CELLULAR ENERGY METABOLISM DURING FETAL DEVELOPMENT

II. Fatty Acid Oxidation by the Developing Heart

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ABSTRACT

In view of the importance of fatty acids as substrates for the mature heart, fatty acid oxidation by fetal and calf heart mitochondria has been investigated. Free fatty acids of 10 carbon units or less which exhibit carnitine-independent transport into mitochondria were effective substrates for oxidative phosphorylation in both fetal and calf heart mitochondria. Efficient oxidative phosphorylation with these substrates was dependent upon the presence of bovine serum albumin in the assay medium to reverse the uncoupling effects of the fatty acids. In the presence of bovine serum albumin, ADP/O ratios were in the range of 3 when short-chain fatty acids and carnitine esters of short- and long-chain fatty acids were substrates. Compared with calf heart mitochondria, fetal heart mitochondria showed decreased carnitine-dependent oxidation of palmityl-CoA. However, the oxidation of palmitylcarnitine was identical in both. These data suggest that the formation of palmitylcarnitine is rate limiting for palmityl-CoA oxidation by the fetal heart mitochondria and that long-chain fatty acids are not readily oxidized by the fetal heart.

INTRODUCTION

Fatty acids are the principal metabolic fuel of the adult heart (1, 2). Although the developing heart exhibits tightly coupled oxidative phosphorylation with citric acid cycle intermediates as substrates (3), little is known of the capacity of the fetal heart to utilize fatty acids as energy-yielding substrates. Fatty acid oxidation by mitochondria isolated from the fetal heart has not yet been explored.

Mitochondria exhibit a permeability barrier to palmityl-CoA and coenzyme A esters of other fatty acids. The formation of carnitine derivatives of the long-chain fatty acids is required for their optimal oxidation, for only in this form do they rapidly penetrate into the inner mitochondrial membrane. The synthesis of such carnitine intermediates is catalyzed by the enzyme carnitine palmityltransferase as is seen in the following two-step reaction (4):

$$\text{Palmityl-CoA} + \text{carnitine} \rightarrow \text{palmitylcarnitine} + \text{CoA} \quad (1)$$

$$\text{Palmitylcarnitine} + \text{CoA} \rightarrow \text{palmityl-CoA} + \text{carnitine} \quad (2)$$

This is an equilibrium reaction in which palmitylcarnitine is formed in the first step of the sequence for transport into the inner mitochondrial membrane (5). Here, it is reconverted to palmityl-CoA in a second step and the palmityl-CoA undergoes β-oxidation thus providing acetyl-CoA which enters the citric acid cycle. In contrast to the long-

chain fatty acids, fatty acids of 10 carbon units or less are oxidized independently of the carnitine transferase system. These fatty acids permeate the mitochondrial membrane freely and undergo β-oxidation after activation to their CoA esters within the inner membrane.

This communication reports data indicating that fetal and calf heart mitochondria show coupled oxidative phosphorylation with short-chain fatty acids and carnitine esters of both long- and short-chain fatty acids as substrates. However, it will be shown that carnitine-dependent oxidation of palmityl-CoA is extremely limited in isolated fetal heart mitochondria.

METHODS

Bovine fetal hearts and calf hearts were obtained from a local slaughterhouse and fetal age was determined by using the scale devised by Winters and Comstock (6). The hearts were chilled on ice within 30 min of beath, and mitochondria were prepared as described previously by the method of Chance and Hagihara with a minor modification (3, 7).

Mitochondria were also prepared with a Brinkman P-20 high sheer homogenizer. For this preparation, 100 g of minced heart in 240 ml of 0.25 M sucrose and 20 mM Tris Cl, pH 7.5, were homogenized for 40 sec at a rheostat setting of 4. The homogenate was centrifuged at 900 *g* for removing nuclei and debris, and the mitochondria were isolated by centrifugation at 6000 *g*. The mitochondria were washed at 6000 *g* until free of blood, and finally suspended in 0.25 M sucrose.

