Malonyl-CoA metabolism in cardiac myocytes

Christian HAMILTON and E. David SAGGERSON¹

Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

(1) Malonyl-CoA is thought to play a signalling role in fuel-selection in cardiac muscle, but the rate at which the concentration of this potential signal can be changed has not previously been investigated. (2) Rapid changes in cellular malonyl-CoA could be observed when rat cardiac myocytes were incubated in glucose-free medium followed by re-addition of 5 mM glucose, or when cells were transferred from a medium containing glucose to a glucose-free medium. On addition of glucose, malonyl-CoA increased by 62 % to a new steady-state level, at a rate of at least 0.4 nmol/g dry wt. per min. The half-time of this change was less than 3 min. After removal of glucose the malonyl-CoA content was estimated to decline by 0.43–0.55 nmol/g dry wt. per min. (3) Malonyl-CoA decarboxylase (MDC) is a possible route for disposal of malonyl-CoA. No evidence was obtained for a cytosolic activity of MDC in rat heart where most of the activity was found in the mitochondrial

fraction. MDC in the mitochondrial matrix was not accessible to extramitochondrial malonyl-CoA. However, approx. 16 % of the MDC activity in mitochondria was overt, in a manner that could not be explained by mitochondrial leakage. It is suggested that this, as yet uncharacterized, overt MDC activity could provide a route for disposal of cytosolic malonyl-CoA in the heart. (4) No activity of the condensing enzyme for the fatty acid elongation system could be detected in any heart subcellular fraction using two assay systems. A previous suggestion [Awan and Saggerson (1993) *Biochem. J.* **295**, 61–66] that this could provide a route for disposal of cytosolic malonyl-CoA in heart should therefore be abandoned.

Key words: fatty acid elongation, malonyl-CoA decarboxylase, mitochondria.

INTRODUCTION

Inhibition, by malonyl-CoA, of the overt form of carnitine palmitoyltransferase (CPT₁) that is associated with the outer membrane of mitochondria has, in conjunction with acetyl-CoA carboxylase (ACC), a pivotal role in tissue fuel-homoeostasis. This concept was originally developed in the context of reciprocal regulation of long-chain fatty acid oxidation and synthesis in the liver [1]. More recently it has emerged that the ACC/malonyl-CoA/CPT₁ system is also important in the regulation of fuel selection in muscle. Heart and skeletal muscle mitochondria contain a CPT₁ isoform that is distinct from liver CPT₁ [2] and is substantially more sensitive to inhibition by malonyl-CoA [3–5]. Muscle tissues also possess a distinct isoform of ACC [6–9]. In an active lipogenic tissue, such as the liver, malonyl-CoA is a metabolic pathway intermediate for fatty acid biosynthesis. By contrast, in heart [10] and skeletal muscles [11] the activity of fatty acid synthase is very low and, despite the suggestion that there may be a small usage of malonyl-CoA for fatty acid elongation in heart [10], it is clear that rather than being a major biosynthetic intermediate malonyl-CoA is essentially a ‘signalling molecule’. In accord with this proposed signalling role, cardiac malonyl-CoA content changes with physiological or pathophysiological condition. In the long-term, heart malonyl-CoA is decreased during the first week after birth [12,13] and during starvation [14]. In the shorter term, in perfused rat heart, malonyl-CoA is increased by insulin [10] (an effect which is opposed by palmitate or adrenaline [10]) and by dichloroacetate [15], whereas it is decreased during ischaemia [16] and, particularly, during post-ischaemic reperfusion [13,16].

For malonyl-CoA to play a convincing role as a signalling molecule, its cellular concentration ought to be susceptible to rapid modulation through changes in the rates of its formation and/or removal. Whilst the role of ACC in the formation of malonyl-CoA is accepted to be firmly based, far less is known about the disposal of malonyl-CoA in muscle tissues. One possibility is that malonyl-CoA is decarboxylated by malonyl-CoA decarboxylase (MDC). If this were the case an extra-mitochondrial MDC activity would have to be present, and information concerning this possibility is incomplete. Two isoforms of MDC have been identified in the goose uropygial gland [17], one targeted to the mitochondrial matrix and the other to the cytosol. Previous studies of MDC in mammals have concentrated on an activity in the liver mitochondrial matrix [18–20] although some soluble MDC activity has been observed in extracts of skeletal muscle, which could not be attributed to mitochondrial leakage [11]. Recently Dyck et al. [13] demonstrated a high activity of MDC in rat and rabbit heart, which increased during postnatal development and appeared to be controlled by protein phosphorylation/dephosphorylation. However, the subcellular distribution of this heart MDC activity was not documented in the study by Dyck et al. [13].

