INTERACTION OF FATTY ACID AND GLUCOSE OXIDATION BY CULTURED HEART CELLS

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ABSTRACT

Chick embryo heart cells in tissue culture actively oxidize [1-14C]palmitate to 14CO2. Fatty acid oxidation by cell monolayers was linear with time and increasing protein concentration. The addition of carnitine to the assay medium resulted in a 30-70% increase in the rate of fatty acid oxidation. The specific activity of palmitic acid oxidation did not change significantly with time in culture and was also the same in rapidly proliferating and density-inhibited cell cultures. Addition of unlabeled glucose to the assay medium resulted in a 50% decrease in 14CO2 production from [1-14C]palmitate. Conversely, palmitate had a similar sparing effect on [14C]glucose oxidation to 14CO2. Lactate production accounted for most of the glucose depleted from the medium and was not inhibited by the presence of palmitate in the assay. Thus, the sparing action of the fatty acids on glucose oxidation appears to be at the mitochondrial level. The results indicate that although chick heart cells in culture are primarily anaerobic, they can oxidize fatty acid actively.

INTRODUCTION

Developing tissues of a variety of species have high energy needs because of requirements of both growth and maintenance of tissue function. We have previously shown that the developing mammalian heart has an impairment of fatty acid oxidation and decreased activities of enzymes necessary for activation and mitochondrial transport of the fatty acids (1, 2). Those observations were consistent with the notion that fetal energy requirements are met primarily through the oxidation of transplacentally derived glucose. Although the chick embryo is also highly dependent on glucose (3), it has an active fatty acid oxidation. In contrast to the rat, the developing chick heart has significant activities of fatty acid-activating enzymes and acylcarnitine transferase (2).

We have investigated the oxidation of glucose by myocardial cells grown in tissue culture (4, 5). It was shown that glucose oxidation to CO2 was much greater in cells in logarithmic growth as compared with nonproliferating myocardial cells showing density-dependent inhibition of growth. This was attributed to higher activity of the pentose phosphate pathway in the dividing cell population, presumably related to increased requirements for ribose phosphate precursors of nucleotides.

The present communication is concerned with fatty acid oxidation by myocardial cells in culture and will describe interactions between fatty acid and glucose oxidation. The results indicate that cultured chick embryo myocardial cells can carry out an active fatty acid oxidation and that, although glucose metabolism by the cells is pri-
mainly anaerobic, glucose can spare fatty acid oxidation and, conversely, fatty acids can spare glucose oxidation to CO₂.

**MATERIALS AND METHODS**

Myocardial cells were isolated from 7- and 13-day chick embryo hearts by a minor modification of the method of DeHaan (6) as described previously (5). The cells were plated in small Falcon flasks (Falcon Plastics, Division of B-D Laboratories, Inc., Oxnard, Calif.) containing 4 ml of Gibco F-12 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% Gibco fetal calf serum and 5% chick embryo extract. Cells were generally plated at a density of 1.5 to 2.5 × 10⁶ per flask and were incubated at 37°C under 5% CO₂. Tissue culture media were changed daily with only the initial medium containing 500 U penicillin (E. R. Squibb & Sons, Princeton, N. J.)/ml.

Fatty acids were complexed to albumin according to the method of Spector and Hoak (7). When [1-¹⁴C]palmitic acid was used, unlabeled palmitic acid was added as a carrier to give a final specific activity of 1 mCi/mmol. The palmitic acid was first adsorbed onto Celite (Johns Manville Products Corp., Celite Div., Denver, Colo.) by dissolving 1 mmol of palmitic acid in hexane. 10 g Celite, 7-9 µm, was spread thinly on the bottom of a large beaker, the solution of palmitic acid in hexane was added to cover the Celite, and the hexane was gently evaporated. Fat-free bovine serum albumin was prepared according to the method of Chen (8). 1 g of the palmitic acid-Celite complex was added to 30 ml of 0.25 mM albumin in calcium-free Krebs-Ringer phosphate buffer, pH 7.4. The mixture was stirred for 80 min at room temperature, after which the supernatant solution was decanted and centrifuged for 10 min at 15,000 g. The sediment was discarded and, after a second centrifugation, the supernatant fraction was passed through a sterile HA millipore filter attached to a syringe, and stored at 3°C. The final concentration of free fatty acid was 1.6 mM as determined by a modification of the method of Trout et al. (9). The molar ratio of fatty acid to albumin was above 6:1.

