Changes in Citric Acid Cycle Flux and Anaplerosis Antedate the Functional Decline in Isolated Rat Hearts Utilizing Acetoacetate

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Abstract

To determine the temporal relationship between changes in contractile performance and flux through the citric acid cycle in hearts oxidizing acetoacetate, we perfused isolated working rat hearts with either glucose or acetoacetate (both 5 mM) and freeze-clamped the tissue at defined times. After 60 min of perfusion, hearts utilizing acetoacetate exhibited lower systolic and diastolic pressures and lower cardiac outputs. The oxidation of acetoacetate increased the tissue content of 2-oxoglutarate and glutamate and decreased the content of succinyl-CoA suggesting inhibition of citric acid cycle flux through 2-oxoglutarate dehydrogenase. Whereas hearts perfused with either acetoacetate or glucose were similar with respect to their function for the first 20 min, changes in tissue metabolites were already observed within 5 min of perfusion at near-physiological workloads. The addition of lactate or propionate, but not acetate, to hearts oxidizing acetoacetate improved contractile performance, although inhibition of 2-oxoglutarate dehydrogenase was probably not diminished. If lactate or propionate were added, malate and citrate accumulated indicating utilization of anaplerotic pathways for the citric acid cycle. We conclude that a decreased rate of flux through 2-oxoglutarate dehydrogenase in hearts oxidizing acetoacetate precedes, and may be responsible for, contractile failure and is not the result of decreased cardiac work. Further, anaplerosis plays an important role in the maintenance of contractile function in hearts utilizing acetoacetate. (*J. Clin. Invest.* 1991. 87:384–390.) Key words: 2-oxoglutarate dehydrogenase • contractile failure • anaplerosis • coenzyme A

Introduction

Prior studies of the metabolism of ketone bodies by heart muscle have demonstrated that neither acetoacetate nor β-hydroxybutyrate alone can support contractile function at near-physiological workloads (1, 2) despite the ability of the heart to utilize readily acetoacetate in preference to glucose (3). It has been further demonstrated that the levels of citric acid cycle intermediates in hearts oxidizing acetoacetate are consistent with inhibition of citric acid cycle flux at the reaction catalyzed by 2-oxoglutarate dehydrogenase (2). In the latter report, tissue metabolites were determined only after a decline in contractile performance was manifest so that the temporal relationship between metabolic and hemodynamic changes could not be determined.

The importance of determining at what point changes in flux through 2-oxoglutarate dehydrogenase occur with respect to functional changes is brought out by a study which demonstrated that 2-oxoglutarate dehydrogenase activity correlates with the rate of citric acid cycle flux, which is determined by the external (i.e., pressure-volume) work of the left ventricle (4). Because 2-oxoglutarate dehydrogenase activity is affected by contractile performance, the changes in citric acid cycle intermediates and therefore 2-oxoglutarate dehydrogenase activity previously reported may represent an effect of contractile failure rather than the cause of decreased performance.

At present two mechanisms are proposed to be responsible for the phenomenon of contractile failure during ketone body metabolism. The first suggests that the activation and metabolism of ketone bodies results in the sequestration of free coenzyme A (CoASH)[1] as acetoacetyl-CoA and acetyl-CoA. Consequently there would be insufficient CoASH to act as cofactor for 2-oxoglutarate dehydrogenase (2). A second mechanism that has been advanced concerns the metabolic fate of succinyl-CoA. While succinyl-CoA may be converted to succinate by the action of either the citric acid cycle enzyme succinyl-CoA synthetase or the enzyme responsible for the activation of acetoacetate, 3-oxoacid-CoA transferase, the affinity of the former for succinyl-CoA is two orders of magnitude higher than the latter. Because of this disparity in affinities, it has been suggested that most of the succinyl-CoA would remain in the citric acid cycle pathway, and therefore an insufficient amount of acetoacetate would be activated decreasing the amount of acetyl-CoA entering the citric acid cycle (5–7).

Earlier work has also suggested that the amino acid asparagine can enter into the citric acid cycle as oxaloacetate increasing the pool size of all citric acid cycle intermediates and thereby improves function during the oxidation of acetoacetate (2). These experiments had been designed with the assumption that inhibition of 2-oxoglutarate dehydrogenase depletes the citric acid cycle of intermediates beyond 2-oxoglutarate. The entry of carbon skeletons into the citric acid cycle as C4 units (rather than as C2 units in the form of acetyl-CoA) represent a pathway of anaplerosis (8). The purpose of anaplerosis is to replenish depleted cycle intermediates, and in the present case, anaplerotic precursors should increase the pool of oxaloacetate available for condensation with acetyl-CoA to form citrate. While the importance of anaplerosis in ketone body metabolism of the heart has not been investigated before, there is evi-

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1. *Abbreviations used in this paper:* CoASH, free coenzyme A; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure.

dence to suggest that the utilization of substrates that can enrich the citric acid cycle (e.g., lactate and asparagine) may play a critical role in maintaining mechanical function (2).