In the present study we used suspensions of cardiac myocytes to investigate the dynamics of changes in malonyl-CoA content in the heart, and established that rapid depletion and restoration of this metabolite occurs when glucose supply is manipulated. We subsequently investigated the possibility of a cytosolic MDC activity in the heart. We found no evidence for this, but did observe that a proportion of the MDC that was associated with the mitochondrial fraction was overt and therefore, in principle,

Abbreviations used: ACC, acetyl-CoA carboxylase; CPT₁, the overt form of carnitine palmitoyltransferase; KHB, Krebs–Henseleit bicarbonate; MDC, malonyl-CoA decarboxylase.

¹ To whom correspondence should be addressed (e-mail saggerson@biochem.ucl.ac.uk).

may be able to decarboxylate cytosolic malonyl-CoA. We also re-investigated the possibility that elongation of fatty acyl-CoA chains might provide a route for disposal of cytosolic malonyl-CoA in heart [10]. We found that the activity reported previously [10] as being elongation activity was unlikely to be so.

MATERIALS AND METHODS

Chemicals

These were obtained and used as described in [11] and [21].

Animals

Male Sprague–Dawley rats (250–300 g) were bred and maintained at University College London at 20–22 °C with constant access to drinking water and Rat & Mouse Breeding Diet (Special Diet Services, Witham, Essex, U.K.). The diet contained (by wt.) 21 % protein, 39 % starches and sugars and 4 % fat. The light/dark cycle was 13 h/11 h with light from 06:00–19:00 h.

Isolation, incubation and extraction of rat cardiac myocytes

This involved disaggregation of the heart by collagenase, essentially as described in [22] and outlined in [21]. The cells were then washed six times in fresh medium and allowed to settle under gravity for 5 min. Disaggregation and washing media contained fatty acid-poor bovine albumin (20 mg/ml) and, normally, 10 mM glucose. Cells obtained after the final wash were normally resuspended in Krebs–Henseleit bicarbonate (KHB) medium containing 1.3 mM CaCl_2 , 5 mM glucose and fatty acid-poor bovine albumin (20 mg/ml), before distribution into incubation flasks. In one series of experiments myocyte preparations were depleted of extracellular glucose. This was done by using only 5 mM glucose in the disaggregation medium and omitting glucose entirely from the last three washes and from the medium in which the cells were resuspended before distribution into incubation flasks. Preparations with less than 70 % viable cells were discarded. Viable cells exhibited a typical rod shape, had visible cross-striations and excluded Trypan Blue.

Myocytes were incubated in plastic scintillation vials at 37 °C under O_2/CO_2 (19:1) in KHB medium containing fatty acid-poor bovine albumin (20 mg/ml). Other additions and incubation times are indicated in the Figure and Tables. Incubations were terminated by the addition of ice-cold HClO_4 to a final concentration of 6 % (w/v). After standing on ice for 30 min the acid-soluble extract was separated from the acid-insoluble pellet by centrifuging for 10 min at 2000 g_{av} . Subsequently, 5 M Bis-Tris propane (pH 6.0) was added to the acid-soluble extract (0.1 ml/ml) followed by final adjustment of the pH of the extract to 6.0 with KOH (10 M and then 1 M). After standing on ice for 30 min, the tubes were centrifuged as above and the supernatants were collected for the assay of malonyl-CoA, which was performed on the same day. The acid-insoluble pellets were treated with alkali to liberate CoA-SH and carnitine from long-chain fatty acyl-CoAs and long-chain acylcarnitines, respectively, and then neutralized and centrifuged as described in [10]. The resulting supernatants were stored at –70 °C for a few days before assaying for CoA-SH and carnitine.

Perfusion, freeze-clamping and extraction of hearts

Heart perfusion at 37 °C using a recirculating Langendorff technique was performed as described by Awan and Saggerson [10] in KHB medium containing 5 mM glucose and fatty acid-poor bovine albumin (20 mg/ml). After 45 min of perfusion, hearts were removed from canulae, immediately crushed between

stainless steel plates precooled in liquid nitrogen and ground to a fine powder under liquid nitrogen. The powder was extracted with ice-cold HClO_4 [6 % (w/v)] to give acid-soluble and -insoluble extracts, which were then treated as described above.