Fatty acid oxidation by cultured myocardial cells was measured using methods described previously (5). Before assay, the growth medium was aspirated directly after incubation and frozen without acid precipitation and subsequent neutralization. Glucose was measured by the glucose oxidase procedure (Glucostat, Worthington Biochemical Corp., Freehold, N. J.). Protein per flask was determined by the method of Lowry et al. (11) after the cells were scraped and dissolved in 1 N NaOH. L-carnitine was a generous gift from the Otsuka Pharmaceutical Co., Osaka, Japan. Isotopes were obtained from I.C.N. Corp., Chemical and Radioisotopes Div., Irvine, Calif.

**RESULTS**

The time-course of ¹⁴CO₂ production from [1-¹⁴C]-palmitate is shown in Fig. 1. The cells were plated at 2.5 × 10⁶ cells per flask and assays were carried out after 3 days in culture. After an initial lag which may relate to recovery of the cells from media and temperature changes, or equilibration of substrate pools, the rate of ¹⁴CO₂ production was linear up to 105 min. As is also shown in Fig. 1, the addition of 1.25 mM carnitine to the assay medium enhanced fatty acid oxidation to CO₂. Fig. 2 shows the effect of increasing concentrations of palmitic acid (at a fixed molar ratio of fatty acid to albumin) on ¹⁴CO₂ production by cells grown at two different densities. For both, oxidation increased...
Figure 1 Time-course of $^{14}$CO$_2$ production from [1-$^{14}$C]palmitate. 14-day embryonic heart cells were plated at an initial density of $9.0 \times 10^5$ cells per flask. The assay was carried out after 3 days in culture. (O---O) The assay medium contained 0.16 mM [1-$^{14}$C] palmitate (sp act 1 mCi/mmol) complexed to bovine serum albumin (molar ratio 6.4) in Krebs-Ringer phosphate buffer, pH 7.4. Other conditions are as described in the text. (•---•) 0.16 mM [1-$^{14}$C] palmitate plus 1.25 mM l-carnitine.

Figure 2 $^{14}$CO$_2$ production as a function of [1$^{14}$C] palmitate concentration. 1.85 mM l-carnitine was present in all assays. (O---O) 0.49 mg protein per flask from an initial planting density of $3 \times 10^5$ cells. (•---•) 0.86 mg protein per flask from an initial plating density of $5 \times 10^5$ cells. Incubation conditions are as in Fig. 1. Assay time was 60 min.

with increasing palmitate concentration up to about 0.1 mM when the substrate reached saturating levels. All subsequent assays used 0.16 mM palmitate.

The effect of cell density on fatty acid oxidation was investigated using cultures plated at densities ranging from 0.4 to $6.0 \times 10^5$ cells per flask (Fig. 3). Assays for palmitate oxidation were carried out after 3 days in culture. Cells plated at high densities are density inhibited at that time whereas those plated at lower densities are still in logarithmic growth (5). As is shown in Fig. 3, production of $^{14}$CO$_2$ from [1-$^{14}$C]palmitate was linear with increasing protein over the density range. The specific activity of palmitate oxidation (nanomoles CO$_2$/milligrams protein) remained constant or increased slightly at higher cell densities. Fatty acid oxidation by hearts from 7-day chick embryos was similar but showed higher specific activity (30%) than observed with 13-day heart cultures.

