, a six-fold increase in malate content is probably distributed in both cytosol and mitochondria. Sundqvist *et al* demonstrated that, when propionate is metabolized by the heart in addition to glucose, the mass action ratio of malic enzymes approaches the equilibrium value. This allows the accumulation of CAC intermediates “until a steady state is reached, under which cataplerosis *via* malic enzymes is possible” [29].

Fig 5 shows the mass isotopomer distribution of propionyl-CoA, methylmalonyl-CoA and succinyl-CoA as a function of the perfusate concentration of [$^{13}\text{C}_3$]propionate. The M3 enrichment of propionyl-CoA and methylmalonyl-CoA plateaued at about 90 and 85%, respectively Figs 5A, 5B). The mass isotopomer distribution of succinyl-CoA showed a much lower proportion of M3 isotopomer (16% at 2 mM [$^{13}\text{C}_3$]propionate) compared to methylmalonyl-CoA (85%). In addition, substantial amounts of M1 and M2 isotopomers of succinyl-CoA were formed. This results from (i) redistribution and loss of label in the CAC, and (ii) dilution of the labeling of CAC intermediate by unlabeled carbon entering the cycle as acetyl-CoA. The latter derived from unlabeled glucose, lactate and pyruvate in the perfusate,

as well as from endogenous substrates such as fatty acids. The small amount of M2 methylmalonyl-CoA (Fig 5B) results from the reversibility of methylmalonyl-CoA mutase.

Fig 6 presents various calculations of relative anaplerosis in the heart, expressed as percent contribution to the catalytic intermediates of the cycle which carry acetyl-CoA as it oxidized. These calculations use the M3 enrichments of propionyl-CoA and succinyl-CoA. The contribution of exogenous M3 propionate to the pool of CAC intermediates (Fig 6, solid squares) was calculated by dividing the M3 enrichment of succinyl-CoA by 0.97, i.e., by the M3 enrichment of [$^{13}\text{C}_3$]propionate in the perfusate. The contribution of exogenous propionate to the CAC intermediates peaked at 17% at 1 mM [$^{13}\text{C}_3$]propionate. Relative anaplerosis from all sources (Fig 6, solid diamonds) was calculated as the M3 enrichment ratio (succinyl-CoA)/(propionyl-CoA). Since heart propionyl-CoA was not labeled at zero [$^{13}\text{C}_3$]propionate concentration, anaplerosis from all sources at zero [$^{13}\text{C}_3$]propionate concentration was extrapolated to 5% of the turnover of CAC intermediates. The third curve (Fig 6, solid triangles), calculated as the difference between the other two curves, represents relative anaplerosis from substrates other than exogenous M3 propionate: glucose, lactate, pyruvate, endogenous amino acids (including those generating unlabeled propionyl-CoA). This contribution decreased to almost zero at [$^{13}\text{C}_3$]propionate concentrations of 1 – 2 mM. Then, [$^{13}\text{C}_3$]propionate accounts for almost all anaplerosis. This finding confirms the effectiveness of anaplerosis from propionyl-CoA reflected by the mass action ratio succinyl-CoA/propionyl-CoA (see above). However, at low propionate concentration, e.g., 0.2 mM, anaplerosis from propionate is similar to that induced by endogenous substrates + glucose + lactate + pyruvate, but adds up to theirs. Comte *et al* had reported that in hearts perfused with 4 mM glucose + 1 mM [U- $^{13}\text{C}_3$]lactate + 0.2 mM [U- $^{13}\text{C}_3$]pyruvate + 0.2 mM octanoate, anaplerosis from pyruvate accounted for 6.3% of the supply of CAC intermediates [31].