Subcellular fractionation

Fractions enriched in heart mitochondria, sarcoplasmic reticulum and cytosol were isolated at 0–4 °C, as described previously [23]. Hearts were rapidly removed and placed into ice-cold 10 mM NaHCO_3 (pH 7.4) containing 4 mM dithiothreitol (buffer A). The hearts were minced with scissors and washed with 10 vol. of buffer A, followed by homogenization in a further 10 vol. of the same buffer using three 10 s bursts of a Polytron homogenizer. The homogenate was centrifuged for 15 min at 330 g_{av} and the resulting supernatant was centrifuged for 15 min at 5000 g_{av} to yield the mitochondrial fraction, which was resuspended in buffer A. The supernatant was centrifuged for 30 min at 23 000 g_{av} to yield a crude pellet that was resuspended in buffer containing 39 mM histidine/HCl (pH 7.4), 0.6 M KCl and 4 mM dithiothreitol, and then re-centrifuged at 23 000 g_{av} for 30 min to yield the sarcoplasmic reticulum fraction (which was depleted of myofibrils). This fraction was resuspended in buffer containing 0.1 M Tris/HCl (pH 7.2), 0.25 M sucrose and 0.3 M KCl. The supernatant from the first 23 000 g_{av} centrifugation was re-centrifuged at 100 000 g_{av} for 60 min. The supernatant yielded the cytosolic fraction. All three fractions were stored at –70 °C until they were used for enzyme assays.

Liver and brain microsomes were obtained by homogenizing freshly-removed tissues in 10 vol. of ice-cold buffer containing 20 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 2 mM EGTA and 0.01 % (w/v) PMSF using a Potter–Elvehjem glass homogenizer fitted with a motor-driven Teflon pestle (radial clearance, 0.19 mm; approx. 450 rev./min). The homogenate was centrifuged at 600 g_{av} for 10 min, the resulting supernatant at 9200 g_{av} for 10 min and the second resulting supernatant at 100 000 g_{av} for 1 h to yield a microsomal fraction that was resuspended in the homogenization buffer and stored at –70 °C.

Enzyme assays

Citrate synthase (EC 4.1.4.7) [24] was assayed spectrophotometrically at 37 °C in buffer containing 0.1 M Tris/HCl (pH 8.0) and 0.1 M sucrose. NAD^+ -dependent malate dehydrogenase (EC 1.1.1.37) was assayed spectrophotometrically at 37 °C in a 1 ml reaction volume containing 0.1 M Tris/HCl (pH 8.0), 0.1 M sucrose, 5 mM NADH, 5 μM rotenone and 5 mM oxaloacetate. Lactate dehydrogenase (EC 1.1.1.27) was assayed spectrophotometrically at 25 °C [25]. MDC (EC 4.1.1.9) was assayed spectrophotometrically in a 1 ml reaction volume at 37 °C in a split-beam spectrophotometer, using a minor modification of the method of Svoronos and Kumar [26], which itself was a modification of the original method described in [27]. The reaction mixture initially contained 0.1 M Tris/HCl (pH 8.0), 0.1 M sucrose, 0.5 mM dithiothreitol, 5 μM rotenone, 10 mM L-malate, 0.5 mM NAD^+ and 8.5 units of malate dehydrogenase. This was preincubated at 37 °C for 7 min to establish an equilibrium between the malate dehydrogenase reactants, after which 4.3 units of citrate synthase were added and the preincubation continued for 1 min. Equal amounts of tissue extract were then added to both cuvettes, followed by addition of 0.3 mM malonyl-CoA to the reaction cuvette. After a further 30 s interval a linear rate of NADH formation was then measured at 340 nm over a period of approx. 10 min.

Fatty acyl-CoA elongation was determined by two methods (I and II). The method I radiochemical assay for fatty acyl-CoA

elongation was carried out as described by Prasad and Cinti [28]. Reactions were carried out at 37 °C in 1 ml of buffer containing 0.1 M Tris/HCl (pH 7.4), bovine albumin (4 mg/ml), 0.4 mM [2-¹⁴C]malonyl-CoA (0.23 µCi/µmol) and 30 µM palmitoyl-CoA, and were initiated by addition of appropriate amounts of tissue extracts. After 30 min, reactions were stopped by the addition of 0.75 ml of 2.7 M KOH in methanol, followed by saponification at 65 °C for 45 min. After acidification with 1 ml of 4 M HCl the products were extracted three times with 2.5 ml of petroleum ether (b.p. 40–60 °C). The petroleum ether fractions were pooled, transferred to glass vials and taken for liquid scintillation counting. The method II radiochemical assay for fatty acyl-CoA elongation was identical except that the solvent in the pooled petroleum ether extracts was allowed to evaporate to dryness at room temperature before the samples were taken for liquid scintillation counting. Activity of the condensing enzyme of the fatty acyl-CoA elongation system was measured spectrophotometrically at 37 °C as described previously [29], in 1 ml of buffer containing 0.1 M Tris/HCl (pH 7.4), bovine albumin (2.6 mg/ml), 40 µM palmitoyl-CoA and 0.25 mg of microsomal protein. A baseline was recorded in a split-beam spectrophotometer by scanning from 400–290 nm against an identical reference cuvette. The reaction was then initiated by addition of 60 µM malonyl-CoA to the sample cuvette, and the relative change in absorbance at 303 nm (due to the formation of an enolate–albumin complex [29]) was followed. Any change in absorbance at 350 nm was subtracted from that observed at 303 nm. Condensing enzyme activity was calculated using the molar extinction coefficient for the enolate–albumin complex of 29 mM⁻¹cm⁻¹ [29].