The first objective of the present study was then to determine whether early changes in the activity of 2-oxoglutarate dehydrogenase as reflected by changes in citric acid cycle intermediates precede the changes in contractile performance in hearts oxidizing ketone bodies. The second goal of this study was to define further the mechanisms responsible for contractile failure during metabolism of acetoacetate as a sole substrate as well as the mechanisms responsible for reversing the failure with competing substrates. The current findings indicate that inhibition of 2-oxoglutarate dehydrogenase does indeed antedate a fall in mechanical performance and that anaplerotic mechanisms that can replenish the citric acid cycle downstream to 2-oxoglutarate dehydrogenase thereby providing oxaloacetate for citrate synthetase play a critical role in generating sufficient flux through spans of the citric acid cycle to maintain sufficient energy production to sustain contractile performance of the heart.

Methods

Hearts from anesthetized (150 mg pentobarbital/kg body weight, i.p.), heparinized (250 U, i.v.) male Sprague-Dawley rats (250–300 g) were excised and placed in ice cold Krebs-Henseleit buffer (9) (Ca^{2+}, 2.5 mM). To flush out blood and allow resumption of contractions, we cannulated the hearts by the aorta and perfused them retrogradely (10) for 5 min with substrate-free Krebs-Henseleit buffer equilibrated with 95% O_2/5% CO_2. The left atrium was then cannulated and the hearts were switched to a recirculating working heart circuit (1) with a preload of 15 cm H_2O and an afterload of 140 cm H_2O. The perfusate in the working heart circuit contained the substrates listed in the tables and figures. Hearts were perfused as working hearts for 5 min before any measurements to allow for stabilization.

Hearts were perfused for 5, 10, 15, 20, or 60 min in the working mode. Hearts perfused for 60 min had a pressure transducer attached to a sidearm of the aortic cannula to record aortic pressure and heart rate. Hearts perfused for 5, 10, 15, or 20 min had a catheter (PE-90; i.d., 0.86 mm; o.d., 1.27 mm) introduced into the left ventricular cavity through the lumen of an 18-gauge needle inserted into the apex. To monitor for any early changes in performance, we attached the catheter to a pressure transducer and measured left ventricular developed pressure (LVDP), left ventricular end diastolic pressure (LVEDP), the first derivative of pressure with respect to time ($+dP/dt_{max}$ and $-dP/dt_{max}$) and heart rate. The high-frequency response cutoff for the differentiator was set at 30 Hz, the setting at which recorder output was not appreciably changed by further adjustment. In addition to pressure measurements, aortic and coronary flows were determined for hearts at the time points indicated in the figures and tables.

Hearts were freeze clamped by aluminum tongs cooled to the temperature of liquid nitrogen (11) after the 5 minutes of retrograde perfusion (time point, −5) or after perfusion in the working mode for the time points indicated in the tables and figures. Hearts were subsequently stored at −70°C until the time of assay. Extraction of the freeze-clamped tissue was performed according to the method of Williamson and Corkey (12) and assayed fluorimetrically for pyruvate, CoASH, acetyl-CoA, and succinyl-CoA (12) and spectrophotometrically for lactate, citrate, 2-oxoglutarate, glutamate, succinate, malate, and aspartate (13).

Values are reported as the mean±SEM. All tissue metabolites are expressed as micromoles per gram dry weight. Student's t test or one-way analysis of variance with Dunnett's test were used to test for statistical significance where appropriate (14).

Results

After 60 min of perfusion, hearts utilizing glucose had a cardiac output of 159.8±17.3 ml/min per g dry, whereas the cardiac output for hearts oxidizing acetoacetate was 52% of that for glucose perfused hearts (Table I). The lower cardiac output for hearts utilizing acetoacetate was mirrored by both decreased systolic and diastolic pressures. After 60 min of perfusion when contractile performance had fallen for hearts perfused with acetoacetate, the tissue content of 2-oxoglutarate as well as its transamination product glutamate were increased when compared to hearts utilizing glucose (Table II); at the same time, the tissue content of succinyl-CoA was decreased by 85%.