Oxidation of palmityl-coenzyme A (CoA) by isolated mitochondria was determined polarographically with a Clark oxygen electrode and was also measured independently by trapping $^{14}CO_2$ evolved during the oxidation of palmityl-1-^{14}C-CoA. In the polarographic experiments, the reaction was carried out in 1 ml of medium containing 0.225 M sucrose, 15 mM inorganic phosphate, 50 mM Tris Cl, 15 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, and 2.5–5 mg of fat-free bovine serum albumin. The albumin was defatted according to the method of Chen (8). Oxidation of palmityl-1-^{14}C-CoA was determined in capped flasks fitted with polypropylene center wells. The reaction was initiated by the addition of mitochondria to the same media used in the polarographic experiments. The final reaction volume was 1.0 ml and contained 0.15 mM ADP (adenosine diphosphate). The reaction was carried out in a Dubnoff (Labline Inc., Chicago, Ill.) shaker and terminated in 5 min by the addition of 0.2 ml of 7% perchloric acid. The flasks were shaken for an additional 45 min after hyamine hydroxide was injected into the center wells to absorb the $^{14}CO_2$. The hyamine hydroxide was then transferred to scintillation vials and the $^{14}CO_2$ was counted in a Beckman scintillation spectrometer. The concentrations of L-carnitine, palmitylcarnitine, and palmityl-CoA used in the assays are given in the figures.

ADP concentration was determined spectrophotometrically, and in the case of polarographic experiments, ADP/0 ratios were calculated as the ratio of ADP added to the reaction to the quantity of oxygen consumed (9). ADP was added initially in some experiments in order to deplete the mitochondria of endogenous substrates. Protein was determined by a modification of the Biuret method (10) or by the Lowry method (11). DL-Palmitylcarnitine and other carnitine derivatives of fatty acids were generous gifts from Dr. John Lowenstein of Brandeis University and palmityl-1-^{14}C-CoA was a gift from Dr. Frank Saur of the Biochemistry Section of the Canada Department of Agriculture. L-Carnitine was generously provided by the Otsuka Pharmaceutical Factory, Osaka, Japan. Other reagents were obtained from commercial sources.

RESULTS

Carnitine-Dependent Oxidation of Palmityl-CoA by Fetal and Calf Heart Mitochondria

Carnitine-dependent oxidation of palmityl-CoA by fetal heart mitochondria was slower than that of calf heart mitochondria. As seen in a polarographic experiment with calf heart mitochondria (Fig. 1 *a*), the addition of carnitine to the medium containing palmityl-CoA resulted in an increase in oxidative rate from 25 n atoms oxygen/mg/min to 80 n atoms/mg/min. There was generally a lag period after the addition of carnitine before oxygen uptake was stimulated maximally. Subsequent addition of palmitylcarnitine to the calf heart mitochondria resulted in only a slight increase in the oxidative rate to 90 n atoms/mg/min. In contrast, the addition of carnitine in an experiment with fetal heart mitochondria had no appreciable effect on the rate of oxidation of palmityl-CoA (Fig 1 *b*). However, the rate of oxidation after the addition of palmitylcarnitine was greatly stimulated. The burst of oxidation seen after the initial addition of ADP to the medium was probably due to the oxidation of endogenous substrates. In some experiments with very young calves, rates obtained with palmityl-CoA plus carnitine were less than with palmitylcarnitine as substrate, but values as low as those seen with the fetal heart mitochondria were never observed. Bovine serum albumin was necessary