Isolation of heart mitochondria and measurement of oxygen uptake

Rat hearts were rapidly removed and placed in ice-cold buffer containing 8.25 mM Tris/HCl (pH 7.2), 0.21 M mannitol and 70 mM sucrose. The hearts were finely chopped with scissors and washed three times to remove blood, followed by ice-cold homogenization for only a few seconds using a Polytron homogenizer. The homogenate was centrifuged at 0–4 °C at 300 *g*_{av} and the resulting supernatant was then centrifuged for 8 min at 3000 *g*_{av} in order to pellet the mitochondria. After three cycles of gentle resuspension in buffer containing 8.25 mM Tris/HCl (pH 7.2), 0.21 M mannitol, 70 mM sucrose and fatty acid-poor bovine albumin (0.5 mg/ml), followed by centrifugation at 3000 *g* for 8 min, the resulting washed mitochondrial fraction was resuspended in ice-cold buffer containing 8.25 mM Tris/HCl (pH 7.2), 0.21 M mannitol and 70 mM sucrose.

Mitochondrial oxygen consumption was measured at 37 °C in a water-jacketed perspex chamber with an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). Measurements were carried out on duplicate samples of approx. 2 mg of mitochondrial protein in 3 ml of medium containing 10 mM Tris/HCl (pH 7.3), 120 mM KCl, 0.5 mM EGTA, fatty acid-poor bovine albumin (1.3 mg/ml), 1 mM potassium phosphate and 1 mM ADP to ensure mitochondrial State 3 conditions. Other additions are indicated in Table 4. The electrode was calibrated on the basis that 3 ml of the medium in air at 37 °C would initially contain 0.66 µmol of O₂.

Measurements of metabolite contents

Malonyl-CoA in HClO₄-soluble extracts was measured essentially by the radiochemical method described previously [30], modified for small amounts of malonyl-CoA, as described in

[11]. CoA-SH, derived from long-chain fatty acyl-CoAs, was measured by a sensitive recycling assay [31]. Carnitine derived from long-chain acylcarnitines was measured by the radiochemical method described in [32].

Measurement of myocyte dry weight and protein contents

Triplicate aliquots of myocyte stock suspensions and resuspension medium (minus cells) were heated at 70 °C in pre-weighed beakers until no further decrease in weight was observed. The dry weight of the myocytes was then obtained by subtracting the dry weight of the resuspension medium.

Protein contents of mitochondria and other subcellular fractions were measured using a bicinchonic acid ('BCA') assay kit supplied by Sigma Chemical Co. using bovine albumin as a standard.

Statistical methods

Values are presented as means ± S.E.M. Statistical significance was calculated using the Student's *t* test for paired or unpaired samples, as appropriate.

RESULTS AND DISCUSSION

Rapid turnover of malonyl-CoA in cardiac myocytes

Our objective was to estimate the speed of formation/removal of malonyl-CoA within cells. This necessitated a rapid sampling approach, which was more feasible using myocyte suspensions than perfused hearts. We were unaware of any previous measurements of malonyl-CoA in cardiac myocytes and we therefore carried out various preliminary experiments in which cells were incubated for 1 h prior to extraction (Tables 1 and 2). These were aimed at establishing whether the malonyl-CoA content of myocytes was reasonably similar to that of the whole heart, whether this could be acutely changed by agents which altered malonyl-CoA in perfused heart [10] and, in particular, which agent(s) to choose to perturb malonyl-CoA content in the time course/turnover study. On the basis that 1 g of whole heart, dry weight, is equivalent to approx. 5 g of tissue, wet weight (J. Mowbray, personal communication), we estimated that the malonyl-CoA contents of myocytes incubated with glucose or glucose + insulin for 1 h ranged from approx. 50% (Table 1) to 100% (Table 2) of the contents observed previously [10] in perfused hearts under similar conditions (although we noted

Table 1 Effects of palmitate and insulin on the contents of malonyl-CoA and fatty acyl-CoA in cardiac myocytes

Myocytes were isolated as described in the Materials and methods section followed by incubation for 1 h with 5 mM glucose and fatty acid-poor bovine albumin (20 mg/ml). Sodium palmitate (0.5 mM) and insulin (10 nM) were added as indicated. Values are means ± S.E.M. * and † indicate *P* < 0.01 and *P* < 0.005, respectively, for the effect of palmitate and ‡ indicates *P* < 0.01 for the effect of insulin (paired tests).