In addition to the changes in metabolites immediately upstream and downstream from the 2-oxoglutarate dehydrogenase reaction, hearts perfused with acetoacetate for 60 min had an increased tissue content of malate and a decreased level of aspartate (Table II). Activation of anaplerotic pathways is suggested by the increase in the tissue content of malate and the fall in the level of aspartate which is the transamination product of oxaloacetate. The conversion of aspartate to oxaloacetate represents one such anaplerotic pathway. Malate, which is at near-equilibrium with oxaloacetate (15), would be elevated either directly by the anaplerotic mechanism mediated by NADP-dependent malic enzyme (16) or by the enrichment of oxaloacetate by aspartate. The tissue content of acetyl-CoA in hearts utilizing acetoacetate was 176% higher than in hearts utilizing glucose (Table II). A complementary fall in the tissue content of CoASH of 59% was also seen in acetoacetate perfused hearts when compared to hearts utilizing glucose as a sole substrate.

If hearts were perfused with either acetoacetate or glucose as the sole substrate for short periods of time (≤ 20 min), the two groups had similar developed pressures, LVEDP, negative dP/dt_{max} and cardiac output (Fig. 1). This finding is in keeping with observations in hearts perfused for 60 min when there was no statistically significant difference in hemodynamic parameters until 45 min (data not presented). In spite of the similarity of left ventricular function for hearts utilizing glucose or acetoacetate during the first 20 min of perfusion, changes in citric acid cycle intermediates begin to occur as early as 5 min. While the tissue content of glutamate, 2-oxoglutarate, and succinyl-CoA remained relatively constant over the first 20 min of perfusion when glucose was the sole substrate (Fig. 2), the tissue contents of glutamate and 2-oxoglutarate were elevated in acetoacetate perfused hearts by 5 and 15 min, respectively. At the same time, succinyl-CoA fell in hearts oxidizing acetoacetate after 10 min of perfusion. The differences in glutamate, 2-oxoglutarate,

Table I. Hemodynamic Characteristics of Isolated Working Rat Hearts after 60 min of Perfusion with Either Glucose or Acetoacetate as the Sole Substrate

Substrate	Aortic pressure		Cardiac output
	Systolic	Diastolic	
	mmHg		ml/min·g dry
Glucose (5 mM)	118.6±2.4	94.2±1.7	159.8±17.3
Acetoacetate (5 mM)	93.1±7.8*	76.0±5.9*	81.6±11.8*

Mean±SEM; * $P < 0.005$, $n = 10$ for both groups.

Table II. Tissue Metabolites from Isolated Working Rat Hearts after 60 min of Perfusion with Either Glucose or Acetoacetate as the Sole Substrate

Substrate	Citrate	2-Oxoglutarate	Glutamate	Succinyl-CoA	Malate	Aspartate	CoASH	Acetyl-CoA
Glucose (5 mM)	1.56±0.22	0.253±0.059	12.96±0.67	0.348±0.026	0.477±0.076	5.18±0.42	0.397±0.038	0.055±0.012
Acetoacetate (5 mM)	1.03±0.26	0.475±0.104*	21.25±0.98‡	0.053±0.021‡	0.757±0.116*	2.96±0.40‡	0.163±0.029‡	0.152±0.024‡

Mean±SEM, all values reported as micromoles per gram dry weight; * $P < 0.05$, ‡ $P < 0.001$, $n = 10$ for both groups.

and succinyl-CoA between the two groups persisted through 20 min of perfusion in the working mode and were similar to the changes seen at 60 min of perfusion after the decline in mechanical function appeared.

As with hearts perfused for 60 min, utilization of acetoacetate for shorter periods of time resulted in changes in malate and aspartate when compared to hearts utilizing glucose as a sole substrate (Fig. 3). The tissue content of malate was consistently higher in hearts oxidizing acetoacetate at 10 min of perfusion. In contrast to tissue glutamate and malate, aspartate levels in acetoacetate perfused hearts fell dramatically and remained at 23–40% of the value for glucose perfused hearts. The difference in aspartate levels was also detected as early as 10 min into perfusion. An unexpected finding was that there was an initial fall in aspartate for perfused hearts regardless of the substrate. Although tissue aspartate values stabilized after 5 min of perfusion for hearts utilizing glucose, aspartate content for hearts perfused with acetoacetate continued to fall such that by 20 min the tissue aspartate content was only 9.7% of the

initial value following Langendorff perfusion. Additionally, the tissue content of citrate in hearts perfused with acetoacetate increased above glucose utilizing hearts as early as 5 min. By 20 min of perfusion, the tissue content of citrate in hearts perfused with acetoacetate was 6.7-fold higher than that of glucose perfused hearts.

