REGULATION OF MALONYL-CoA CONCENTRATION AND TURNOVER IN THE NORMAL HEART

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Running title: Turnover of malonyl-CoA in rat heart

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ABBREVIATIONS

ACC, acetyl-CoA carboxylase; MCD, malonyl-CoA decarboxylase; MPE, molar percent enrichment.

FOOTNOTE

1. Mass isotopomers are designated as Mn, where n is the number of atomic mass units above the molecular weight of the unlabeled isotopomer M.

ACKNOWLEDGMENTS

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ABSTRACT

The goal of this study was to test the relationship between malonyl-CoA concentration and its turnover measured in isolated rat hearts perfused with NaH$^{13}$CO$_3$. This turnover is a direct measurement of the flux of acetyl-CoA carboxylation in the intact heart. It also reflects the rate of malonyl-CoA decarboxylation, i.e., the only known fate of malonyl-CoA in the heart.

Conditions were selected to result in stable malonyl-CoA concentrations ranging from 1.5 to 5 nmol·g wet wt$^{-1}$. The malonyl-CoA concentration was directly correlated with the turnover of malonyl-CoA, ranging from 0.7 to 4.2 nmol·min$^{-1}$·g wet wt$^{-1}$, (slope = 0.98, $r^2 = 0.94$). The $V_{\text{max}}$ activities of acetyl-CoA carboxylase and of malonyl-CoA decarboxylase exceeded the rate of malonyl-CoA turnover by two orders of magnitude, and did not correlate with either concentration or turnover of malonyl-CoA. However, conditions of perfusion which increased acetyl-CoA supply resulted in higher turnover and concentration, demonstrating that malonyl-CoA turnover is regulated by the supply of acetyl-CoA. The only condition where the activity of malonyl-CoA decarboxylase regulated malonyl-CoA kinetics was when the enzyme was pharmacologically inhibited, resulting in increased malonyl-CoA concentration and decreased turnover.

Our data show that, in the absence of enzyme inhibitors, the rate of acetyl-CoA carboxylation is the main determinant of the malonyl-CoA concentration in the heart.
INTRODUCTION

Malonyl-CoA is a key intermediate of fatty acid synthesis in lipogenic organs. It is also an inhibitor of carnitine-palmitoyltransferase I, a regulator of fatty acid oxidation in most tissues (1-3). A number of studies have documented the modulation by malonyl-CoA of fatty acid oxidation in the heart under physiological and pathological conditions, such as maturation (4), diabetes (5), increased cardiac work (6), and postischemic reperfusion (7). In the heart, malonyl-CoA formed by cytosolic acetyl-CoA carboxylase (ACC) is, as far as is known, disposed only via decarboxylation catalyzed by malonyl-CoA decarboxylase (MCD). Thus, cytosolic acetyl-CoA and malonyl-CoA are the two components of a substrate cycle which regulates carnitine palmitoyltransferase-I activity and the rate of mitochondrial fatty acid oxidation in the heart.

No information is available on the relationship between the concentration of malonyl-CoA and its turnover, i.e., with the flux of ACC in the intact heart. The rate of turnover of malonyl-CoA is likely to be regulated by the cytosolic acetyl-CoA concentration and by the activities of ACC and MCD. The concentrations of cytosolic acetyl-CoA and malonyl-CoA in the heart are much lower than the Km of the two enzymes for their respective substrates (6;8). Thus, it is unlikely that the modulation of ACC and MCD activities exerts a tight control on malonyl-CoA turnover.

It was previously shown that the myocardial content of malonyl-CoA is elevated when acetyl-CoA levels are increased by either activation of pyruvate dehydrogenase (8;9) or by perfusing the heart with octanoate instead of palmitate or oleate (10;11). The increase in malonyl-CoA content was independent of ACC activity. It is not clear whether the higher concentration of malonyl-CoA in the presence of octanoate reflected (i) an increase in acetyl-CoA carboxylation
fueled by an increase in cytosolic acetyl-CoA concentration, or/and (ii) a decrease in the flux of malonyl-CoA decarboxylation.

