Regulation of fatty acid oxidation of the heart by MCD and ACC during contractile stimulation

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Goodwin, Gary W., and Heinrich Taegtmeyer. Regulation of fatty acid oxidation of the heart by MCD and ACC during contractile stimulation. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E772–E777, 1999.—We tested the hypothesis that the level of malonyl-CoA, as well as the corresponding rate of total fatty acid oxidation, is regulated by the opposing actions of acetyl-CoA carboxylase (ACC) and malonyl-CoA decarboxylase (MCD). We used isolated working rat hearts perfused under physiological conditions. MCD in heart homogenates was measured specifically by $^{13}$CO$_2$ production from [3-14C]malonyl-CoA, and ACC was measured specifically based on the portion of total carboxylase that is citrate sensitive. Increased heart work (1 µM epinephrine + 40% increase in afterload) elicited a 40% increase in total β-oxidation of exogenous plus endogenous lipids, accompanied by a 33% decrease in malonyl-CoA. The basal activity and citrate sensitivity of ACC (reflecting its phosphorylation state) and citrate content were unchanged. AMP levels were also unchanged. MCD activity, when measured at a subsaturating concentration of malonyl-CoA (50 µM), was increased by 55%. We conclude that physiological increments in AMP during the work transition are insufficient to promote ACC phosphorylation by AMP-stimulated protein kinase. Rather, increased fatty acid oxidation results from increased malonyl-CoA degradation by MCD.

FATTY ACIDS are the principal respiratory substrate for the heart (18, 24), and the rate of total fatty acid oxidation is increased by contractile stimulation (7). Both exogenous and endogenous (triglycerides within heart myocytes) fats are utilized for mitochondrial β-oxidation, the latter by way of turnover of the triglyceride pool (7, 20). The flux-generating step, as well as a regulated step for triglyceride utilization, is lipolysis (9, 16, 25). Flux for total fatty acid oxidation is regulated at the level of entry of long-chain fatty acyl-CoA esters into mitochondria at carnitine palmitoyltransferase I (CPT-I, reviewed in Ref. 14).

CPT-I is inhibited by malonyl-CoA, which acts as a downstream signaling molecule for an elaborate scheme of cellular regulation designed to adjust rates of fatty acid oxidation to diverse signals for metabolic demand in addition to signals for the availability of respiratory substrates. The paradigm that is developing for skeletal muscle is that metabolic demand is signaled by a kinase cascade (reviewed in Ref. 8), whereas substrate availability is signaled by allosteric regulation of acetyl-CoA carboxylase (19), which is responsible for malonyl-CoA synthesis in the cytosol.

The kinase cascade (AMP-stimulated protein kinase and its upstream kinase) senses the status of AMP and phosphocreatine (8, 17). Stimulation of AMP kinase (AMPK) and AMPK kinase by AMP or relief of inhibition by phosphocreatine causes phosphorylation and inactivation of acetyl-CoA carboxylase (ACC), reduced malonyl-CoA levels, and increased flux through CPT-I. The other signaling pathway that feeds into malonyl-CoA, which senses the availability of carbohydrates for respiration, is allosteric stimulation of ACC by citrate (21, 27). Citrate is also a precursor for acetyl-CoA synthesis in the cytosol, providing substrate for ACC.

The aforementioned regulatory pathways feed into malonyl-CoA synthesis by ACC. Little is known about the opposing pathways responsible for the removal of malonyl-CoA. In muscle, which exhibits little or no de novo lipogenesis (3), malonyl-CoA decarboxylase (MCD) seems to be a major pathway for malonyl-CoA disposal (2) and is therefore a likely candidate for regulation of β-oxidation.

The objective of the present study was to define the pathways responsible for regulation of malonyl-CoA levels and, hence, of fatty acid oxidation of the heart in relation to contractile state. Specifically, we tested for regulation of ACC by the aforementioned signaling pathways and also tested for regulation of MCD. Regulation of ACC in relation to contractile state has not previously been examined in heart to our knowledge. These are the first measurements of MCD in relation to contractile state in any tissue to our knowledge. We postulate reciprocal regulation of opposing pathways for malonyl-CoA synthesis and degradation.