The changes in the [lactate]/[pyruvate] ratio (L/P) during the first 20 min of perfusion are shown on the bottom graph of Fig. 3. Small and relatively minor changes in the redox state as expressed by the L/P were similar for both groups. Together with the fact that the perfusion is a recirculating preparation (which allows β-hydroxybutyrate released by the heart to compete with acetoacetate as a reducing substrate) argues against either acidosis or some significant change in the redox state being responsible for the observed fall in mechanical performance.

Fig. 4 depicts the early changes in CoASH and acetyl-CoA in hearts oxidizing glucose or acetoacetate as a sole substrate. As early as 5 min into perfusion, there is a significantly greater amount of CoASH (0.282±0.020 vs. 0.160±0.019 μmol/g dry weight) in hearts utilizing glucose. This difference becomes even more dramatic with time with CoASH levels in hearts oxidizing acetoacetate falling from 57% of the value for glucose perfused hearts at 5 min to 36% of the corresponding value found in hearts perfused with glucose after 20 min of perfusion.
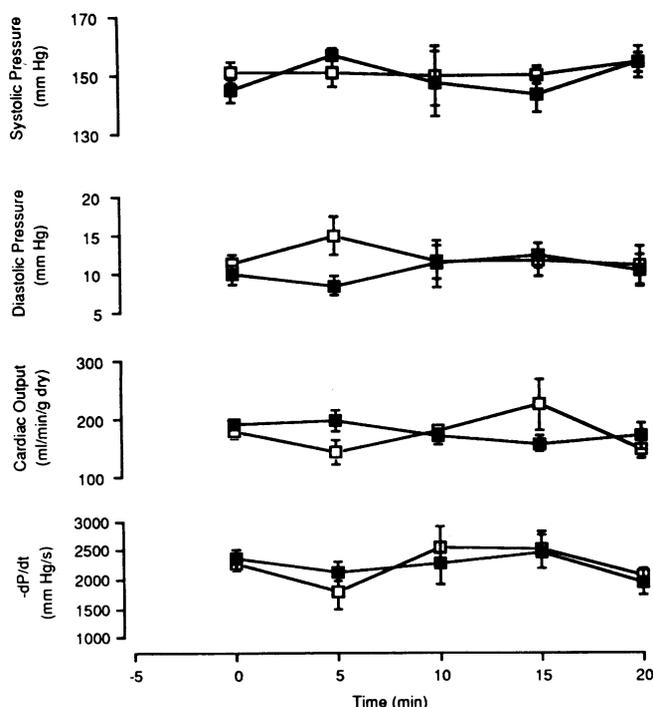
Figure 1. Hemodynamic characteristics of hearts perfused with glucose or acetoacetate. After retrograde perfusion (time, −5), isolated rat hearts were perfused as a working preparation with either glucose (5 mM, open squares) or acetoacetate (5 mM, solid squares) with a catheter inserted into the left ventricular cavity to monitor contractile parameters. Values reported as mean±SEM, $n = 6$ for both groups.
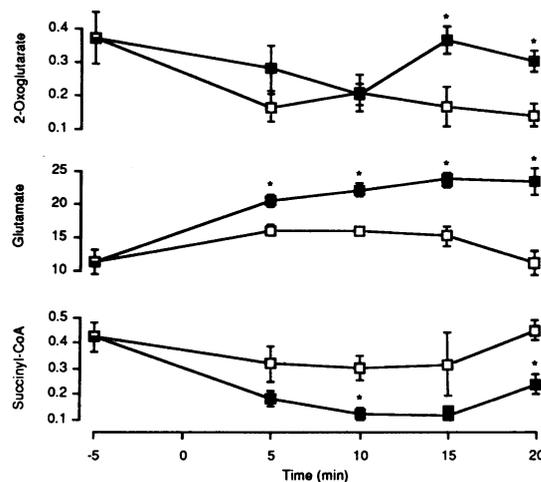
Figure 2. Heart content of metabolites affected by 2-oxoglutarate dehydrogenase with hearts perfused with glucose or acetoacetate. Hearts were perfused with either glucose (5 mM, open squares) or acetoacetate (solid squares). The experimental design is as described in Fig. 1. All values are reported as mean±SEM, $n = 6$ for both groups and the units are micromoles per gram dry weight, *$P < 0.05$ compared to glucose perfused hearts at the same time point.
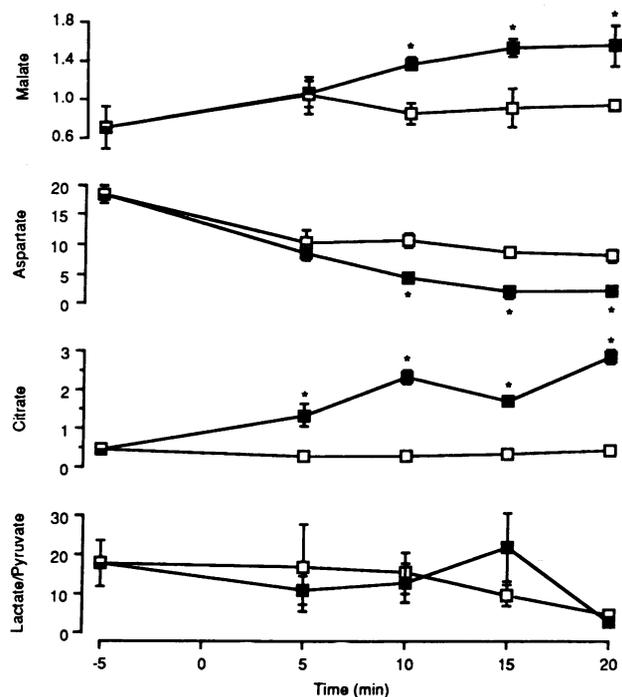
Figure 3. Intermediary metabolites from hearts perfused with glucose or acetoacetate. Hearts were perfused with either glucose (5 mM, *open squares*) or acetoacetate (*solid squares*). The experimental design is as described in Fig. 1. All values are reported as mean±SEM, n = 6 for both groups and the units are micromoles per gram dry weight, *$P < 0.05$ compared to glucose perfused hearts at the same time point.

