Relative importance of malonyl CoA and carnitine in maturation of fatty acid oxidation in newborn rabbit heart

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Relative importance of malonyl CoA and carnitine in maturation of fatty acid oxidation in newborn rabbit heart. Am J Physiol Heart Circ Physiol 284: H283–H289, 2003. First published September 5, 2002; 10.1152/ajpheart.00461.2002.—After birth, a dramatic increase in fatty acid oxidation occurs in the heart, which has been attributed to an increase in L-carnitine content and a switch from the liver (L) to muscle (M) isoform of carnitine palmitoiltransferase (CPT)-1. However, because M-CPT-1 is more sensitive to inhibition by malonyl CoA, a potent endogenous regulator of fatty acid oxidation, a switch to the M-CPT-1 isoform should theoretically decrease fatty acid oxidation. Because of this discrepancy, we assessed the contributions of myocardial L-carnitine content and CPT-1 isoform expression and kinetics to the maturation of fatty acid oxidation in newborn rabbit hearts. Although fatty acid oxidation rates increased between 1 and 14 days after birth, myocardial L-carnitine concentrations did not increase. Changes in the expression of L-CPT-1 or M-CPT-1 mRNA after birth also did not parallel the increase in fatty acid oxidation seen in the newborn heart. In 1-day-old rabbit hearts, malonyl CoA levels are very high but decrease dramatically by 7 days after birth (5, 18, 19). These changes in malonyl CoA can be explained by alterations in the control of both malonyl CoA synthesis and degradation (5, 16, 18, 19).

Fatty acid oxidation in the newborn heart matures rapidly after birth (16, 29). For example, in 1-day-old rabbit hearts, fatty acid oxidation rates are very low and contribute <10% of the overall ATP production (17). However, by 7 days of age, fatty acid oxidation dramatically increases and becomes the predominant source of energy production. What is responsible for this increase in fatty acid oxidation has not been completely delineated, although an increase in the mitochondrial uptake of fatty acids has been implicated in this process (1, 9, 18). The transport of fatty acids into the mitochondria is primarily controlled at the level of carnitine palmitoyltransferase-1 (CPT-1) (20). CPT-1 is in turn inhibited by malonyl CoA and stimulated by L-carnitine (1, 20, 22, 27, 28). The heart expresses two isoforms of CPT-1, L-CPT-1 and M-CPT-1, with L-CPT-1 being less sensitive to inhibition by malonyl CoA and requiring lower L-carnitine concentrations for maximal stimulation (20, 22, 27, 28).

The dramatic increase in fatty acid oxidation seen in the newborn heart has been attributed to an increase in L-carnitine levels and a switch from the L-CPT-1 isoform to the M-CPT-1 isoform (1). However, because M-CPT-1 is more sensitive to inhibition by malonyl CoA, this switch should actually lead to a decrease in fatty acid oxidation rates, not an increase as previously observed (17, 18, 29). An alternate explanation for the increase in fatty acid oxidation is a decrease in myocardial malonyl CoA levels after birth, resulting in a decrease in malonyl CoA inhibition of CPT-1. Indeed, we have previously shown that after birth, malonyl CoA levels do dramatically decrease in the newborn heart. In 1-day-old rabbit hearts, malonyl CoA levels are very high but decrease dramatically by 7 days after birth (5, 18, 19). Malonyl CoA is synthesized in the heart by acetyl CoA carboxylase (ACC), the activity of which decreases in the heart after birth (18). ACC is in turn phosphorylated and inactivated by AMP-activated protein kinase (AMPK) (12), the activity and expression of which increases in the heart after birth (19). Malonyl CoA is degraded in the heart by decarboxylation to acetyl CoA by malonyl CoA decarboxylase (MCD) (16). The relative importance of changes in MCD and ACC control of malonyl CoA versus changes in L-carnitine control of CPT-1 or alterations in CPT-1 activity/expression to the increase in fatty acid oxidation in the newborn heart is not clear.