We included two improvements over existing studies of regulation of heart fatty acid oxidation. First, we postulate that CPT-I regulates flux for total β-oxidation of exogenous plus endogenous (triacylglyceride derived) fatty acids. We measured exogenous oleate oxidation by a conventional method (PH$_2$O production from [9,10-
citrate, which is an important feature of the regulation of non-specific carboxylase activity (27, 28). Pyruvate carboxylase and/or propionyl-CoA carboxylase are bio-

-METHODS-

Sources of materials. [3-14C]malonyl-CoA (not commercially available) was synthesized enzymatically from acetyl-

CoA, ATP, [14C]NaHCO3, and purified ACC, as described by Kolattukudy et al. (10). After overnight incubation at room temperature, glucose (10 mM) and yeast hexoki-

nase (desalted, 1 U) were added to remove unreacted ATP, which cochromatographs with malonyl-CoA, and then the reaction was adjusted to pH 3 with HCl to remove unreacted 14CO2. The product was purified on DEAE-cellulose (1 × 10 cm) equilibrated with 3 mM HCl, as described by Kolattukudy et al. The column was developed with a gradient of LiCl (0 to 0.2 M in 3 mM HCl) over a 5-column volume. [3-14C]malonyl-CoA eluted as the only peak of radioactivity, coinciding with a sharp, well-resolved peak of absorbance at 260 nM. The specific activity (7 Ci/µmol calculated from the A260) was the same as that of the [14C]NaHCO3 starting material (6.8 Ci/µmol). The yield of [3-14C]malonyl-CoA from the limiting reagent (acetyl-CoA) was 20% (3 µCi). ACC used for this synthesis (also unavailable commercially) was puri-

fied from fresh rat liver after the enzyme was induced (4 rats were fasted for 2 days and then fed bread for 3 days), with the first two steps of the "alternate procedure" described by Ahmad and Ahmad (1), followed by chromatography on DEAE-cellulose, as described by Tanabe et al. (26). We obtained 12 U (specific activity 2.1 U/mg protein). The sources of other materials were given previously (7) or were from Sigma (St. Louis, MO) or Boehringer Mannheim (Indianapo-

lis, IN).

Heart perfusions and perfusion protocol. We used hearts from perfusions of our previous study (7). Procedures for the working heart perfusions and for metabolic flux determina-

tion were described in detail in that study (7). The perfusate was Krebs-Henseleit buffer with 5 mM glucose, 40 µU/ml insulin, 0.5 mM lactate, and 0.4 mM oleate prebound to 3% albumin. The free Ca2+ concentration was 1.4 mM. Rat hearts were freeze-clamped on the cannula with aluminum tongs cooled in liquid N2. "Unstimulated" refers to hearts freeze-

clampd just before stimulation of contracile activity. "Stimu-

lation" refers to hearts freeze-clamped after 20 min of contrac-

tile stimulation, resulting from raising the afterload from 100 to 140 cmH2O and by adding of 1 µM epinephrine to the perfusate.

Analytical procedures. Frozen hearts, stored at −70°C, were weighed and ground to a fine powder under liquid N2, and a portion was taken for dry weight determination. Metabolites were measured in freshly prepared 6% perchloric acid extracts of heart, adjusted to pH 5 with buffered KOH. AMP and citrate were measured with established enzymatic methods (5). Malonyl-CoA was measured radiochemically with purified rat liver fatty acid synthase (1.1 U/mg protein) and [acetyl-3H]acetyl-CoA (Sigma). We included a malonyl-