The goal of the present investigation was to test the hypothesis that myocardial malonyl-CoA concentration is proportional to the rate of acetyl-CoA carboxylation under physiological conditions. We recently developed a mass spectrometric technique for measuring the malonyl-CoA turnover in isolated organs, based on the time course of its labeling from NaH$^{13}$CO$_3$ (12). The malonyl-CoA concentration can also be assayed in the same samples by isotope dilution using an internal standard of [U-$^{13}$C$_3$]malonyl-CoA. We used these techniques to study the relationship between malonyl-CoA concentration in rat hearts under physiological conditions, and during pharmacological inhibition of MCD. In addition, we measured the malonyl-CoA concentration and turnover in the hearts of anesthetized pigs.
EXPERIMENTAL PROCEDURES

Materials: Chemicals and biochemicals were obtained from Sigma-Aldrich. NaH\textsuperscript{13}CO\textsubscript{3}, and [U-\textsuperscript{13}C\textsubscript{3}]malonic acid were purchased from Isotec (Miamisburg, OH). A standard of [U-\textsuperscript{13}C\textsubscript{3}]malonyl-CoA was prepared and purified by HPLC (12). Two inhibitors of malonyl-CoA decarboxylase, CBM-300864 and CBM-301940 were kindly provided by Chugai Pharma USA.

Organ perfusion experiments
Hearts from Sprague-Dawley rats (180-220g, fed, overnight-fasted, or two-day fasted) were perfused in the Langendorf mode with non-recirculating bicarbonate buffer containing combinations of substrates (glucose, lactate, pyruvate, oleate, and/or octanoate) which would induce a wide range of malonyl-CoA concentrations (Table 1). After 15 min of equilibration, the unlabeled bicarbonate in the perfusate was replaced by 40% enriched [\textsuperscript{13}C]bicarbonate, and the hearts were quick frozen after 0.5 to 30 min of tracer infusion. In some groups of experiments, other compounds (inhibitors of MCD, DMSO) were added to the perfusate as indicated in Table I.

In vivo pig experiments
Six anesthetized pigs were thoracotomized and fitted with a pump-controlled bypass between the femoral artery and the left anterior descending coronary artery (13). NaH\textsuperscript{13}CO\textsubscript{3} was infused into the bypass at a rate calculated to achieve a 15% enrichment of bicarbonate/CO\textsubscript{2} in the infused myocardial territory. The enrichment of bicarbonate/CO\textsubscript{2} was measured (12) in samples of venous
blood from the infused territory just before taking punch biopsies of the myocardium at 1, 10 and 60 min (two pigs at each time point)