Incubation conditions	Metabolite content (nmol/g dry wt. of myocytes)	
	Malonyl-CoA (<i>n</i> = 6)	Fatty acyl-CoA (<i>n</i> = 4)
Control	6.7 ± 0.6	161 ± 19
With palmitate	4.3 ± 0.9*	178 ± 16
With insulin	8.4 ± 0.8‡	148 ± 15
With insulin + palmitate	5.0 ± 0.8†	177 ± 15

Table 2 Effect of glucose concentration on the malonyl-CoA content of cardiac myocytes

Cells were isolated as described in the Materials and methods section, except that glucose was omitted from the sixth and final wash of the cells. Myocytes were incubated for 60 min in KHB medium containing albumin (20 mg/ml) and the indicated concentration of glucose with or without 10 nM insulin. The values are means $\pm$ S.E.M. for 5 separate myocyte preparations. * and § indicate $P < 0.005$ and $P < 0.025$, respectively, for comparison of 1 mM glucose with zero glucose. † and || indicate $P < 0.01$ and $P < 0.005$, respectively, for comparison of 2.5 mM glucose with 1.0 mM glucose. ‡ and ¶ indicate $P < 0.01$ and $P < 0.005$, respectively, for comparison of 5 mM glucose with 2.5 mM glucose. ** and †† indicate $P < 0.05$ and $P < 0.025$, respectively, for effects of insulin. Paired tests were used throughout.

Glucose concentration (mM)	Malonyl-CoA content (nmol/g dry wt. of myocytes)	
	Without insulin	With insulin
0	6.6 $\pm$ 0.9	7.4 $\pm$ 1.7
1.0	8.5 $\pm$ 1.1*	9.6 $\pm$ 1.9§
2.5	10.6 $\pm$ 1.4†	12.1 $\pm$ 1.6 ,**
5.0	13.8 $\pm$ 1.1‡	15.3 $\pm$ 1.4¶,††

malonyl-CoA contents that were approx. one-third of those in heart in a further series of experiments, see Figure 1 below). Myocyte contents of long-chain fatty acyl-CoA (161 ± 19 nmol/g dry wt., Table 1) or of long-chain fatty acylcarnitine [335 ± 16 nmol/g dry wt. ($n = 6$), results not shown] were comparable to measurements that were made in perfused hearts at the same time as this myocyte study. In hearts perfused with 5 mM glucose, fatty acyl-CoA content ($n = 6$) was 29 ± 5 nmol/g wet wt. (equivalent to approx. 145 nmol/g dry wt.) and fatty acylcarnitine content ($n = 6$) was 81 ± 12 nmol/g wet wt. (equivalent to approx. 405 nmol/g dry wt.). In myocytes, palmitate, and to a lesser extent insulin, caused changes in malonyl-CoA that were similar to those seen in perfused heart [10]. Insulin significantly increased malonyl-CoA levels, whereas palmitate both decreased its content and abolished the effect of insulin (Table 1). Awan and Saggerson [10] showed that adrenaline halved the malonyl-CoA content of perfused heart (when glucose and insulin were present). Therefore in other preliminary experiments (results not shown) we investigated the effects of adrenergic agents with myocytes incubated for 1 h with 5 mM glucose and 10 nM insulin (malonyl-CoA content = 13 ± 1 nmol/g dry wt.). Surprisingly, there was no decrease at all in malonyl-CoA content on addition of 0.1, 0.25, 0.5 or 2 μ M adrenaline ($n = 6$). Likewise the β -adrenergic agonist isoprenaline had no effect on myocyte malonyl-CoA levels when tested at 0.1, 0.25 and 1 μ M ($n = 5$, results not shown) despite the fact that this agonist caused a significant increase in glycerol release by the cells. Finally, the α -adrenergic agonist phenylephrine also had no effect on malonyl-CoA levels when tested at 0.25, 0.5 and 1 μ M ($n = 4$, results not shown).

As shown in Table 2, changing the glucose supply caused up to a 2-fold increase in malonyl-CoA levels during 1 h incubations in the presence or absence of insulin. Since, of the agents tested, glucose caused the greatest effect on malonyl-CoA we decided to use it as the perturbing agent in time course experiments.