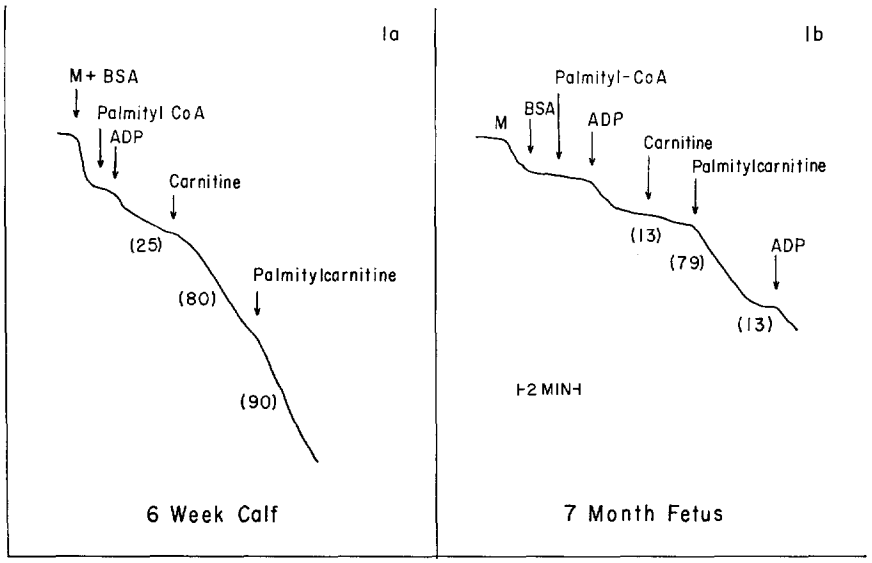

FIGURE 1 *a* Polarographic tracing of 5–6-wk-old calf heart mitochondria with palmityl-CoA plus carnitine as substrate. 1.0 mg of mitochondria (*M*) was used in the assay. Palmityl-CoA and L-carnitine concentrations were 50 μM and 1.0 mM, respectively. Palmitylcarnitine concentration was 100 μM. 0.292 μmole of adenosine diphosphate (ADP) was added as indicated. Other conditions are as described in the text.

FIGURE 1 *b* Polarographic tracing of bovine fetal heart mitochondria with palmityl-CoA plus carnitine as substrates. 0.75 mg of mitochondria (*M*) was used in the assay. Other conditions are the same as in Fig. 1 *a*.

in the medium and presumably served to protect the mitochondria from the uncoupling effects of the fatty acids and the detergent action of the long-chain fatty acid carnitine esters. This will be discussed below. The addition of exogenous coenzyme A did not affect the rate of palmityl-CoA oxidation by either calf or fetal heart mitochondria. Fig. 2 illustrates the high degree of acceptor-controlled (ADP-stimulated) respiration shown by the fetal heart mitochondria with palmitylcarnitine as substrate. ADP/O ratios with palmitylcarnitine approximated the generally accepted value for fatty acids in both the calf and fetal heart mitochondria.

As an independent measure of carnitine-dependent palmityl-CoA oxidation by fetal and calf heart mitochondria, experiments were carried out with palmityl-1-^{14}C-CoA plus carnitine as substrate. In these experiments, $^{14}CO_2$ production by fetal heart mitochondria was 25–30% of the values observed with the calf mitochondria. In both, oxidation was negligible in the absence of carnitine. These data indicate that the conversion of palmityl-CoA to palmitylcarnitine is slow in the fetal heart mitochondria. Presumably, fetal heart mitochondria are capable of converting palmitylcarnitine to palmityl-CoA at the site of β-oxidation. Although a wide gestational range of bovine fetal material was investigated, we were unable to find any relationship between fetal age and mitochondrial fatty acid oxidation.

Observations were also made with other tissues. Fetal guinea pig heart mitochondria also exhibited decreased carnitine-dependent oxidation of palmityl-CoA. However, mitochondria isolated from fetal guinea pig livers and chick embryo hearts of various ages showed rates of oxidation with palmityl-CoA plus carnitine as substrate that were comparable to the rates obtained with adult controls.