Concurrently, the acetyl-CoA content of acetoacetate perfused hearts increased from 214% at 5 min to 375% at 20 min of the acetyl-CoA content of glucose perfused hearts. Both groups experienced an early, transient increase in the tissue content of acetyl-CoA that is most likely due to the utilization of endogenous triglyceride stores during the period of substrate-free Langendorff perfusion. This observation is supported by the
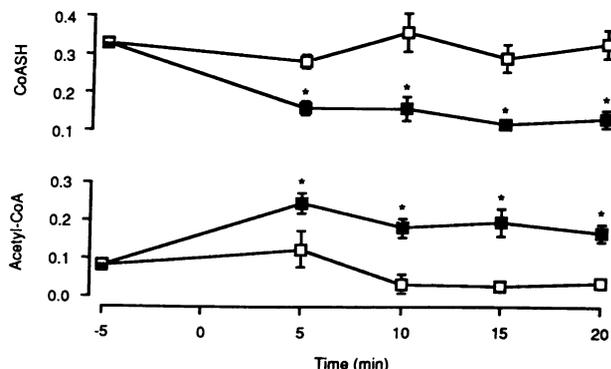
Figure 4. Tissue content of free CoASH and acetyl-CoA from hearts perfused with glucose or acetoacetate. Hearts were perfused with either glucose (5 mM, *open squares*) or acetoacetate (*solid squares*). The experimental design is as described in Fig. 1. All values are reported as mean±SEM, n = 6 for both groups and the units are micromoles per gram dry weight, *$P < 0.05$ compared to glucose perfused hearts at the same time point.

appearance of glycerol in the perfusate at the concentration of ~ 8 μM for both groups (data not presented).

Because of the dramatic fall in tissue aspartate content as well as the accumulation of malate and citrate in hearts utilizing acetoacetate, anaplerosis was assumed to play some role in the relationship between contractile function and acetoacetate metabolism. To determine the relative importance of anaplerotic pathways in maintaining stable contractile function in hearts oxidizing acetoacetate, several competing substrates were added to acetoacetate for hearts perfused for 60 minutes. To this end, hearts were perfused with acetoacetate plus either lactate (10 mM), propionate (2 mM), or acetate (10 mM). Lactate was chosen as one competing substrate because of the ability of its oxidized form, pyruvate, to enter the citric acid cycle by conversion to the C4 compound malate through the action of NADP-dependent malic enzyme. As the levels of acetyl-CoA and CoASH indicate a dramatic increase in the [acetyl-CoA]/[CoASH] ratio in hearts oxidizing acetoacetate, pyruvate dehydrogenase (PDH) is likely to be only minimally active (17) and very little lactate should enter the citric acid cycle via the action of PDH. Propionate was chosen as a anaplerotic substrate because of its ability to enter the citric acid cycle immediately downstream from 2-oxoglutarate dehydrogenase as succinyl-CoA. A final substrate to be added to acetoacetate was acetate. This particular substrate does not enter the citric acid cycle through anaplerotic pathways, rather it is activated by acetyl-CoA synthase to acetyl-CoA which can then condense with oxaloacetate to form citrate. The purpose of adding acetate as a competing substrate was to determine the effect of a second source of acetyl-CoA on contractile performance.