*Analytical Procedures*

The concentration and $^{13}$C-labeling of malonyl-CoA were assayed as described previously (12). We developed a gas chromatography-mass spectrometric assay for acetyl-CoA carboxylase activity because, in our hands, the assay with NaH$^{14}$CO$_3$ had high blanks. Also, this assay measures non-acid-volatile radioactivity as the product and does not specifically identify malonyl-CoA. Our assay involves (i) incubating the tissue extract with acetyl-CoA and NaH$^{13}$CO$_3$ (ii) spiking the reaction mixture with an internal standard of [U-$^{13}$C$_3$]malonyl-CoA, (iii) hydrolyzing malonyl-CoA to malonate, and (iv) isotope ratio gas chromatography-mass spectrometric analysis of the di-tert-butyldimethylsilyl derivative of malonate. The assay is conducted with labeled NaH$^{13}$CO$_3$ to minimize the background resulting from traces of malonate in the tissue extract. We ran the assay on a 800 g supernatant of a tissue extract prepared by homogenizing 50 mg of powdered heart tissue in 0.15 ml of buffer containing 0.05 M Tris-HCl pH 7.5, 0.25 M mannitol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol (w/w), 1 mM DTT, and a 1:1000 dilution of mammalian cell protease inhibitor cocktail (Sigma). The 165 µl assay mixture contained 60 mM Tris acetate pH 7.5, 1 mg/ml of dialyzed fatty acid-free bovine serum albumin (Intergen), 2 mM mercaptoethanol, 5 mM magnesium acetate, 1.06 mM acetyl-CoA, and 0 or 10 mM citrate. After 2 min preincubation at 37°C, the reaction was started by adding an amount of extract containing 25 µg of protein from the supernatant of the whole tissue extract. After 4 min of incubation, the reaction was stopped with 25µl of 10% perchloric acid and the sample was spiked with 2 nmol [U-$^{13}$C$_3$]malonyl-CoA.
After centrifugation, the acid extract was brought to pH 12 with 2 M KOH and incubated at 40° for 1 hr to hydrolyze the CoA esters. After centrifuging KClO₄, the supernatant was brought to pH 1 with 1 M HCl and evaporated in a Savant vacuum centrifuge. The residue was treated to prepare di-tert-butyldimethylsilyl malonate which was assayed by gas chromatography-mass spectrometry (12). The amount of M1 malonyl-CoA formed was calculated from the M1/M3 mass isotopomer¹ ratio of malonate, and the ACC activity expressed in nmol•min⁻¹•(g wet weight)⁻¹. The activity of MCD in heart tissue was assayed by the method of Kerner and Hoppel (14). Since rapid changes in the Km of MCD have been reported in rat heart (6), we measured MCD activity at a low substrate concentration (0.01 mM malonyl-CoA; ~5 to 10% of maximal activity) and under near Vmax conditions (0.3 mM malonyl-CoA).

Calculations and statistics
For each group of perfusions with NaH¹³CO₃, the data of the ¹³C-labeling of malonyl-CoA were fitted to an exponential regression:

\[ \text{MPE} = \text{MPE}_{(t=\infty)}[1 - e^{-kt}] \]

where MPE and MPE_{(t=\infty)} are the molar percent enrichments of malonyl-CoA at any time and at infinite time, respectively. The kinetic constant of the pool, k (min⁻¹), was multiplied by the average malonyl-CoA concentration of the group to yield the turnover of the pool (nmol•min⁻¹•g⁻¹).

To test whether the MCD inhibitors significantly affected the kinetic constant of the malonyl-CoA pool, we performed a log rank test (15) (Chi-square distribution with one degree of freedom), assuming that the labeling of malonyl-CoA is described by the above exponential. Adjustment for multiple comparisons was done by Holm’s procedure (16).
RESULTS

In 8 groups of rat hearts perfused under the conditions listed in Table 1 and without inhibitors (groups 1-8), the malonyl-CoA concentrations ranged from 1.5 nmol·g⁻¹ (hearts from fed rats perfused with 1 µM epinephrine) to 5 nmol·g⁻¹ (hearts from two-day starved rats perfused with 0.8 mM octanoate). Groups 1-8 are listed in Table 1 in the order of increasing malonyl-CoA concentrations measured in the tissue. Within each group of perfusions, the malonyl-CoA concentration remained stable with the duration of the experiment (CV < 16%). The ACC and MCD activities measured in tissue extracts were not different among the groups, thus there was no correlation between malonyl-CoA concentration and enzyme activities (Table 2).

After 15 min of equilibration, we replaced the unlabeled bicarbonate in the perfusate with 40% enriched NaH¹³CO₃. Fig 1A shows the time course of malonyl-CoA labeling in three of the groups of heart perfusions (groups 1, 2, 8). The data were fitted to an exponential regression to calculate the kinetic constant and half-life of the malonyl-CoA pool. In the absence of inhibitors (groups 1-8 of Table 1), the half-lives of malonyl-CoA labeling ranged from 0.76 to 1.27 min (Fig 1). The kinetic constant of each group was multiplied by the average malonyl-CoA concentration of the group to yield the turnover of malonyl-CoA in the group. Fig 2 (solid symbols) shows the linear correlation between the concentration and turnover of malonyl-CoA in groups 1 to 8 of hearts perfused under various physiological conditions (slope = 0.98; r² = 0.94).