Oxidation of Free Fatty Acids and Their Carnitine Derivatives by Fetal and Calf Heart Mitochondria

In experiments with both fetal and calf heart mitochondria, the rates of oxidation of free fatty acids of C-12 chain length and higher were low

compared with the rates of oxidation with their acylcarnitine esters (Table I). This reflects the very slow rate of penetration of long-chain fatty acids into the inner mitochondrial membrane and the resultant decrease in their rate of activation. It has been shown that short-chain fatty acid oxidation by liver mitochondria is not carnitine dependent (12). When capric, octanoic, and caproic acid (C-10, C-8, C-6) and their carnitine derivatives were substrates for fetal and calf heart mitochondria, the oxidative rates of the fatty acids were equivalent to or slightly higher than the rates observed with the carnitine derivatives (Table I). There were no differences in the capacity of fetal and calf heart mitochondria to oxidize these substrates. Both exhibited efficient oxidative phosphorylation with the shorter chain fatty acids as substrates when BSA was present in the medium. Respiratory control values of over 6 were routinely obtained and ADP/O ratios were in the expected range. The fatty acid showing the highest oxidative rate in both the fetal and calf heart mitochondria was capric acid (C-10). Fig. 3 shows the excellent degree of acceptor-controlled respiration observed in the fetal mitochondria with capric acid and caprinylcarnitine as substrates.

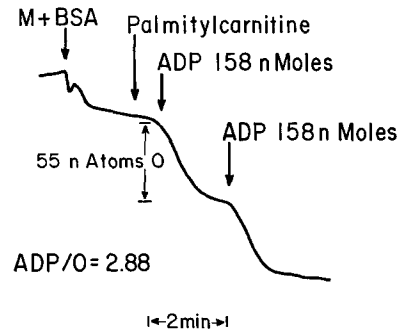

FIGURE 2 Polarographic tracing of fetal heart mitochondria with palmitylcarnitine as substrate. 0.75 mg of mitochondria (*M*) was used in the assay. Palmitylcarnitine concentration was 100 μM. Other conditions are as described in the text.

TABLE I

Oxidation of Fatty Acids and Carnitine Derivatives by Fetal Heart Mitochondria

1.0 mg of mitochondria was used in each assay. The concentrations of fatty acids and acylcarnitines were 0.34 mM and 0.1 mM, respectively. These concentrations gave maximal rates of oxygen uptake during state 3 respiration. Other conditions are as in the text.

Substrate	Rate of oxidation
	n Atoms O/min/mg
Palmitic acid (C-16)	13.5
Palmitylcarnitine	80.0
Myristic acid (C-14)	6.5
Myristylcarnitine	80.0
Lauric acid (C-12)	32.5
Laurylcarnitine	71.0
Capric acid (C-10)	75.0
Caprinylcarnitine	65.0
Octanoic acid (C-8)	67.5
Octanoylcarnitine	60.0
Caproic acid (C-6)	47.5
Caproylcarnitine	40.0

Effects of Bovine Serum Albumin on Fatty Acid Oxidation by Fetal and Calf Heart Mitochondria

Fat-free bovine serum albumin (BSA) markedly improved the respiratory control values and ADP/O ratios of fetal and calf heart mitochondria utilizing free fatty acids as substrates. A representative tracing with capric acid as substrate in the calf heart mitochondria is shown in Fig. 4. The addition of BSA to the reaction media resulted in a striking improvement in the respiratory control value and an increase of the ADP/O ratio from 1.7 to 3.1. Similar data were obtained with C-6, C-8, and to some extent with C-12. The oxidation of long-chain fatty acids by heart mitochondria in the absence of carnitine were also dependent on the presence of BSA in the medium. It has been shown that high concentrations of fatty acids inhibit fatty acid oxidation by liver mitochondria (13, 14). Such fatty acid inhibition occurs when a critical ratio of fatty acid to protein in the assay is exceeded. The inhibitory level of fatty acid can be lowered by addition of BSA to the assay medium (Fig. 5).

DISCUSSION

These investigations were carried out for determining the ability of fetal heart mitochondria to utilize fatty acids as substrates for oxidative phosphorylation. Wittels and Bressler (15) reported that newborn rat heart homogenates had an impaired capacity to oxidize long-chain fatty acids because of low levels of both carnitine palmityltransferase and carnitine. There is also in vivo