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In addition to fatty acid oxidation, glucose oxidation is an important source of energy for the adult heart. In contrast to fatty acid oxidation after birth, rates of glucose oxidation are low in the newborn heart (17) and do not fully mature until after weaning (29). Pyruvate decarboxylation is the key rate-limiting irreversible step in glucose oxidation and is catalyzed by the pyruvate dehydrogenase (PDH) multienzyme complex. Activity of PDH is controlled by kinase-mediated inactivation, responsive to acetyl CoA-to-CoA and NADH-to-NAD+ ratios and phosphatase-mediated activation, which responds to mitochondrial calcium and magnesium concentrations (23). In the adult heart, fatty acids are potent inhibitors of glucose oxidation in the heart, secondary to an increase in the acetyl CoA-to-CoA and NADH-to-NAD+ ratios that result from the oxidation of fatty acids (23). Whether glucose oxidation remains low in the newborn heart due to a delay in the maturation of the enzymes involved in glucose oxidation or to the increase in fatty acid oxidation is not known.

The first aim of this study was to determine the relative contributions of CPT-1 isoform expression, myocardial l-carnitine content, and the kinetics of CPT-1 to the maturation of fatty acid oxidation in the newborn heart. The second aim of this study was to determine whether the low glucose oxidation rates seen in the newborn period are due to a low PDH activity and/or a low capacity for glucose oxidation.

METHODS

Animals. Newborn New Zealand White rabbits between 1 and 14 days of age were used in this study.

Heart tissue isolation. Newborn rabbits were injected with 60 mg/kg ip pentobarbital sodium. Hearts from anesthetized rabbits were removed and placed in ice-cold Krebs-Henseleit solution. Hearts were then used for either the preparation of mitochondria, cannulated for isolated heart perfusions, or direction of acyl carnitine formation using palmitoyl CoA and carnitine as substrates (8). The final concentrations in the assay were 75 μM palmitoyl CoA, 0.01–1.5 mM carnitine (0.1–20 μCi/μmol l-[14C]carnitine), 4 mM ATP, 4 mM MgCl2, 0.25 mM glutathione, 40 μg/ml rotenone, 2 mM KCN, 15 mM KCl, 1% (wt/vol) BSA, and 105 mM Tris·HCl; pH 7.4. Two hearts were pooled for preparation of the crude mitochondrial fraction (0.5 ml) and were homogenized in 2 ml of homogenization buffer and centrifuged at 6,000 g for 15 min. The resultant pellet was carefully resuspended in 2 ml of homogenization buffer and centrifuged at 6,000 g for 15 min. The resultant pellet (crude mitochondria) was gently resuspended in 2 ml of homogenization buffer. The crude mitochondrial fraction (0.5 ml) was layered onto 9 ml of 30% Percoll and centrifuged at 50,000 g for 60 min at 4°C. The bottom mitochondrial protein band was collected.

Assay of CPT-1 activity. CPT-1 activity was assayed in the direction of acyl carnitine formation using palmitoyl CoA and carnitine as substrates (8). The final concentrations in the assay were 75 μM palmitoyl CoA, 0.01–1.5 mM carnitine (0.1–20 μCi/μmol l-[14C]carnitine), 4 mM ATP, 4 mM MgCl2, 0.25 mM glutathione, 40 μg/ml rotenone, 2 mM KCN, 15 mM KCl, 1% (wt/vol) BSA, and 105 mM Tris·HCl; pH 7.4. For malonyl CoA inhibition curves, the two substrates of CPT-1, palmitoyl CoA (75 μM) and l-carnitine (0.2 mM), were used at saturating concentrations in the presence of varying concentrations of malonyl CoA ranging from 0 to 1 μM.