The effect of two inhibitors of MCD, CBM-300864 and CBM-301940 was tested in hearts perfused with buffer containing 1% DMSO. In the control group (group 6), 1% DMSO did not affect the relationship between concentration and turnover of malonyl-CoA (Fig 2). The two
inhibitors increased the concentration of malonyl-CoA (Table 2), and 30 µM CBM-300864 significantly decreased the turnover of malonyl-CoA (Fig 1B, Fig 2, open symbols).

We measured the malonyl-CoA concentration and turnover in the hearts of 6 anesthetized pigs to get data from an animal species whose metabolic rate per kg is close to that of adult humans. The malonyl-CoA concentration was 1.3 ± 0.3 nmol/g wet wt, and its turnover was 0.15 nmol·min⁻¹·g wet wt⁻¹. Thus, in live pig hearts the malonyl-CoA concentration is comparable to that of the perfused rat heart, but the rate of turnover is about 70-80% lower.
DISCUSSION

This study was conducted to gain insight on the dynamics of the malonyl-CoA pool which plays a major role in controlling fatty acid oxidation in the heart. To date, the regulation of malonyl-CoA metabolism in the heart has been studied (i) by defining the factors that modulate the activities of ACC and MCD, and (ii) by correlating malonyl-CoA concentrations with activities of carnitine-palmitoyltransferase I and with rates of fatty acid oxidation. Our study was aimed at correlating malonyl-CoA concentrations with rates of acetyl-CoA carboxylation calculated from the kinetics of labeling of malonyl-CoA from NaH\(^{13}\)CO\(_3\) in intact hearts. The turnover of malonyl-CoA, assayed with NaH\(^{13}\)CO\(_3\), is a measurement of the rate of acetyl-CoA carboxylation in the intact heart. Fig 2 shows that the malonyl-CoA concentrations in rat hearts perfused in the absence of inhibitors of MCD is directly proportional to the rate of acetyl-CoA carboxylation with a slope of 0.98 (Groups 1-8). The activities of ACC and MCD exceeded the rate of malonyl-CoA turnover by two orders of magnitude, and did not correlate with either concentration or turnover of malonyl-CoA. The only condition where the activity of MCD regulated malonyl-CoA kinetics was when the enzyme was pharmacologically inhibited, which caused an increased malonyl-CoA concentration and decreased turnover. Thus, under a wide range of physiological conditions, the rate of malonyl-CoA decarboxylation appears to adjust to the malonyl-CoA concentration.

In the heart, the only known fate of malonyl-CoA is its decarboxylation to acetyl-CoA by MCD. Therefore when the malonyl-CoA concentration is stable (as was the case in our experiments), the isotopic turnover of malonyl-CoA represents, not only the flux of acetyl-CoA carboxylation, but also the flux of malonyl-CoA decarboxylation. It was recently demonstrated that MCD
inhibition with CBM-300863 and CBM-301940 increases malonyl-CoA concentration (17). In
the present study we demonstrated that the rise in malonyl-CoA with MCD inhibition was due to
a decrease in malonyl-CoA turnover (Figs 1 and 2, compare groups 9-11 to group 6, the DMSO
control). The data of groups 9-11 no longer followed the relationship between malonyl-CoA
concentration and turnover of groups 1-8. The two inhibitors did not affect the activity of ACC
assayed in rat heart extract (not shown). It appears that the inhibitors indirectly inhibited acetyl-
CoA carboxylation by increasing product inhibition on ACC, presumably without affecting the
cytosolic acetyl-CoA concentration.