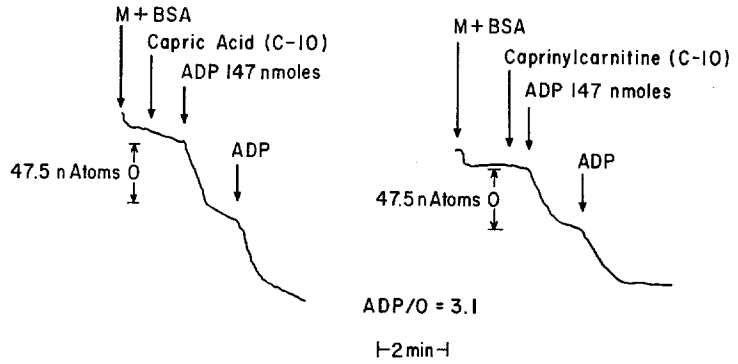

FIGURE 3 Polarographic tracing of bovine fetal heart mitochondria with capric acid and caprinylcarnitine as substrates. Mitochondria were isolated from a 7-month bovine fetus. Capric acid oxidation is shown on the left, and caprinylcarnitine oxidation is shown on the right. 0.75 mg of mitochondria (*M*) was used in the assays. The concentrations of capric acid and caprinylcarnitine were 0.34 mM and 0.1 mM, respectively. Other conditions are as in the text.

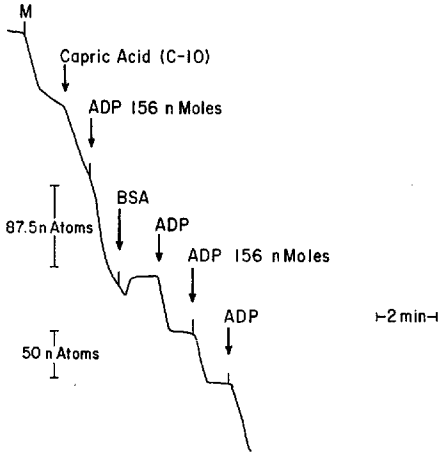

FIGURE 4 Effect of albumin on capric acid oxidation by bovine heart mitochondria. Mitochondria were prepared from the heart of a 6-wk-old calf. The capric acid concentration was 0.34 mM. 0.75 mg of mitochondria (*M*) was used in the assay. 5.0 mg of bovine serum albumin (*BSA*) were added to the assay where indicated. Other conditions are as in the text. Similar data were observed with fetal mitochondria.

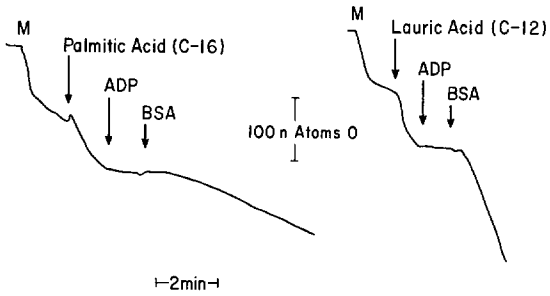

FIGURE 5 Effect of albumin on palmitic and lauric acid oxidation by heart mitochondria (6-wk-old calf). 0.75 mg of mitochondria (*M*) was used in the assays. The concentrations of palmitic acid and lauric acid were 0.34 mM. 5.0 mg of bovine serum albumin (*BSA*) were added to the assays where indicated. Other conditions are as in the text.

evidence that fatty acids are poor substrates in the young heart. In experiments with puppies, Breuer et al. (16) found that free fatty acids were not extracted from the coronary arteries of these animals until after the 2nd week of life. On the other hand, glucose oxidation by newborn rat heart homogenates has been shown to be much more active than in the adult heart (15). This suggests that glucose may be preferentially oxidized by the developing heart. It has been well established that anaerobic glycolysis and the hexose monophosphate shunt are active metabolic pathways during early development (17–21). The presence of abundant glycogen in the fetal heart is another indication of the importance of carbohydrate as a substrate (22).

The observations presented here indicate that fetal bovine heart mitochondria show impaired oxidation of palmityl-CoA and presumably coenzyme A derivatives of other long-chain fatty acids, but exhibit efficient oxidative phosphorylation with long-chain acylcarnitines as substrates. These results suggest that in the fetal heart mitochondria, the first step in the carnitine palmityltransferase sequence is rate limiting.