The experiment summarized in Figure 1 was conceived as one in which a 'glucose-free' steady-state was established, followed by addition of glucose in order to see how quickly the malonyl-CoA content increased. It was thought to be inadvisable to omit glucose from the entire procedure for isolation of the myocytes. On the other hand we wanted to ensure an initial glucose-free incubation phase. Therefore we lowered the glucose concen-

Figure 1 Time course of myocyte malonyl-CoA content following removal and re-addition of glucose

Myocytes were isolated and washed so as to deplete intracellular glucose (see the Materials and methods section). The cells were then immediately transferred to flasks containing KHB medium and albumin (20 mg/ml), but no glucose. After incubation, with sampling as indicated, 5 mM glucose was added to the remaining flasks at 20 min and incubation continued for the indicated times. The values are means $\pm$ S.E.M. of 5 independent preparations. A, B and C indicate $P < 0.025$, $P < 0.005$ and $P < 0.0005$, respectively, versus the zero time values and D indicates $P < 0.005$ versus the 20 min value (paired tests).

tration to 5 mM in the collagenase digestion and early washing stages and omitted it entirely from the last three washes (each of which took approx. 5 min as the cells were allowed to settle under gravity). Surprisingly, following these procedures the malonyl-CoA content still declined after the cells were added to the glucose-free incubation flasks, and only reached a new steady-state sometime within the following 10 min. We do not know whether this reflected an inbuilt delay in malonyl-CoA removal (because glucose units are provided from glycogen breakdown or possibly because the pathway for malonyl-CoA removal has first to be activated) or whether it might have been an effect caused by dilution of the cells into the incubation flasks. Whatever its cause, it allowed a minimum estimate of approx. 0.3 nmol/g dry wt. per min to be made of the rate at which the cellular malonyl-CoA content could be decreased. After re-introduction of 5 mM glucose at 20 min, malonyl-CoA content was unchanged for the first 3 min. This was presumably the time required for glucose transport into the myocytes followed by its intracellular metabolism and the activation/inactivation of malonyl-CoA synthesis/degradation respectively. Thereafter the malonyl-CoA content increased by 62% to a new steady-state, with a half-time of less than 3 min. A minimum estimate of the rate of increase in malonyl-CoA content during the 23–26 min time interval was approx. 0.4 nmol/g wet wt. per min. It was consistently noted that this new steady-state malonyl-CoA content was lower than the zero time value. At present we have no explanation for this, but it suggests that the balance between malonyl-CoA synthesis and disposal is more in favour of the latter after this cycle of glucose deprivation and re-addition.

A further experiment was performed in order to obtain an estimate of the rate of malonyl-CoA removal. In this case myocytes were isolated in the normal manner, with 10 mM

Table 3 Distribution of MDC, citrate synthase and lactate dehydrogenase between subcellular fractions obtained from rat heart

Heart subcellular fractions were isolated by the method of Wientzek and Katz [23], as described in the Materials and methods section. The values are means $\pm$ S.E.M. from 4 separate preparations and are expressed as percentages of the total enzyme activity that was obtained in the post-330 g supernatant.

Fraction	Malonyl-CoA decarboxylase	Citrate synthase	Lactate dehydrogenase
Mitochondrial	71 $\pm$ 2	65 $\pm$ 3	0.5 $\pm$ 0
Sarcoplasmic reticulum	5 $\pm$ 0	2 $\pm$ 0	0.5 $\pm$ 0
Cytosolic	24 $\pm$ 2	33 $\pm$ 2	99 $\pm$ 0

glucose present (see the Materials and methods section) and then incubated for 30 min in KHB/albumin medium containing 5 mM glucose. At this stage some flasks were extracted for measurement of malonyl-CoA (10.3 ± 0.8 nmol/g dry wt., $n = 7$). The contents of other flasks were centrifuged at $100 g_{av}$ for 2 min and the resulting pellets were immediately resuspended in KHB/albumin medium with or without 5 mM glucose and returned to the incubation bath. At intervals after the start of the centrifugation procedure (6 min and 12 min) these flasks were extracted for measurement of malonyl-CoA. The values obtained were: 6 min with glucose, 8.7 ± 1.4 ; 6 min without glucose, 7.8 ± 1.6 ; 12 min with glucose, 10.7 ± 1.0 ; 12 min without glucose 5.1 ± 1.0 (all values are given as nmol/g dry wt., $n = 7$). After 6 min the omission of glucose had no significant effect on the malonyl-CoA content. However, after 12 min the 52% decrease in malonyl-CoA was statistically significant ($P < 0.01$ by a paired test). These data allowed estimates of malonyl-CoA removal of 0.43–0.55 nmol per min/g dry wt. of cells.