MCD activity. MCD activity was measured in rabbit heart tissue using a radiometric assay as described previously (5). Heart perfusions. Isolated working hearts obtained from 1- and 7-day-old rabbits were used for direct measurement of fatty acid and glucose oxidation as described previously (5, 14, 16, 18). Hearts were perfused with Krebs-Henseleit solution containing 11 mM glucose and 0.4 mM palmitate, prebound to 3% BSA. The perfusate pressure was 60 mmHg, and the time of perfusion was 40 min. Rates of fatty acid and glucose oxidation were measured in separate sets of hearts. Glucose oxidation rates were determined as previously described by trapping and measuring 14CO2 released by the metabolism of [U-14C]glucose in the presence or absence of 0.4 mM palmitate (14). Steady-state rates of palmitate oxidation were measured hearts by quantitatively collecting 14CO2 produced by hearts perfused with [1-14C]palmitate. Metabolic values were normalized for heart mass (dry wt).

PDH activity. PDH activity (both activated and total activity) was measured using a revised protocol (2) based on the radiometric assay described by Constantin-Teodosiu et al. (3).
RESULTS AND DISCUSSION

L-Carnitine content in newborn hearts. The amounts of free carnitine, long-chain acyl carnitine, and short-chain acyl carnitine in 1-, 4-, 10-, and 14-day-old rabbits are shown in Table 1. Myocardial free carnitine content did not vary dramatically among these four age groups. An increase in short-chain acyl carnitine and a decrease in long-chain acyl carnitine was observed during maturation. However, total carnitine content did not vary dramatically among the four age groups. The cellular concentrations of carnitine were calculated based on a cytosolic space of 2 ml/g dry wt in heart muscle and assuming an equal distribution of carnitine between different subcellular compartments, as described by Idell-Wenger et al. (13). The myocardial carnitine concentrations were as follows: 1 day old, 1.83 mM; 4 days old, 1.36 mM; 10 days old, 2.09 mM; and 14 days old, 2.03 mM. Free carnitine concentrations were as follows: 1 day old, 0.6 mM; 4 days old, 0.55 mM; 10 days old, 0.95 mM; and 14 days old, 0.95 mM. These concentrations are all above the \( K_m \) for either CPT-1 isoform expressed in the heart, making it unlikely that carnitine concentration was limiting for CPT-1 activity in the newborn period.

Values for the \( K_m \) for carnitine have previously been determined in various tissues from the rat, guinea pig, dog, and human (22). These values have been found to have a wide range for both isoforms of CPT-1, with the rat liver showing the lowest \( K_m \) for carnitine (32 \( \mu \)M) and the dog heart the highest (695 \( \mu \)M) (22). In a study by Cook et al. (4), regulation of CPT-1 gene isoforms has not been correlated with fatty acid oxidation in the fetal or newborn rat heart. However, previous studies using tissue homogenates, isolated mitochondria, or isolated perfused organs have shown that fatty acid oxidation of the hearts from rats, rabbits, pigs, and calves is very low. The capacity of the heart to oxidize fatty acids increases shortly after birth in these species (for an extensive review, see Ref. 9). Species differences, if any, should be limited with level of gene and/or mRNA expression and not reflected by differences in the maturation of fatty acid oxidation.

CPT-1 mRNA expression in the newborn heart. mRNA expression of M-CPT-1 and L-CPT-1 in newborn rabbit hearts is shown in Fig. 1. The expression of L-CPT-1 mRNA was increased in the first 3 days after birth then returned to 1-day-old levels by day 10 (Fig. 1, top). In contrast, M-CPT-1 expression was very low in 1-, 3-, and 7-day-old hearts but increased in 10- and 14-day-old hearts (Fig. 1, bottom). While the expression pattern of mRNA of the two isoforms of CPT-1 did show changes in the developing rabbit heart, it is unlikely a switch in CPT-1 from the L-CPT-1 to M-CPT-1 isoform can explain the increase in fatty acid oxidation observed during this period. The maturation of fatty acid oxidation in the newborn heart occurs between 1 and 7 days (from 22.6 ± 5.6 nmol·g dry wt\(^{-1}\)·min\(^{-1}\) in 1-day-old hearts to 299 ± 46 nmol·g dry wt\(^{-1}\)·min\(^{-1}\) in 7-day-old hearts). This increase in fatty acid oxidation precedes the increase in M-CPT-1 expression we observed in these hearts.