Palmityl-CoA + carnitine
→ palmitylcarnitine + CoA (1)

Palmitylcarnitine + CoA
→ palmityl-CoA + carnitine (2)

Oxidation with palmityl-CoA plus carnitine as substrate involving steps 1 and 2 is markedly limited in the fetal mitochondria when compared with the oxidation of these substrates by the calf mitochondria. However, the transport of palmitylcarnitine and its rate of oxidation (step 2) by the fetal mitochondria is equivalent to the rate observed with the calf heart mitochondria.

It has been shown that the formation of palmitylcarnitine controls the oxidation of palmityl-CoA (23, 24). Bremer and Norum (23), in experiments with rat liver mitochondria, observed a lag period before the maximal rate of oxidation was achieved when palmityl-CoA and carnitine were used as substrates. A similar lag period was observed in our experiments with calf heart mitochondria. This lag indicates that palmityl-CoA oxidation is controlled by the level of accumulation of the palmitylcarnitine intermediate. Our results suggest that the critical level of palmitylcarnitine necessary for the subsequent oxidation of palmityl-CoA is not reached in fetal heart mitochondria when palmityl-CoA and carnitine are substrates.

Carnitine palmityltransferase activity is inhibited by high levels of palmityl-CoA (23). Albumin has been shown to decrease this inhibition by binding excess palmityl-CoA. In view of the high levels of albumin used in our experiments, substrate inhibition of the transferase activity of fetal mitochondria by the levels of palmityl-CoA used in our experiments is unlikely. Albumin was highly effective in protecting fetal and calf heart mitochondria from the uncoupling effects and inhibition of oxidation caused by both short-chain and long-chain fatty acids. Such protection given mitochondria against the uncoupling effects of fatty acids by albumin is well known (13, 25).

As fetal heart mitochondria show deficient conversion of palmityl-CoA to palmitylcarnitine, energy metabolism in the fetal heart is likely to depend on the availability of carbohydrate. Ballard and Oliver (26) have shown that the fetal ruminant exhibits active gluconeogenesis so that glucose can be made available to the fetal heart by that pathway as well as through placental transport.

Shorter chain fatty acids, which freely permeate the inner mitochondrial membrane, were oxidized equally well by fetal and calf heart mitochondria. Therefore, these compounds could serve as effective substrates for the fetal heart. Another possible metabolic fate of fatty acids transported into mitochondria is to provide substrates for fatty acid elongation (27–29). Fatty acid synthesis by this pathway involves the addition of acetyl-CoA to endogenous mitochondrial fatty acids. The role of the elongation pathway in the fetal heart is unknown. However, it is possible that shorter chain fatty acids which do not exhibit carnitine-dependent transport may serve as substrates for fatty acid elongation in fetal mitochondria. As fatty acids synthesized by the elongation pathway may be incorporated into the lipid of the mitochondrial membrane, this pathway could have considerable importance in the developing heart.

It is not clear from this study exactly when calf mitochondria develop the capacity to oxidize palmityl-CoA. Adaptive changes favoring long-chain fatty acid oxidation may occur after exposure of the suckling calf to the high fatty acid content of milk or may relate to development of the rumen microflora. It is of interest that carnitine-supported oxidation of palmityl-CoA is very active in the embryonic chick heart. This may reflect the high lipid content of the yolk.

Differences in the membrane composition of fetal liver mitochondria compared with adult liver mitochondria have been reported (30). Fetal rat liver mitochondria show a higher lipid-to-protein ratio than is seen in mitochondria isolated from the mature animal. Thus, the biochemical alterations reported in this communication may accompany and possibly relate to such differences in membrane composition.

This research was supported by the United States Public Health Service grant 1-RO1-HD03610-01 and the Shriners Burns Institute. The authors are grateful for helpful discussions with Dr. D. R. Sanadi and Dr. John Lowenstein. The technical assistance of Miss Jane U. Edwards is deeply appreciated.

Received for publication 30 July 1969, and in revised form 7 October 1969.

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