Table I

| L-carnitine | $^{14}$CO$_2$ nmol/mg protein |
|-------------|-----------------------------|
| 0           | 3.39                        |
| 0.03        | 3.04                        |
| 0.1         | 4.67                        |
| 0.3         | 3.99                        |
| 1.0         | 4.54                        |
| 2.5         | 4.81                        |
| 10.0        | 4.83                        |

13-day embryonic heart cells were plated at an initial density of $1.5 \times 10^5$ cells per flask. The assay was carried out after 3 days in culture in a total volume of 2.0 ml per flask. Palmitate concentration was 0.16 mM. The incubation time was 1 h. Other conditions are as described in the text.
As shown above, the addition of carnitine to the assay medium stimulated palmitate oxidation. Table I shows that stimulation of palmitate oxidation to $^{14}$CO$_2$ was obtained with carnitine concentrations above 0.1 mM. The percentage of stimulation of palmitate oxidation by added carnitine varied from 30 to 90% of basal levels in different experiments, but did not correlate with the age of embryos used or the density of the cell cultures. A safe concentration of carnitine (1.25 mM) was used in subsequent experiments to eliminate possible differences in endogenous carnitine levels.

Production of $^{14}$CO$_2$ by homogenates of cultured cells was compared with oxidation by intact cells. Cells scraped from the flasks and then homogenized showed reduced levels of oxidation of $[^{14}$C]-glucose (uniformly labeled) and $[1^{14}$C]palmitate when assayed with added carnitine. However, as shown in Table II, the homogenates were far more dependent upon exogenous carnitine than were intact cells.

Fatty acid oxidation was also measured in cells to which 1 mM carnitine had been added to the growth medium 24 h before assay. As is shown in Fig. 4, cells grown in the presence of added carnitine gave rates of $^{14}$CO$_2$ production from $[^{14}$C]-palmitate higher than controls, but similar to values obtained for controls assayed in the presence of carnitine. Palmitate oxidation by cells grown in the presence of carnitine was not stimulated further by added carnitine in the assay medium.

Having established that cultures of chick heart cells oxidize both palmitate and glucose to CO$_2$, we also investigated the effects of glucose on...
[1-14C]palmitate oxidation and of palmitate on [14C]glucose oxidation. As is shown in Fig. 5, added glucose decreased the oxidation of [1-14C]palmitate to CO2 up to 50%. Conversely, the addition of unlabeled palmitate to the assay media containing [14C]glucose (uniformly labeled) decreased 14CO2 production from glucose to a similar degree. Carnitine alone had no effect on glucose oxidation. 14CO2 production from [14C]glucose (uniformly labeled) is expressed in counts per minute rather than nanomoles because calculation of the yield from glucose is more complex than the simple substrate-product relationship in the case of palmitic acid. These results indicate that palmitate can spare glucose oxidation and that, conversely, glucose can spare palmitate oxidation in chick heart cell cultures.

Measurements were made of lactate production and glucose depletion during a 1-h incubation. As is shown in Fig. 6, there was some lactate production by cells incubated only in Krebs-Ringer phosphate buffer. This probably reflects glycogenolysis or the presence of other endogenous substrates such as glutamine. Lactate production increased markedly when glucose was included in the assay medium. Glucose depletion and lactate production both increased with increased glucose concentrations (Table III). The addition of palmitate to the assay medium did not have a sparing effect on lactate production by cells incubated with buffer or with added glucose (Fig. 6). In fact, under the latter conditions lactate production increased.

Figure 5  (A) Effect of glucose on 14CO2 production from [1-14C]palmitate. Cells were plated at an initial density of 1.5 x 10^6 per flask and assayed after 3 days in culture. The assay media contained 0.16 mM [1-14C]palmitate and 1.25 mM carnitine. Assays were performed with [1-14C]palmitate alone (control) and in the presence of 1 mM glucose. Assay time was 60 min. (B) Effect of palmitic acid on 14CO2 production from [14C]glucose (uniformly labeled). Assays were performed with [14C]glucose (uniformly labeled) alone (control) and in the presence of 0.16 mM palmitate. 1.25 mM carnitine was used throughout. Cells were plated at an initial density of 1.5 x 10^6 per flask assayed after 3 days in culture. Assay time was 60 min.