Disposal of malonyl-CoA

Table 3 shows that although 24% of rat heart MDC activity was in the cytosolic fraction this could, most probably, all be attributed to release from the matrix of damaged mitochondria, because 33% of citrate synthase activity was present in the same fraction. Myocyte malonyl-CoA can be rapidly removed (Figure 1) and its only known metabolic source is the cytosolic enzyme ACC. This necessitates either the unlikely possibility that cytosolic malonyl-CoA can in some way access MDC in the mitochondrial matrix or that a proportion of the MDC recovered in the mitochondrial fraction (Table 3) is not a matrix enzyme but is overt, and therefore accessible to cytosolic malonyl-CoA. We have investigated both possibilities.

We prepared rat heart mitochondria by a protocol that ensures a high level of functional integrity as evidenced by good respiratory control. With only malate present as a carbon substrate these mitochondria respired at approx. 50% of their maximal State 3 rate. On further addition of a source of acetyl-CoA, such as pyruvate or acetylcarnitine, the rate of respiration was almost doubled (Table 4). By contrast, there was absolutely no increase in respiration when malonyl-CoA was added, even though this was provided at the high concentration of 1 mM. The unlikely possibility that externally-added malonyl-CoA might somehow become a substrate for the MDC activity in the mitochondrial matrix and hence provide intramitochondrial acetyl-CoA could therefore be firmly discounted.

When 'intact' heart mitochondria, freshly isolated by the same method as that used to obtain mitochondria for the respiratory studies, were incubated under conditions for assay of MDC, we observed an overt MDC activity of 1.4 ± 0.1 nmol/min per mg of mitochondrial protein. After ultrasonication of the mitochondria (ten 5 s bursts at 4 °C) the measurable MDC activity increased to

Table 4 Oxygen consumption by rat heart mitochondria under State 3 conditions

Freshly-isolated mitochondria were incubated in an oxygen electrode chamber at 37 °C for 5 min as described in the Materials and methods section. 1 mM ADP was present. The values are means $\pm$ S.E.M. from 4 separate mitochondrial preparations.

Additions to incubation	Mitochondrial oxygen consumption (nmol/min per mg of protein)
3 mM Malate	46 $\pm$ 2
3 mM Malate + 3 mM pyruvate	88 $\pm$ 2
3 mM Malate + 5 mM acetylcarnitine	84 $\pm$ 2
3 mM Malate + 1 mM carnitine + 1 mM malonyl-CoA	46 $\pm$ 2

5.9 ± 0.5 nmol/min per mg of protein ($n = 7$ in both cases). If MDC activity was all derived from the mitochondrial matrix this would mean that $26 \pm 2\%$ of these supposedly intact mitochondria were ruptured or were at least sufficiently leaky to admit malonyl-CoA. However, a significantly smaller proportion ($P < 0.001$), i.e. $11 \pm 1\%$ ($n = 7$), of the citrate synthase activity in freshly isolated mitochondria was accessible to its assay substrates. Also, 11% of NAD⁺-malate dehydrogenase activity was found to be overt prior to ultrasonication ($n = 2$). Unless 'leaky' mitochondria admit malonyl-CoA far more readily than they admit the substrates for citrate synthase and malate dehydrogenase, or MDC escapes from the matrix of damaged mitochondria far more readily than these other enzymes we have to conclude that the heart mitochondrial preparation contains MDC activity (approx. 0.8 nmol/min per mg of mitochondrial protein) that is located external to the mitochondrial inner membrane (i.e. on the outer side of the inner membrane, in the inter-membrane space or associated with the outer membrane). The other possibility that must be considered is that this MDC activity is associated with contaminating membranes of non-mitochondrial origin. Irrespective of its detailed location, the key possibility is that this MDC activity may be able to use cytosolic malonyl-CoA as its substrate.

The observation that a particulate extract from cardiac myocytes converted radiolabel from [2-¹⁴C]malonyl-CoA into light-petroleum-soluble material in an assay containing palmitoyl-CoA led Awan and Saggerson [10] to suggest that these cells contained a fatty acyl-CoA elongation pathway similar to the one that has been extensively studied in liver and brain microsomes [33]. Awan and Saggerson suggested that this pathway offered a route for disposal of cytosolic malonyl-CoA in the heart and, possibly, that the condensing enzyme of the system could be a 'sink' for the binding of malonyl-CoA. However, re-investigation now provides no evidence for the elongation pathway in heart. Using the spectrophotometric assay described

Table 5 Comparison of assay methods for fatty acyl-CoA elongation

Assays were carried out as described in the Materials and methods section. Values are means $\pm$ S.E.M. n.d., not detectable.