When calculating relative anaplerosis from labeled propionate from the labeling ratio succinyl-CoA/propionyl-CoA, one must chose the labeling of propionate so that the mass isotopomer of succinyl-CoA used in the calculation cannot be formed by recycling via α -ketoglutarate dehydrogenase. This condition is satisfied by the use of [$^{13}\text{C}_3$]propionate, since M3 succinyl-CoA can only arise from M3 propionyl-CoA. Of the two sources of succinyl-CoA, i.e., anaplerosis from M3 propionyl-CoA and recycling in the CAC (via α -ketoglutarate dehydrogenase), the latter cannot yield M3 succinyl-CoA. Therefore, all M3 succinyl-CoA is derived from anaplerosis. In another study, we perfused hearts with [3- ^{13}C]propionate instead of [$^{13}\text{C}_3$]propionate. Then, apparent relative anaplerosis was about 50% instead of 16% (not shown). Such high and improbable relative anaplerosis from [3- ^{13}C]propionate, also reported by Jeffrey *et al* [32], is overestimated because some M1 succinyl-CoA is formed via α -ketoglutarate dehydrogenase. This emphasizes the need to select a proper labeling pattern of the substrate used to trace anaplerosis.

Fig 7 shows the M3 labeling of CAC intermediates as a function of the [$^{13}\text{C}_3$]propionate concentration. Note that fumarate and malate are more labeled than their precursors succinyl-CoA and succinate. This unexpected finding cannot be explained by labeling of malate *via* malic enzyme since the labeling of pyruvate was much lower than that of malate (see below). Note that propionyl-CoA is much more M3 labeled that CAC intermediates, including fumarate and malate (compare Figs 5A and 7). This discrepancy was also observed in perfusions with constant 0.25 mM [$^{13}\text{C}_3$]propionate for increasing times (Fig 2B), and in earlier orientation experiments (not shown). We have carefully checked for the presence of unknown compounds that would yield signals at M3 of fumarate and malate. This was excluded by demonstrating that in hearts perfused with unlabeled propionate, the mass isotopomer distributions of fumarate and malate were identical to theoretical distributions. These analyses require d long chromatographic runs (about 60 min), as previously reported [22]. One is tempted to speculate that either (i) some unknown reaction(s) link propionyl-CoA to malate, possibly with partial

channeling of labeled intermediates [33], (ii) the labeling of some pools of intermediates is not homogeneous, or (iii) metabolites are micro-compartmented in a way that does not allow quick isotopic equilibrium. The latter mechanism had been proposed for the incomplete isotopic equilibration of pyruvate and lactate [34,35]. This matter requires further investigation.

Fig 8 shows the mass isotopomer distribution of pyruvate and lactate in heart tissue (panels A, B), and in the effluent perfusate (panels C, D). The labeling of pyruvate from [$^{13}\text{C}_3$]propionate in a non-gluconeogenic organ (i.e., in the absence of phosphoenolpyruvate carboxykinase) can only result from the activity of malic enzyme. Through this enzyme, M3 malate (a mixture of [1,2,3- $^{13}\text{C}_3$]- and [2,3,4- $^{13}\text{C}_3$]malate) yields M2 and M3 pyruvate which equilibrates with lactate. M2 and M1 malate yield M2, M1 and unlabeled pyruvate. The mass isotopomers of lactate are less abundant than the corresponding isotopomers of pyruvate. This is because of the large size of the lactate pool derived from glycolysis and from the non-recirculating perfusate. The lower enrichments of pyruvate and lactate in the effluent perfusate, compared to tissue, also results from the incomplete equilibration of extra- and intracellular metabolites via cytosolic lactate dehydrogenase.

The up to 2.5% M3 labeling of tissue pyruvate raised the possibility that acetyl-CoA could be labeled *via* pyruvate dehydrogenase. So, we assayed the mass isotopomer distribution of the acetyl moiety of citrate [21]. The latter is a proxy of mitochondrial acetyl-CoA. We could not find any label in the acetyl moiety of citrate assayed as acetylthiophenol. Since the baseline M2 enrichment of acetylthiophenol is 4.5%, we could have detected a M2 enrichment of acetylthiophenol as low as 1%. Therefore, we conclude that mitochondrial acetyl-CoA was for all practical purposes unlabeled, and that the labeling of CAC intermediates did not include labeled carbons derived from acetyl-CoA.