CoA internal standard for each determination, as described by McGarry et al. (15). ACC assays. We initially measured carboxylase activity after fractionating heart homogenates with polyethylene glycol 8000 (fraction precipitating between 2.5 and 6%) as described by Kudo et al. (11). This procedure did not reduce non-citrate-dependent carboxylase activity (see also Fig. 3 of Ref. 11) and was abandoned. The procedure we used was adapted from Witters et al. (29). The frozen, powdered tissue (50 mg) was dispersed into 9 vol (wt/vol) of cold homogeniza-

tion buffer, and the icy slurry was mixed with a glass rod. Phenylmethylsulfonyl fluoride (0.1 mM) was added to the slurry from a 0.1 M solution in methanol. The slurry was further homogenized with several passes of a tight-fitting Teflon Potter until thawed and then was centrifuged at 40,000 g for 30 min. The supernatant was dialyzed overnight against 100 vol of cold homogenization buffer consisting of 50 mM KH2PO4, 50 mM KF, 5 mM Na2HPO4, 1 mM EDTA, and 1 mg/ml BSA (fatty acid free), adjusted to pH 7.0 with KOH. The buffer was freshly supplemented with 1 mM dithiothreitol, 1 mM benzamidine, 1 µM leupeptin, 1 µM pepstatin-A, and 1 µg/ml aprotinin. ACC assays were based on acetyl-CoA-dependent 14CO2 fixation. The assay buffer was 50 mM HEPES, 2.1 mM ATP, 5 mM creatine phosphate, 1 mg/ml BSA (fatty acid free), and 10 mM Mg-aceate, adjusted to pH 7.5 at 30°C with KOH. The buffer was further supplemented with 0.3 mM acetyl-CoA, 1 mM dithiothreitol, and 0.1 mg/ml creatine kinase (Boehringer). Assays were performed without (in duplicate) or with the indicated additions of Mg-loading citrate, with a stock solution containing equimolar K3-

citrate and Mg-aceate, pH 7.5. The reaction volume was 50 µl, and the sample volume was 10 µl. After thermal equilibra-

tion to 30°C, reactions were started by the addition of 14CO2 was measured radiochemically with purified rat liver fatty acid synthase (1.1 U/mg protein) and [acetyl-

CoA, ATP, [14C]NaHCO3, and purified ACC, essentially as described by Kolattukudy et al. (10). After overnight incubation at room temperature, glucose (10 mM) and yeast hexoki-

nase (desalted, 1 U) were added to remove unreacted ATP, which cochromatographs with malonyl-CoA, and then the reaction was adjusted to pH 3 with HCl to remove unreacted 14CO2. The product was purified on DEAE-cellulose (1 × 10 cm) equilibrated with 3 mM HCl, as described by Kolattukudy et al. The column was developed with a gradient of LiCl (0 to 0.2 M in 3 mM HCl) over a 5-column volume. [3-14C]malonyl-CoA eluted as the only peak of radioactivity, coinciding with a sharp, well-resolved peak of absorbance at 260 nM. The specific activity (7 Ci/µmol calculated from the A260) was the same as that of the [14C]NaHCO3 starting material (6.8 Ci/µmol). The yield of [3-14C]malonyl-CoA from the limiting reagent (acetyl-CoA) was 20% (3 µCi). ACC used for this synthesis (also unavailable commercially) was puri-

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After thermal equilibration to 30°C, reactions were initiated with malonyl-CoA (10,000 dpm, final concentration of 0.05 or 0.2 mM), the reaction tube was placed in a glass scintillation vial containing 1 ml of hyamine hydroxide (1 M in methanol), and the vial was fitted with a serum cap. Reactions were stopped after 10 min by injection of 0.2 ml of 6% perchloric acid. The vials were shaken overnight to collect $^{14}$CO$_2$ and then were taken for scintillation counting after the addition of 10 ml of scintillation mixture. Values were corrected for blanks with homogenization buffer in place of tissue homogenate. Blanks were equivalent to background radioactivity (20 dpm). We established that $^{14}$CO$_2$ production was linear with respect to time and amount of tissue extract used in the assays.

Data are expressed as means ± SE. Statistical comparison was by Student’s t-test for unpaired data. $P < 0.05$ was considered significant.