Tissue/subcellular fraction	Type of assay for fatty acyl-CoA elongation		
	Radiochemical I (nmol/min per mg protein)	Spectrophotometric (nmol/min per mg protein)	Radiochemical II (nmol/min per mg protein)
Heart mitochondria	0.33 $\pm$ 0.06 (<i>n</i> = 5)	n.d. (<i>n</i> = 5)	n.d. (<i>n</i> = 5)
Heart sarcoplasmic reticulum	0.52 $\pm$ 0.05 (<i>n</i> = 5)	n.d. (<i>n</i> = 5)	n.d. (<i>n</i> = 5)
Heart cytosol	0.31 $\pm$ 0.10 (<i>n</i> = 5)	n.d. (<i>n</i> = 5)	n.d. (<i>n</i> = 5)
Liver microsomes	0.58 $\pm$ 0.02 (<i>n</i> = 4)	0.44 $\pm$ 0.01 (<i>n</i> = 4)	0.39 $\pm$ 0.09 (<i>n</i> = 4)
Brain microsomes	0.83 (<i>n</i> = 1)	0.43 (<i>n</i> = 1)	0.36 $\pm$ 0.12 (<i>n</i> = 3)

in [29], which detects the condensing enzyme of the system, we found no activity in heart mitochondrial or sarcoplasmic reticulum fractions (Table 5). By contrast, significant activities were demonstrated in rat liver and brain microsomes using this assay. These were comparable to literature values (0.44 and 0.33 nmol/min per mg of protein for liver and brain microsomes respectively [33]). When the radiochemical assay described in [28], as used by Awan and Saggerson [10], was employed (radiochemical method I, Table 5) significant apparent activity was observed in heart mitochondrial and sarcoplasmic reticulum fractions, as well as activities in liver and brain microsomes, which were higher than those measured by the spectrophotometric assay. The idea that radiochemical method I was measuring some sort of artefact was strengthened by the detection of apparent elongation activity in the heart cytosolic fraction. The radiochemical assay was modified by evaporating the light-petroleum extracts to dryness before scintillation counting (radiochemical method II). Using this assay no activity was apparent in any heart fractions and the activities in liver and brain microsomes were similar to those measured by the spectrophotometric assay (Table 5). The artefact detected by the radiochemical method was extracted into light-petroleum from acid-aqueous medium and was volatile at room temperature. It is possible that it was [2-¹⁴C]acetic acid, because MDC activity in tissue fractions will generate [2-¹⁴C]acetyl-CoA. In turn, this could be deacylated enzymically or chemically (during the saponification procedure) to acetate, which will have some solubility in light-petroleum after acidification. Our inability to detect elongation activity is in accord with Hagve and Sprecher [34] who could find no evidence for formation of elongation or desaturation products after incubating cardiac myocytes with radiolabelled 18:2, 18:3, 20:4 or 20:5 fatty acids. The finding that the content of C₁₈ fatty acids relative to C₁₆ fatty acids in the heart is nearly twice that found in plasma [33] must therefore be due to selective uptake from the circulation rather than cardiac elongation activity.

Concluding remarks

Using the simple, but unphysiological, stratagem of grossly changing extracellular glucose we have been able to demonstrate that the cardiac myocyte has the enzymic machinery to rapidly raise its malonyl-CoA content. Likewise these cells are able to rapidly lower the concentration of this molecule. There should therefore be the potential for cellular malonyl-CoA to respond rapidly to fuel and hormonal signals. Unfortunately we have not been able to demonstrate any examples of the latter, because the malonyl-CoA content in our cell preparations was not par-

ticularly responsive to insulin and, unexpectedly, was unresponsive to adrenergic agents.

We demonstrated some MDC activity (approx. 16 % of the total activity) that was unlikely to have arisen from a mitochondrial matrix source, together with a clear demonstration that extramitochondrial malonyl-CoA cannot access MDC in the mitochondrial matrix. Both of these findings are in keeping with the notion that MDC could provide a route for the rapid disposal of malonyl-CoA, but considerable further work is required to characterize, locate and understand the regulation of any non-matrix form of MDC.

The conclusion that fatty acyl-CoA elongation activity is not detectable in the heart leads to the abandonment of the suggestion in [10] that the condensing enzyme of this system could act as the cellular binding 'sink' for malonyl-CoA. Therefore other undefined cellular components must constitute this 'sink', which is absolutely necessary because 'global' concentrations of malonyl-CoA in the heart are very much higher than those that inhibit heart CPT₁ [3–5,35,36].

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