Table 1. Carnitine content in newborn rabbit hearts

<table>
<thead>
<tr>
<th>Age, days</th>
<th>Free Carnitine, nmol/g dry wt</th>
<th>Short-Chain Acyl Carnitine, nmol/g dry wt</th>
<th>Long-Chain Acyl Carnitine, nmol/g dry wt</th>
<th>Total Carnitine, nmol/g dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,205 ± 148</td>
<td>690 ± 42</td>
<td>1,771 ± 138</td>
<td>3,666 ± 255†</td>
</tr>
<tr>
<td>4</td>
<td>1,149 ± 134</td>
<td>850 ± 138</td>
<td>728 ± 127†</td>
<td>2,727 ± 148</td>
</tr>
<tr>
<td>10</td>
<td>1,894 ± 318</td>
<td>1,955 ± 300†</td>
<td>338 ± 80ª</td>
<td>4,186 ± 431†</td>
</tr>
<tr>
<td>14</td>
<td>1,853 ± 639</td>
<td>1,967 ± 330†</td>
<td>250 ± 83ª†</td>
<td>4,070 ± 256†</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 6 \) hearts/group. Carnitine concentrations were measured in newborn rabbit hearts as described in METHODS. *Significantly different from 1-day-old hearts; † significantly different from 4-day-old hearts.

Statistical analysis. Data are expressed as means ± SE. Comparisons were performed using Student’s \( t \)-test. Significance was set at \( P < 0.05 \). For groups of four or more, ANOVA followed by the Tukey-Kramer multiple-comparisons test was used.

Fig. 1. Expression of mRNA for the carnitine palmitoyl transferase-1 (CPT-1) isoforms. Expression was measured using RT-PCR ELISA as described in METHODS. Top: L-CPT-1. Results are expressed relative to the level in 1-day-old hearts. All experiments were carried out in duplicate, and results are the mean determinations of 4 different rabbit hearts for each age. Bottom: M-CPT-1. Results are expressed relative to the level in 1-day-old hearts. All experiments were carried out in duplicate, and results are the mean determinations of 4 different rabbit hearts for each age. *Significantly different from 1-day-old hearts.
It is possible that the increase in L-CPT-1 that occurred during the first few days after birth may have some role to play in the maturation of fatty acid oxidation in this period. However, this increase was transient, with L-CPT-1 expression appearing to decrease as M-CPT-1 expression increased. The recent study by Cook et al. (4) suggests a differential regulation of the two CPT-1 genes in the rat heart. These investigators showed a decline in the expression of the L-CPT-1 isoform during maturation and no change in the expression of M-CPT-1 (4). These results differ somewhat from our results, although neither expression pattern could explain the observed increase in fatty acid oxidation seen after birth.

**Kinetics of CPT-1 in newborn rabbit heart mitochondria.** The effects of various L-carnitine concentrations on CPT-1 activity in 1-, 7-, and 10-day-old rabbit hearts is shown in Fig. 2. In heart mitochondria obtained from all three age groups, increasing L-carnitine concentration resulted in an increase in CPT-1 activity. In 1- and 7-day-old hearts, the $V_{\text{max}}$ for CPT-1 was similar despite a 10-fold increase in fatty acid oxidation during this period. However, in 10-day-old hearts, CPT-1 activity at higher L-carnitine concentrations was significantly greater than rates in 1- and 7-day-old hearts. This may reflect the increased expression of M-CPT-1 observed in these hearts compared with 1-day-old hearts (Fig. 1, bottom).

Values for the $K_m$ for carnitine were obtained from Eadie-Hofstee plots (Fig. 2, right). Of interest was the observation that CPT-1 activity in mitochondria from 1- and 10-day-old hearts appeared to express two different $K_m$s for L-carnitine, whereas the best fit for 7-day-old hearts was a single $K_m$ for L-carnitine. The reasons for the different $K_m$ characteristics are not clear and cannot be readily explained by the differential expression patterns of L-CPT-1 (which has a low $K_m$ for L-carnitine) or M-CPT-1 (which has a high $K_m$ for L-carnitine) in the newborn heart.