RESULTS

Malonyl-CoA levels and the regulation of total β-oxidation. Table 1 gives rates of exogenous oleate oxidation ($^3$H$_2$O from [9,10-$^3$H]oleate), total fatty acid oxidation of exogenous plus endogenous lipids, and the level of malonyl-CoA in the hearts before and after stimulation of heart contractile activity. These are the values that we reported previously (7). Values for total β-oxidation were calculated from oxygen consumption and total carbohydrate oxidation and are completely independent of the reported measures for oleate oxidation. Contractile stimulation caused a 75% increase in hydraulic power (7) and a 33% decrease in the tissue content of malonyl-CoA. The decrease in malonyl-CoA caused a 40% increase in total fatty acid oxidation. The increase in exogenous oleate oxidation (20%) was not significant. These results are consistent with our hypothesis that CPT-I regulates flux for total carboxylase activity of exogenous plus endogenous lipids. An increase in the contribution of endogenous lipids to total fatty acid oxidation is to be expected based on the stimulation of hormone-sensitive lipase within heart myocytes by phosphorylation by protein kinase A (9, 16, 25).

The decrease in malonyl-CoA does not result from phosphorylation of ACC. To explain the decrease in malonyl-CoA, we examined regulation of ACC. For this, homogenates of the whole heart were prepared under conditions that protect the phosphorylation state of ACC (we included fluoride, phosphate, and pyrophosphate to inhibit phosphatases and EDTA to inhibit kinases in case any ATP remains in the homogenate). We also dialyzed the preparation to remove endogenous citrate. Most of total carboxylase activity in the hearts at physiological levels of citrate (roughly 0.2 mM) was not citrate dependent. The activity without added citrate was 0.59 ± 0.17 nmol·min$^{-1}$·mg protein$^{-1}$ in the unstimulated state and 0.87 ± 0.07 nmol·min$^{-1}$·mg protein$^{-1}$ in the stimulated state. This is the expected result. Carboxylation of acetyl-CoA in the absence of citrate results from the actions of propionyl-CoA carboxylase and/or pyruvate carboxylase, which are both active in heart (27, 28). To measure the activity of ACC specifically, we calculated the citrate-dependent portion of total carboxylase activity by subtracting the activity measured without citrate from the activity measured with added Mg-citrate. Figure 1 shows ACC activity over a range of concentrations of Mg-citrate added to the assay. The basal activity at a given citrate concentration and the citrate sensitivity of the enzyme, which reflect the phosphorylation state of ACC by AMPK, were not different between the two groups. Therefore, the phosphorylation state of ACC does not explain the decrease in malonyl-CoA levels of the heart resulting from contractile stimulation.

The lack of change in phosphorylation state of ACC by AMPK reflects the fact that, for normoxic heart, AMP does not increase sufficiently during contractile stimulation to activate the AMP-kinase cascade. The level of AMP in the hearts was 1.49 ± 0.33 µmol/g dry wt before stimulation (n = 5) and 1.44 ± 0.15 µmol/g dry wt after stimulation (n = 15).

Another explanation for the decrease in malonyl-CoA could be that the content of citrate in the cytosol decreased, because cytosolic citrate stimulates ACC.

Table 1. Fatty acid oxidation (exogenous and total) and content of malonyl-CoA before and after contractile stimulation.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Contractile State</th>
<th>Unstimulated (n = 5)</th>
<th>Stimulated (n = 5 or 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleate oxidation, µmol·min$^{-1}$·g dry wt$^{-1}$</td>
<td></td>
<td>1.33 ± 0.21</td>
<td>1.61 ± 0.15</td>
</tr>
<tr>
<td>Oleate oxidation, µmol·min$^{-1}$·g dry wt$^{-1}$</td>
<td></td>
<td>1.31 ± 0.04</td>
<td>1.83 ± 0.03*</td>
</tr>
<tr>
<td>Malonyl-CoA, nmol/g dry wt</td>
<td></td>
<td>9.1 ± 1.7</td>
<td>6.1 ± 1.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 15 only for malonyl-CoA during stimulation. Oleate oxidation was measured directly ($^3$H$_2$O from [9,10-$^3$H]oleate), and total β-oxidation was calculated from oxygen consumption not resulting from carbohydrate oxidation. *P < 0.05 vs. unstimulated contractile state.
The content of citrate specifically in the cytosolic compartment is difficult to determine because citrate partitions between mitochondria and cytosol. However, the whole tissue content of citrate did not decrease (it actually increased slightly from 2.05 ± 0.19 µmol/g dry wt before stimulation (n = 5) to 2.42 ± 0.09 µmol/g dry wt after stimulation (n = 15)). This suggests, but does not prove, that the decrease in malonyl-CoA did not occur because the content of citrate in the cytosol decreased.