To test whether any label from M3 propionate could be channeled to mitochondrial acetyl-CoA, we perfused one heart with 1 mM [$^{13}\text{C}_3$]propionate as only substrate. In the tissue, we assayed the MID of malate (M1 = 10.4, M2 = 21.0, M3 = 45.5), pyruvate (M1 = 10.3, M2 = 19.7, M3 = 16.2) and the acetyl moiety of citrate (M2 = 8.1%). These MIDs are compatible with the sequence: malate $\rightarrow$ pyruvate $\rightarrow$ acetyl-CoA. Thus under conditions of energy deprivation, the heart converted some propionate carbon to acetyl-CoA which was used as an energy source. Note that, even under these extreme conditions, only 8% of the acetyl-CoA used in the CAC came from the only exogenous substrate, while 92% derived probably mostly from endogenous fatty acids. We did not investigate in detail anaplerosis from [$^{13}\text{C}_3$]propionate as sole substrate because this condition boosts both cataplerosis and anaplerosis. For each molecule of acetyl-CoA produced from [$^{13}\text{C}_3$]propionate, one malate molecule must leave the CAC and be processed by malic enzyme and pyruvate dehydrogenase. This represents a net cataplerosis which must be compensated by additional anaplerosis from [$^{13}\text{C}_3$]propionate. Therefore, both anaplerosis and cataplerosis are probably boosted to unphysiological levels.

In conclusion, our data illustrate the effectiveness of anaplerosis from propionate in the rat heart perfused under our conditions. It would be impossible to stimulate cardiac anaplerosis by oral propionate or glycerol-propionate, because the liver takes up practically all portal vein propionate in a single blood pass [12]. However, propionyl-CoA can be supplied to the heart by ingestion of an odd-medium-chain triglyceride, such as triheptanoin [10]. Heptanoate, derived from enteric hydrolysis of triheptanoin, is not entirely taken up by the liver. In addition, part of the heptanoate taken up by the liver is converted to C₅-ketone bodies (β -hydroxypentanoate + β -ketopentanoate) which are converted to propionyl-CoA in the heart via the very active 3-oxoacid-CoA transferase of this organ [36]. The improvement of the cardiac condition of patients with long-chain fatty acid oxidation disorders under dietary treatment with anaplerotic triheptanoin [10], compared with trioctanoin, is probably related to the

anaplerotic effect of propionyl-CoA derived from heptanoate. This is presently being tested in perfused hearts from mice with long-chain fatty acid oxidation disorders [37].

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Abbreviations

CAC

citric acid cycle

GC-MS

gas chromatography-mass spectrometry

Fig 1. Anaplerosis from propionate

Numbered enzymes are: 1, propionyl-CoA carboxylase; 2, methylmalonyl-CoA racemase; 3, methylmalonyl-CoA mutase.

Fig 2. Time course of labeling of intermediates of the propionyl-CoA pathway and of the citric acid cycle

Hearts were perfused with 0.25 mM [$^{13}\text{C}_3$]propionate for increasing amounts times.

Fig 3. Tissue contents of intermediates of the propionyl-CoA pathway as a function of the $[^{13}\text{C}_3]$ propionate concentration
 The panels show the contents of propionyl-CoA (A), methylmalonyl-CoA (B), succinyl-CoA (C), as well as the mass action ratio $[\text{succinyl-CoA}]/[\text{propionyl-CoA}]$ (D).

Fig 4. Tissue contents of citric acid cycle intermediates as a function of the [¹³C₃]propionate concentration

The solid squares refer to the total content of intermediates. The solid diamonds refer to the malate content.

Fig 5. Mass isotopomer distribution of propionyl-CoA (panel A), methylmalonyl-CoA (panel b), and succinyl-CoA (panel C), as a function of the $[^{13}\text{C}_3]$ propionate concentration.

Fig 6. Calculations of anaplerosis in rat hearts

Relative rates of anaplerosis are expressed as percent of the turnover of citric acid cycle intermediates. Solid diamonds: anaplerosis from all sources. Solid squares: anaplerosis from exogenous $[^{13}\text{C}_3]$ propionate. Solid triangles: anaplerosis from other substrates.

Fig 7. M3 labeling of citric acid cycle intermediates as a function of the [¹³C₃]propionate concentration

Note the high labeling of fumarate and malate compared to their precursors succinyl-CoA and succinate ($p < 0.01$).

Fig 8.
Mass isotopomer distributions of tissue pyruvate (panel A), tissue lactate (panel B), effluent pyruvate (panel C), and effluent lactate (panel D).