In conclusion, our data demonstrate that, in the absence of inhibitors, the rate of acetyl-CoA
carboxylation varies with the mix of substrates offered to the heart, but appears to be
independent of the ACC and MCD activities measured under Vmax conditions. There is
evidence that most of the tissue content of acetyl-CoA is mitochondrial. Therefore, the cytosolic
concentration of acetyl-CoA must be in the low µM range. Since the Km of ACC for acetyl-
CoA is much higher than the cytosolic concentration of acetyl-CoA, the latter must be a key
short-term modulator of the ACC flux, as previously hypothesized (8;10;18). Future studies will
investigate the respective roles of the various sources of cytosolic acetyl-CoA, which include not
only mitochondria, but also peroxisomes (19).
Table 1. Perfusion conditions and malonyl-CoA content of isolated rat hearts

In all groups, the non-recirculating perfusate contained 50 µM carnitine and 3 nM insulin, as well as 3% bovine serum albumin (BSA, except where indicated). The status of the rats refers to them being fed, overnight-fasted (ONF), or two-day starved (2DS). The first 8 groups of perfusions are listed in the order of increasing malonyl-CoA concentrations measured in the tissue. Group 6 (1% DMSO) is the control for groups 9, 10, 11 (inhibitors of MCD) (n = 7 for each group) (*p<0.01 compared to group 6).

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat status</th>
<th>Concentrations of compounds added to the perfusate</th>
<th>[Malonyl-CoA] nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fed</td>
<td>7.5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Fed</td>
<td>7.5</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>2DS</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fed</td>
<td>7.5</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>ONF</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>ONF</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>Fed</td>
<td>5.5</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>2DS</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>ONF</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>ONF</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>ONF</td>
<td>4</td>
<td>0.5</td>
</tr>
</tbody>
</table>
## Table 2

### Activities of ACC and MCD in perfused rat hearts

The activities, expressed in nmol min⁻¹ (g wet weight)⁻¹, were assayed in six of the groups of perfused rat hearts described in Table 1. The data are mean ± SE (n = 7 for each group).

<table>
<thead>
<tr>
<th>Group</th>
<th>ACC activity</th>
<th>MCD activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM citrate</td>
<td>10 mM citrate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>167 ± 5.5</td>
<td>203 ± 5.4</td>
</tr>
<tr>
<td>2</td>
<td>169 ± 9.5</td>
<td>222 ± 16</td>
</tr>
<tr>
<td>3</td>
<td>123 ± 11</td>
<td>176 ± 7.6</td>
</tr>
<tr>
<td>4</td>
<td>166 ± 5.2</td>
<td>214 ± 9.4</td>
</tr>
<tr>
<td>7</td>
<td>152 ± 10</td>
<td>219 ± 11</td>
</tr>
<tr>
<td>8</td>
<td>164 ± 6.4</td>
<td>232 ± 14</td>
</tr>
</tbody>
</table>
LEGENDS FOR FIGURES

Fig 1. (Top Panel): Time course of malonyl-CoA labeling from 40% enriched NaH\(^{13}\)CO\(_3\) in groups 1, 2, and 8 of perfused rat hearts (Table 1). (Bottom Panel): Time course of malonyl-CoA labeling from 40% enriched NaH\(^{13}\)CO\(_3\) in hearts perfused with buffer containing 1% DMSO and 0, 5 µM, and 30 µM of CBM-300864, or 10 µM of CBM-301940 (groups 6, 9, 10, 11 of Table 1). Each symbol corresponds to one perfused heart.

Fig 2. Correlation between the flux of acetyl-CoA carboxylation and malonyl-CoA concentration in perfused rat hearts. Each numbered symbol refers to one group of perfused hearts described in Table 1. The linear regression was computed from the data of groups 1 to 8.
Figure 1

M1 Malonyl-CoA MPE

Duration of $\text{H}^{13}\text{CO}_3^-$ infusion (min)

- Group 1, $T_{1/2} = 1.24\text{ min}$
- Group 2, $T_{1/2} = 1.15\text{ min}$
- Group 8, $T_{1/2} = 0.80\text{ min}$
- Group 6, $T_{1/2} = 1.27\text{ min}$
- Group 9, $T_{1/2} = 5.37\text{ min}$
- Group 10, $T_{1/2} = 6.34\text{ min}$
- Group 11, $T_{1/2} = 2.22\text{ min}$
Figure 2.

\[ y = 0.98x + 0.65 \]

\[ R^2 = 0.94 \]
Reference List