The decrease in malonyl-CoA with contractile stimulation could result from stimulation of malonyl-CoA degradation by MCD. Another potential level of regulation of malonyl-CoA is by the rate of degradation back to acetyl-CoA, catalyzed by MCD. Table 2 gives activities for MCD in whole heart homogenates measured at two concentrations of malonyl-CoA: 0.2 mM and 50 µM. The latter concentration is the Michaelis-Menten constant (K_m) of purified rat liver mitochondrial MCD (10) and is a subsaturating concentration in our heart homogenates, giving roughly one-half of the activity obtained with 0.2 mM substrate. The rationale for measuring the activity at high and low concentrations is because the ambient concentration of malonyl-CoA in heart cytosol (a few µM) is well below the K_m of MCD. Under this condition, the flux for MCD is proportional to the ratio of maximum velocity (V_max) to K_m. Acute regulation of MCD flux could be achieved, in principle, equally by way of the K_m or the V_max. Regulation by way of the K_m would not be detected based on measurements with a high concentration of malonyl-CoA.

There was no change in the activity of MCD on contractile stimulation when measured with 0.2 mM malonyl-CoA. There was a 55% increase in MCD activity on contractile stimulation when measured with 50 µM malonyl-CoA (Table 2). This suggests that the apparent K_m of the enzyme for malonyl-CoA decreased. We then calculated values for apparent K_m and V_max of MCD in the tissue homogenates, assuming hyperbolic kinetics (Table 2). The apparent K_m was decreased 77% after stimulation of contractile activity. Taken together, our data suggest that increased MCD flux at an ambient concentration of malonyl-CoA contributed to the observed decrease in malonyl-CoA levels and the increase in total fatty acid oxidation.

### Table 2. Malonyl-CoA decarboxylase activity before and after contractile stimulation

<table>
<thead>
<tr>
<th>Contractile State</th>
<th>Parameter Measured</th>
<th>Unstimulated (n = 5)</th>
<th>Stimulated (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCD activity with 50 µM malonyl-CoA</td>
<td>0.94 ± 0.05</td>
<td>1.46 ± 0.10*</td>
</tr>
<tr>
<td></td>
<td>MCD activity with 0.2 mM malonyl-CoA</td>
<td>2.23 ± 0.14</td>
<td>2.34 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Apparent V_max</td>
<td>4.1 ± 0.9</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Apparent K_m, µM</td>
<td>250 ± 90</td>
<td>58 ± 14*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Units for enzyme activity are µmol·min⁻¹·g⁻¹·dry wt⁻¹. Values for apparent maximum velocity (V_max) and Michaelis-Menten constant (K_m) were calculated with the K_m equation. *P < 0.05 vs. unstimulated contractile state.

### DISCUSSION

Our data suggest that increased degradation of malonyl-CoA by acute stimulation of MCD is responsible for the observed decrease in the tissue content of malonyl-CoA and, hence, an increase in total fatty acid oxidation of the heart on stimulation of contractile activity. This is based on the observation that the decrease in malonyl-CoA was correlated with the change in the activity of MCD but not of ACC. We could not rule out the possibility that reduced cytosolic citrate contributed to diminished synthesis of malonyl-CoA by ACC, although the slight increase in total tissue citrate suggests that this was not the case.