Fig. 5 represents the pressure-volume work at 60 min for hearts utilizing acetoacetate (5 mM) either alone or with one of the three competing substrates. Pressure-volume work as determined by the product of the mean aortic pressure and cardiac output normalized to the dry weight of the heart is the major determinant of the external work performed by the heart (1) and is therefore an indicator of the contractile function of the left ventricle. While the addition of acetate does not improve performance significantly after 60 min of perfusion, the addition of either lactate or propionate improved function by 94 and 85%, respectively.

Despite the improvement in performance with the addition of either lactate or propionate there was no improvement in
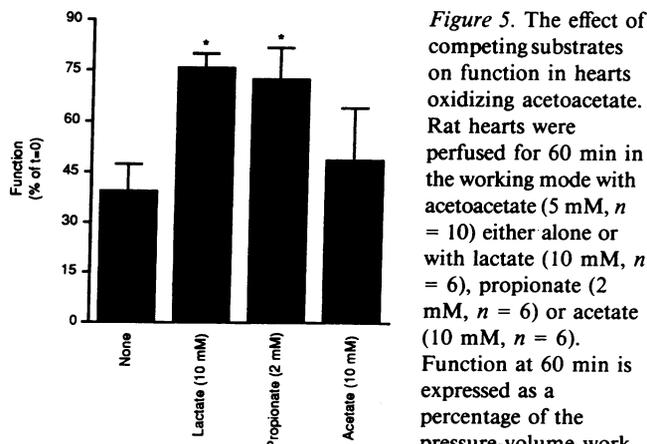
Figure 5. The effect of competing substrates on function in hearts oxidizing acetoacetate. Rat hearts were perfused for 60 min in the working mode with acetoacetate (5 mM, n = 10) either alone or with lactate (10 mM, n = 6), propionate (2 mM, n = 6) or acetate (10 mM, n = 6). Function at 60 min is expressed as a percentage of the pressure-volume work performed at the beginning of the perfusion. Results reported as mean±SEM, *$P < 0.05$.

flux through 2-oxoglutarate dehydrogenase as suggested by the further accumulation of 2-oxoglutarate when compared to hearts utilizing acetoacetate as a sole substrate (Table III). The tissue content of 2-oxoglutarate for hearts utilizing acetoacetate and acetate was also similar to that for acetoacetate perfused hearts. In addition, the tissue content of glutamate was similar irrespective of the addition of competing substrate to acetoacetate.

When either lactate or propionate was present with acetoacetate, there were changes in the levels of certain metabolites that were consistent with enrichment of the citric acid cycle by anaplerosis. Addition of lactate resulted in a 2.3- and a threefold increase in malate and aspartate respectively (Fig. 6). The tissue content of citrate for hearts perfused with lactate in addition to acetoacetate was 3.7 times greater than the citrate content of hearts perfused with acetoacetate alone. If propionate was present along with acetoacetate, the tissue content of succinate, malate, and citrate rose by 79, 585, and 778%, respectively. The addition of acetate to acetoacetate had no significant effect on the tissue content of succinate, malate, aspartate, or citrate. Unexpectedly, the addition of propionate, in contrast to lactate, did not increase the tissue content of aspartate.

Discussion

Although impairment in myocardial contractile function by acetoacetate was first described almost 30 years ago (18), there have been very few studies either in vivo or in vitro to characterize the metabolic component of this mechanical failure (2, 19, 20). The study by Breuer et al. (20) in the instrumented, anesthetized dog represents the only in vivo study attempting to determine if ketone bodies, if present as a sole substrate, would impair contractile function. Although the investigators were unable to demonstrate impairment of function, the use of insulin/ketone body infusions in their experiments to produce severe hypoglycemia (1.2–1.8 mM) and ketonemia (1.3–3.3 mM) cannot completely remove other competing substrates that may improve function. Further, insulin infusions have been shown to have positive inotropic effects in the mammalian myocardium (21). Likewise, other in vivo studies (22) have failed to demonstrate impaired function during the peripheral infusion of acetoacetate because competing substrates were always present. In the same context, it is of interest that Zimmermann et al. (18), after their initial observation on the reversible negative inotropic effect of acetoacetate in the isolated rat heart, were also able to show the same phenomenon in the cross-clamped dog heart perfused in situ with acetoacetate. Because it is technically impossible to have complete control over

the substrates presented to the heart in vivo, the isolated perfused working heart preparation was utilized in the present study not only to document the inability of ketone bodies to support full contractile activity but also to determine how competing substrates improve function in hearts utilizing ketone bodies.