Figure 6  Influence of glucose and palmitic acid on lactate production by cultured heart cells. Cells were plated at an initial density of 1.5 x 10^6 per flask and assayed after 3 days in culture. The buffer was Krebs-Ringer phosphate. The concentrations of glucose and palmitate were 1 mM and 0.16 mM, respectively. 1.25 mM carnitine was added when palmitate was present. Assay time was 60 min.
Cells were plated at 1.25 × 10^6 per flask and assayed after 4 days in culture. All incubations were for 1 h as described in the text. Lactate above basal level was calculated by subtracting the lactate produced by cells in buffer alone, from the total lactate produced by cells incubated with glucose. Preincubated cells were incubated at 37°C on the shaking water bath for 1 h in Krebs-Ringer phosphate buffer and then assayed under usual conditions.

slightly. Thus, the sparing of glucose oxidation by palmitate was observed only with respect to CO₂ production; lactate production from exogenous glucose as well as that from endogenous sources was not inhibited by added palmitate.

Measurements of glucose depletion and lactate production were also carried out after the cells had been preincubated for 1 h in an attempt to reduce endogenous substrates, particularly glycogen. As is shown in Table III, lactate production by preincubated cells assayed in the absence of substrate was less than in nonpreincubated cultures. Glucose depletion was higher in the reincubated cultures. Since each glucose molecule can yield two lactates, it appears that lactate production accounts for most of the glucose removed from the assay medium. By comparison, levels of ¹⁴CO₂ production are far lower than those of lactate production (4). The results indicate that lactate is the main product of glucose metabolism under our assay conditions.

**DISCUSSION**

Our results have demonstrated that chick myocardial cells grown in tissue culture actively metabolize palmitic acid to CO₂. Before they can be oxidized, palmitate and other long chain fatty acids are activated to their corresponding coenzyme A esters by palmitoyl-CoA synthetase. As coenzyme A esters do not permeate the mitochondrial membrane, the transport and subsequent oxidation of palmitoyl-CoA is dependent on the reversible formation of palmitoylcarnitine (12, 13) which serves as the mitochondrial transport intermediate. Once transport has taken place the palmitoylcarnitine is reconverted to palmitoyl-CoA and β-oxidation proceeds. These steps are catalyzed by the enzyme palmitoylcarnitine translocase which is present on both sides of the mitochondrial membrane barrier (14, 15).

We have shown previously that embryonic chick myocardium can carry out fatty acid oxidation and has high activities of palmitoyl-CoA synthetase and palmitoylcarnitine transferase (1). Casillas and Newburgh (16) have reported substantial levels of free carnitine and acylcarnitine esters in the developing chick heart. It is therefore reasonable that cultured chick myocardial cells can carry out fatty acid oxidation. The use of tissue culture material for the investigation of fatty acid oxidation provides a homogeneous source of tissue which can be manipulated in a precise and controlled fashion and makes possible measurement of metabolic processes on intact cells still adherent to the tissue culture flask surface (5).

Ehrlich ascites tumor cell suspensions (17) and Hela cell suspensions (18) have been shown to oxidize long chain fatty acids. Spector (19) reported that oxidation of palmitic acid reflected the capacity of cells to oxidize a spectrum of different chain length fatty acids. Spector also showed (20) that palmitic acid oxidation by intact Ehrlich

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**Table III**

*Lactate Production and Glucose Depletion by Heart Cells*

| Substrates             | Total lactate produced µmol | Lactate above basal levels µmol | Glucose depleted µmol |
|------------------------|----------------------------|--------------------------------|-----------------------|
| Buffer alone           | 0.16                       |                                |                       |
| 0.25 mM glucose        | 0.28                       | 0.12                           | 0.05                  |
| 1.0 mM glucose         | 0.62                       | 0.46                           | 0.21                  |
| Buffer alone, after preincubation | 0.06 |                                |                       |
| 0.25 mM glucose, after preincubation | 0.26 | 0.20                           | 0.13                  |
| 1 mM glucose, after preincubation | 0.61 | 0.55                           | 0.32                  |

Cells were plated at 1.25 × 10^6 per flask and assayed after 4 days in culture. All incubations were for 1 h as described in the text. Lactate above basal level was calculated by subtracting the lactate produced by cells in buffer alone, from the total lactate produced by cells incubated with glucose. Preincubated cells were incubated at 37°C on the shaking water bath for 1 h in Krebs-Ringer phosphate buffer and then assayed under usual conditions.
ascites tumor cells was not stimulated by carnitine but that oxidation of disrupted cells showed a clear carnitine dependency. Presumably under his conditions endogenous carnitine was diluted during the homogenization procedure and became rate limiting for palmitate oxidation. Under our conditions exogenous carnitine always stimulated fatty acid oxidation by intact cells, although carnitine stimulation of palmitate oxidation of heart cell homogenates was more pronounced (Table II). If carnitine was included in the growth medium the addition of exogenous carnitine to the assay medium did not stimulate palmitate oxidation further (Fig. 4). This indicates that saturating levels of carnitine were present at the time of the assay only if the chick heart cells had been grown in medium supplemented with carnitine.