Of significance to this study is that even the highest $K_m$ values are below the concentrations of L-carnitine to which the newborn heart is exposed. Furthermore, there is no correlation between $K_m$ values for L-carnitine and the ability of the newborn heart to oxidize

![Fig. 2. CPT-1 activity and corresponding Eadie-Hofstee plots at different ages. CPT-1 activity (left) was measured in mitochondria of newborn rabbit heart as described in METHODS. Values are means ± SE of 5 experiments in which 2 hearts were pooled to increase the mitochondrial yield for 1-day-old rabbits, 5 hearts for 7-day-old rabbits, and 8 hearts for 10-day-old rabbits. Right: Eadie-Hofstee plots used to determine the $K_m$ for carnitine. *Significantly different from 1-day-old hearts at compared carnitine concentrations; †significantly different from 7-day-old hearts at compared carnitine concentrations.](http://ajpheart.physiology.org/)
fatty acids (14, 17, 18). As a result, increased myocardial concentrations of L-carnitine, changes in CPT-1 isoform expression, and/or changes in the affinity of CPT-1 for L-carnitine are unlikely to be responsible for the dramatic increase in fatty acid oxidation seen in the newborn period.

It has been previously shown that electrical stimulation of isolated rat neonatal cardiac myocytes results in increased expression of M-CPT-1 and a greater sensitivity of CPT-1 to malonyl CoA inhibition (30). We found no difference in CPT-1 activity or sensitivity to inhibition by malonyl CoA in 7-day-old hearts compared with 1-day-old hearts. This is not surprising because we did not observe increased M-CPT-1 expression until 10 days after birth. It is therefore unlikely that a switch in CPT-1 isoform is responsible for the elevated levels of fatty acid oxidation seen in 7-day-old hearts.

**Malonyl CoA inhibition of CPT-1 in rabbit heart mitochondria.** We also determined whether changes in the sensitivity of CPT-1 to inhibition by malonyl CoA occur in the newborn heart. Malonyl CoA inhibition of CPT-1 activity was measured in mitochondria obtained from 1- and 10-day-old hearts (Fig. 3). In these experiments, mitochondria were exposed to 200 μM L-carnitine. Similar to the findings shown in Fig. 2, CPT-1 activity in the absence of malonyl CoA was higher in mitochondria from 10-day-old hearts compared with 1-day-old hearts. The addition of malonyl CoA resulted in a marked inhibition of CPT-1 activity (~95%). Despite this marked inhibition by malonyl CoA, there was no significant difference in the sensitivity of CPT-1 to malonyl CoA inhibition between 1- and 10-day-old hearts. The IC50 values for malonyl CoA were 4 and 5 nM in 1- and 10-day-old rabbit hearts, respectively.

Although the sensitivity to malonyl CoA did not differ between these two age groups, previous studies from our laboratory have demonstrated that there is a dramatic reduction in malonyl CoA levels between 1- and 7-day-old hearts (122.0 ± 8.3 nmol/g dry wt in 1-day-old rabbit hearts vs. 1.4 ± 0.5 nmol/g dry wt in 7-day-old rabbit hearts). This decrease in malonyl CoA is accompanied by a 10-fold increase in fatty acid oxidation (1, 3). As a result, our data support the concept that it is a drop in malonyl CoA levels in the newborn heart that is probably the most important determinant of flux through CPT-1 and the increase in fatty acid oxidation in the newborn heart.