The present study focuses on two aspects of the relationship between function and metabolism in the mammalian heart utilizing ketone bodies, namely the temporal changes in tissue metabolites as they relate to function, and based on these findings, the critical role anaplerotic pathways play in supplementing citric acid cycle flux past the block at 2-oxoglutarate dehydrogenase. The results indicate that the inhibition of 2-oxoglutarate dehydrogenase occurs by 10 min during the utilization of acetoacetate as a sole substrate and before any functional changes are manifest. That inhibition of 2-oxoglutarate dehydrogenase precedes contractile dysfunction argues for a mechanism in which functional changes, in part, are caused by the decreased flux through the citric acid cycle rather than decreased flux resulting from decreased cardiac work (4). The present findings are important in light of the earlier report by Cooney et al. (4) who have argued that flux through 2-oxoglutarate dehydrogenase is the rate limiting step for flux through the citric acid cycle flux in rat heart.

Two hypotheses have been advanced to explain the inability of acetoacetate to maintain contractile function. The first concerns the sequestration of CoASH as acetyl-CoA and acetoacetyl-CoA thereby limiting the amount of CoASH available as cofactor for 2-oxoglutarate dehydrogenase (2). While the present study confirms an early and sustained fall in the tissue content of CoASH if acetoacetate was the sole substrate, the effective intramitochondrial concentration of CoASH is ~ 1 mM (23) which is well above the apparent K_m CoA of 25 μM (24). The above estimates of intramitochondrial CoASH assume that the distribution of CoASH remains 5% cytosolic/
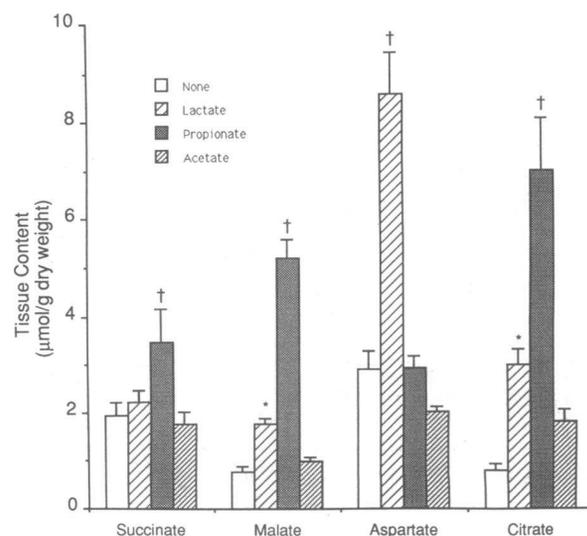
Figure 6. Citric acid cycle intermediates in hearts perfused for 60 min with acetoacetate and a competing substrate. Perfusions were performed as described in Fig. 5 with the competing substrates present at the concentration listed. Metabolites are reported as micromoles per gram dry weight and represent the mean±SEM, *$P < 0.05$, †$P < 0.01$.

95% mitochondrial. It must be emphasized that the effect of binding of CoASH to mitochondrial protein has not been taken into account, although ~ 98% of free CoASH would have to be associated with protein to bring the effective mitochondrial concentration of free, unbound CoASH down to 25 μM. Whereas it has been shown that CoASH can be transported into the mitochondria by a process that is dependent on the mitochondrial membrane electrical gradient (25, 26), the rate of efflux of CoASH into the cytosolic compartment is unknown (although under the conditions used in the present study, it is unlikely there is a dramatic shift in CoASH out of the mitochondrial matrix).

The second theory that has been advanced to explain decreased citric acid flux mediated by ketone body metabolism concerns the activation of acetoacetate to acetoacetyl-CoA. Based on computer modeling of citric acid cycle flux, a majority of succinyl-CoA, the compound that donates its coenzyme A moiety to acetoacetate, remains in the citric acid cycle forming succinate via the action of succinyl-CoA synthase (5-7, 27, 28). As a result, insufficient amounts of acetoacetate are activated and therefore insufficient amounts of acetyl-CoA enter the citric acid cycle. The present study would suggest the opposite, because acetyl-CoA levels increase after only 5 min of utilization of acetoacetate and remain elevated throughout the entire perfusion period.