We have shown previously that glucose oxidation to CO₂ by embryonic chick heart cells depended on the state of growth of the cell population (5). The specific activity of glucose oxidation by cells in logarithmic growth was as much as 10 times higher than that in more dense cell populations showing density inhibition. This decrease in glucose oxidation to CO₂ was shown to be due to a decrease in the activity of the pentose phosphate pathway, perhaps related to decreased requirements for ribose precursors of nucleotides. The specific activity of lactate production also decreased with density-dependent growth inhibition. In contrast to the changes observed for glucose oxidation, the specific activity of oxidation of [1-¹⁴C]palmitate to CO₂ was constant or even increased slightly with increased density of the cell population.

Heart cells grown in monolayer tissue culture have been shown to become primarily anaerobic and to develop a greater dependence on glycolysis. Cahn (21) has shown that the isozymes of lactate dehydrogenase show a shift from the heart to the muscle type in myocardial cell cultures. We have made similar observations. As the muscle isoenzyme shows less inhibition with increasing pyruvate concentrations than the heart form, glycolysis is favored. Lactate production from cells incubated in Krebs-Ringer phosphate without added substrates can be attributed to glycolysis. Lactate production from endogenous substrates decreased after a 1-h incubation of the cells in substrate-free buffer. Heart cells contain abundant periodic acid-Schiff positive droplets which decrease in amount during the incubation (4). More recently, cultured heart cells have been found to have 40-60 µg glycogen/mg protein (Rosenthal and Warshaw, unpublished observation). Thus endogenous lactate production appears to be principally derived from glycogen. Despite the endogenous lactate production, addition of glucose to the assay medium resulted in increased lactate production; the increase was sufficient to account for most of the glucose depleted from the assay medium.

The oxidation of palmitate did not inhibit lactate production either from exogenous glucose or from endogenous reserves. On the other hand, ¹⁴CO₂ production from [¹⁴C]glucose was decreased up to 50% if unlabeled palmitate was present in the assay medium, suggesting that fatty acids can spare glucose oxidation to CO₂ but not glucose metabolism to lactate. This is in contrast to the observation of Spector and Steinberg (22) that free fatty acids did not decrease glucose oxidation to CO₂ in Ehrlich ascites tumor cells. Unlabeled glucose also had a sparing effect on the oxidation of [1-¹⁴C]palmitate to CO₂. Similar results have been obtained using tumor cells (17, 18, 22).

Fatty acids are the principal metabolic fuel of the adult mammalian heart and it has been shown clearly in the perfused heart that infusion of long chain fatty acids results in decreased glucose oxidation (23). The mechanism by which fatty acids inhibit glucose oxidation has been related to inhibition of phosphofructokinase and pyruvate dehydrogenase by increases in intracellular citrate and acetyl-CoA associated with increased fatty acid oxidation (24, 25). Since addition of palmitate in our assays only spared glucose oxidation to CO₂ and not lactate production, it is unlikely that inhibition of phosphofructokinase is responsible for the decrease in glucose oxidation. However, since we consistently observed a slight increase in lactate production when palmitate was included in the medium, the sparing effect of fatty acids on glucose oxidation by the fetal heart cells may be related to the inhibition of pyruvate dehydrogenase (26).

Our results indicate that chick heart cells in culture do not show absolute substrate preferences for either glucose or fatty acids and that substrate competition occurs at the level of oxidative metabolism. Despite our observation that lactate production from glucose far exceed CO₂ production and evidence that the cells were adapting to anaerobic metabolism, the cells maintain an active fatty acid oxidation.
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