**Malonyl CoA degradation in the newborn heart.** We have previously demonstrated that acetyl CoA carboxylase is the key source of malonyl CoA synthesis in the heart (25) and that the decrease in malonyl CoA seen in the newborn heart is accompanied by a decrease in ACC activity (18). Because MCD is the key mechanism by which malonyl CoA is degraded in the heart (5, 6, 11), we measured MCD activity changes in newborn rabbit hearts of different ages. As shown in Fig. 4, the activity of MCD increased in the heart in an age-dependent manner. This increase in MCD activity was almost maximally increased in 7-day-old hearts, a time period in which malonyl CoA levels dramatically decrease (18) and fatty acid oxidation rates dramatically increase (17). This suggests that a drop in malonyl CoA and a decrease in malonyl CoA inhibition of CPT-1 may be one of the key mechanisms responsible for the maturation of fatty acid oxidation after birth.

The data from this study and our previous studies (5, 18) suggest that an increase in ACC activity and a decrease in MCD activity are primarily responsible for the dramatic decrease in myocardial malonyl CoA levels in the newborn heart. The decrease in ACC activity in the newborn heart appears to be primarily due to an increased activity and expression of AMPK in the newborn heart (15, 19). AMPK activity/expression increases dramatically in the immediate newborn period, resulting in a phosphorylation and inactivation of ACC (15, 19). The increase in MCD activity may be entirely...
due to increased MCD protein expression in the heart after birth (5). Alternatively, it is also possible that the increase in AMPK after birth may activate MCD. Saha et al. (26) have shown that AMPK can phosphorylate and activate skeletal muscle MCD in skeletal muscle. However, we and others have not been able to reproduce this finding in either the adult heart (7) or skeletal muscle (10). Whether AMPK regulates MCD in the newborn heart remains to be determined. However, based on the data provided here, we propose the scheme shown in Fig. 5.

**Fatty acid control of glucose oxidation in the newborn heart.** Whereas fatty acid oxidation increases dramatically after birth (probably due to a decrease in malonyl CoA levels), glucose oxidation (the other main source of acetyl CoA for the tricarboxylic acid cycle) does not increase during this period (17). Whether this is due to an increase in fatty acid inhibition of glucose oxidation or due to a delayed maturation of the enzymes controlling glucose oxidation is not clear. We therefore determined what happens to the activity of myocardial PDH, the rate-limiting enzyme for glucose oxidation, in the newborn period. We measured both the active phosphorylated form of PDH as well as total PDH activity (Fig. 6). A progressive age-dependent increase in active PDH and total PDH was observed between 1-, 7-, and 10-day-old hearts, suggesting that the capacity for glucose oxidation increases in the newborn period.

To determine whether the increase in PDH was accompanied by an increase in glucose oxidation, isolated working hearts from 1- and 7-day-old hearts were used to measure glucose oxidation (Fig. 7). Perfusion of hearts in the absence of fatty acids, a condition that measures the maximal capacity of the heart to oxidize glucose, showed that the capacity of the heart to oxidize glucose increases dramatically between 1 and 7 days of age. This increase is consistent with the increase in PDH activity observed in Fig. 6. However, if hearts were perfused with physiologically relevant levels of fatty acids, glucose oxidation rates were very low in both 1- and 7-day-old hearts (Fig. 7). The dramatic increase in glucose oxidation observed in glucose-perfused hearts was completely prevented. This suggests that the newborn heart rapidly acquires the capability of oxidizing glucose but that glucose oxidation is inhib-
affected due to the increase in fatty acid oxidation observed during this period. Therefore, the decrease in malonyl CoA observed after birth not only accelerates fatty acid oxidation but also inhibits glucose oxidation during this period. This inhibition of glucose oxidation likely occurs as a result of an inhibition of PDH that occurs as a result of the increase in fatty acid oxidation.

In summary, our results suggest that malonyl CoA has a key role in the maturation of fatty acid oxidation after birth. We demonstrate that a decrease in malonyl CoA, as opposed to changes in L-carnitine content, is responsible for the increase in fatty acid oxidation in the newborn heart. The decrease in malonyl CoA not only increases fatty acid oxidation but also results in glucose oxidation rates remaining low in the newborn period. These results highlight the key role of malonyl CoA in the control of cardiac energy metabolism.

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