Known modulators of 2-oxoglutarate dehydrogenase include succinyl-CoA (29), Ca^{2+}, and ATP (30). Whereas it is unknown what changes occur in mitochondrial Ca^{2+} and ATP content, oxidation of acetoacetate causes a fall in succinyl-CoA, indicating that feedback inhibition is not playing a part in decreased flux. The present investigation can offer no further mechanisms to explain the inhibition of flux through 2-oxoglutarate dehydrogenase, which appears to be the first, and possibly most important, metabolic insult, in causing a decline in contractile performance. It does, however, bear out the important role enrichment of the citric acid cycle by C3 units plays in stabilizing contractile performance. At least two anaplerotic pathways appear to be vital in maintaining function during the oxidation of acetoacetate. Both of these pathways share the common characteristics of feeding into the citric acid cycle in the second span from succinyl-CoA to oxaloacetate, that is, the span beginning immediately after the reaction catalyzed by 2-oxoglutarate dehydrogenase.

Measurements of arterio-venous differences performed by Breuer et al. (20) in the dog heart utilizing acetoacetate in vivo indicate a positive correlation between the arterial concentrations of ketone bodies and the citric acid cycle intermediates citrate, aconitate and 2-oxoglutarate, and a negative correlation between arterial ketone body concentrations and oxaloacetate concentrations. Whereas the findings must be considered qualitative, they are consistent with inhibition of 2-oxoglutarate dehydrogenase.

As was shown by the present study and others, the utilization of propionate increases both succinate and malate in rat hearts (31). As malate is in near equilibrium with oxaloacetate through malate dehydrogenase (15), the addition of propionate causes the accumulation of oxaloacetate facilitating further condensation of oxaloacetate with acetyl-CoA derived from acetoacetate. This allows citric acid cycle flux to continue up to the reaction catalyzed by 2-oxoglutarate dehydrogenase. The

additional flux through supplementation by propionate is supported by the finding of further accumulation of 2-oxoglutarate above that for hearts utilizing acetoacetate alone.

Unlike propionate, lactate can enter the citric acid cycle as pyruvate following pathways of either oxidative decarboxylation via PDH or carboxylation to either malate via NADP-dependent malic enzyme or oxaloacetate via pyruvate carboxylase. During the oxidation of acetoacetate when the [acetyl-CoA]/[CoASH] increases 10-fold, the anaplerotic pathway will predominate as PDH activity is reduced by 80-85% (17). Although pyruvate carboxylase is thought to have only a low, but measurable, activity in heart muscle (32), significant enrichment of the C6 of citrate from [1-^{14}C] pyruvate occurs during utilization of glucose, pyruvate and acetate (33). The label in C6 of citrate under these conditions can only come from pyruvate after carboxylation. The above two findings can be reconciled by the existence of another mechanism for pyruvate carboxylation. It has been suggested that NADP-dependent malic enzyme is responsible for pyruvate carboxylation observed in heart muscle (16). The possible anaplerotic role for NADP-dependent malic enzyme is supported by the distribution of malate, 2-oxoglutarate, isocitrate, and pyruvate across the mitochondrial membrane (34).

In the present study, it appears likely that pyruvate carboxylation is indeed occurring at a higher rate than the data of Davis et al. suggest (32). The tissue content of both malate and aspartate are increased consistent with enrichment of the citric acid cycle pool of malate and oxaloacetate and equilibration with aspartate. In addition, citrate and 2-oxoglutarate are also elevated suggesting that entry of lactate into the C4 pool of the citric acid cycle provides further substrate necessary for continued flux through citrate synthase and up to the inhibited 2-oxoglutarate dehydrogenase. Further work with [1-^{14}C] pyruvate will be necessary to characterize more fully the mechanism of pyruvate carboxylation in the heart perfused with acetoacetate.

In contrast to both lactate and propionate, acetate did not improve performance in hearts utilizing acetoacetate. Acetate alone has been shown to be utilized with no significant functional impairment in isolated working rat hearts (2) but, as was demonstrated in the present study, it does not enrich the citric acid cycle pool content of malate or citrate as it is converted directly to acetyl-CoA by acetyl-CoA synthase. The results indicate that acetate does not have a deleterious effect in and of itself in hearts utilizing acetoacetate, rather it does not provide a source for the necessary C4 units to support continued flux through the span of the citric acid cycle from oxaloacetate to 2-oxoglutarate.

In summary, the present study demonstrates early changes in tissue metabolites in the isolated working rat heart oxidizing acetoacetate that suggest a metabolic disruption of the citric acid cycle that precedes (and most likely is responsible for) mechanical failure of the left ventricle. While the cause of the inhibition of 2-oxoglutarate dehydrogenase still remains unknown, the results indicate that enrichment of the citric acid cycle by anaplerotic pathways is necessary to provide a sufficient supply of C4 carbon units entering the citric acid cycle to maintain flux through essential spans of the citric acid cycle in an attempt to compensate for diminished flux through 2-oxoglutarate dehydrogenase.

Acknowledgments

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