We postulated reciprocal regulation of ACC and MCD. Contrary to the hypothesis, we did not find inactivation of ACC during contractile stimulation. Inactivation of ACC in heart was anticipated from observations in skeletal muscle. Contractile stimulation of skeletal muscle causes phosphorylation and inactivation of ACC because AMPK becomes stimulated by the rise in AMP (28). We propose that the difference between heart and skeletal muscle in this respect is because the dynamic range of the contractile state of the heart is not as large as that of skeletal muscle. Unlike skeletal muscle, the heart exhibits high basal contractile activity and did not exhibit increased AMP during stimulation in our study. Presumably, the large dynamic range of the contractile state of skeletal muscle will contribute to larger transient shifts in high-energy phosphates, contributing to increased β-oxidation resulting from stimulation of AMPK.

We interpreted ACC activity as the portion of total carboxylase activity that is citrate dependent. This was necessary because most of the activity contributing to acetyl-CoA carboxylation in heart homogenates is not relevant to the regulation of malonyl-CoA levels in the cytosol. Propionyl-CoA carboxylase and/or pyruvate carboxylase contribute to acetyl-CoA carboxylation in vitro, is abundant in heart mitochondria, and is not readily separable from ACC without a specific antibody (21, 27, 28). Nonspecific carboxylation explains high citrate-independent acetyl-CoA carboxylation in a previous study of heart ACC (11) because ACC_b (the 280-kDa isoform that predominates in heart and skeletal muscle) has nearly complete dependence on citrate for activity. This is based on observations with purified enzyme (27) and from Vavvas et al. (28), who recently measured ACC specifically in skeletal muscle by immunoprecipitating the enzyme in a catalytically active form. Our interpretation suggests that the decrease in carboxylation of acetyl-CoA in reperfused hearts, reported by Kudo et al. (11), did not result from a specific decrease in ACC (it appears that the citrate-dependent portion of total carboxylase actually increased slightly). Rather, the absence of a change in ACC during reperfusion in that study seems more consistent with their observation that fatty acid oxidation was also unchanged during reperfusion, when measured in absolute terms (11).
We observed an increase in the activity of MCD when measured at a low, but not a high, concentration of malonyl-CoA. This suggests that the enzyme was regulated by way of the $K_m$. Because the ambient concentration of malonyl-CoA in the heart (a few $\mu$M) is much less than the $K_m$ of MCD for malonyl-CoA (~50 $\mu$M), enzyme activity measured at low concentrations of substrate is more relevant to flux through the enzyme under ambient conditions and the $K_m$ becomes a valid parameter for enzyme regulation. Changes in enzyme expression are expected to change the $V_{\text{max}}$ as well, but the duration of our experiment (20 min between stimulated and stimulated hearts) was probably too brief for differences in enzyme expression to manifest.

We did not discern the subcellular localization of MCD in this study (we measured the activity in the whole heart). MCD is thought to exist in both cytosolic and mitochondrial compartments in skeletal muscle (2) and heart (12). Dyck et al. (6) recently reported MCD activity in rat heart mitochondria, but it is not clear if mitochondrial MCD could regulate malonyl-CoA levels in the cytosol. On the basis that malonyl-CoA is not a substrate for carnitine acetyltransf erase, Scholte (22) concluded that mitochondrial MCD cannot affect extra mitochondrial malonyl-CoA. MCD appears to exist in mitochondria to prevent inhibition of pyruvate carboxylase (23) and methylmalonyl-CoA mutase (4) by malonyl-CoA synthesized by pyruvate carboxylase and/or propionyl-CoA carboxylase. This role is not necessarily inconsistent with the regulation of cytosolic levels of malonyl-CoA by mitochondrial MCD. Nevertheless, it seems likely that cytosolic MCD is relevant to regulation of fatty acid oxidation.

In summary, our results suggest that acute regulation of total fatty acid oxidation of the heart during contractile stimulation is regulated by the rate of disposal of malonyl-CoA catalyzed by MCD. We did not find evidence for regulation of ACC in this circumstance. Changes in AMP during contractile stimulation of normoxic heart are insufficient to affect malonyl-CoA levels through the AMP-kinase cascade.

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