

THE EFFECTS OF METABOLIC INHIBITORS ON CULTURED RAT HEART CELLS

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INTRODUCTION

Cells derived from neonatal rat heart tissue may be cultured *in vitro*. These cells retain the ability to spontaneously beat or contract. As the cells age *in vitro*, the beating rate gradually decreases and eventually stops. This loss of function has been described as dedifferentiation (1).

In the intact, adult, mammalian heart, lipids are the primary source of metabolic energy (2). Lipids are also required for the maintenance of beating in cultured heart cells; if lipids are removed from the growth medium, the cells lose the ability to beat (3). However, neonatal rat heart tissue is largely dependent on glucose metabolism (4). As the heart cells age *in vitro*, there is a shift in the respiratory quotient (RQ) from 0.86 at day 0 to 0.96 at day 14. This suggests that the heart cells switch from lipid to carbohydrate metabolism as they age *in vitro* (5). Glucose metabolism via the pentose phosphate pathway has been demonstrated in chick heart embryos (6) and in cultured heart cells (7). The glycolytic pathway for glucose metabolism is also functional in cultured rat heart cells (5).

Glucose metabolism in a number of cells and animal tissues is inhibited by the glucose analog, 2-deoxy-D-glucose (8, 9). This compound is transported into the cell (10) where it is phosphorylated by hexokinase to form 2-deoxyglucose-6-phosphate. This compound then acts as a competitive inhibitor of the glycolytic enzyme, phosphoglucosomerase (8, 11). An inhibitory effect of 2-deoxyglucose-6-phosphate on glucose-6-phosphate dehydrogenase has also been reported (8).

MATERIALS AND METHODS

The hearts from 1-3-day old neonatal rats of Wistar albino strain mothers were cultured in 60 × 15 mm plastic petri dishes (Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) as described by McCarl and Margossian (12). Each plate received the equivalent of the cells from one heart. The cultured cells were incubated in a modified Puck's

medium (13) at 37°C in a 95% air, 5% CO₂ atmosphere saturated with moisture. Every third day the growth medium on the cells was routinely replaced with 4.0 ml of fresh growth medium. The used growth medium from 20-30 plates was pooled and assayed for glucose uptake and lactate production.

Antimycin A, oligomycin, and rotenone (Sigma Chemical Co., St. Louis, Mo.) were added to the growth medium as 10 μl of a solution of the inhibitors in 95% ethanol. The final concentration of these inhibitors in the growth medium was 1×10^{-7} M. Control plates received 10 μl of 95% ethanol. The 2-deoxyglucose-treated cultures received growth medium containing 30 mM 2-deoxyglucose starting with the first medium change at day 3.

Cell protein was determined by the method of Lowry as modified by Oyama and Eagle (14). Lactate was assayed by an enzymatic procedure using lactate dehydrogenase (Boehringer Mannheim Corp., New York) (15). Glucose was assayed by a glucose oxidase:peroxidase:o-dianisidine procedure (Glucostat reagent kit, Worthington Biochemical Corp., Freehold, N. J.).

Since 2-deoxyglucose also reacts in the glucose oxidase assay (16), glucose in the presence of 2-deoxyglucose was assayed by combining two assay techniques. 2-Deoxyglucose was first independently assayed by a specific assay described by Waravdekar and Saslaw (17). The absorbancy that this amount of 2-deoxyglucose would produce using the Glucostat assay was then determined from an appropriate standard curve. This value was then subtracted from the total absorbancy derived from a Glucostat assay of glucose in the presence of 2-deoxyglucose to yield the absorbancy due to the glucose alone.

RESULTS

After culturing, approximately 24 h are required for the heart cells to become acclimated to their *in vitro* environment. During this time, the cells become attached to the bottom of the culture dish, flatten out of their spheroid shape, and initiate beating. Soon afterwards, cell division commences as evidenced by the appearance of mitotic figures. By the time of the first medium change on day 3, the cells have grown together to form a mono-

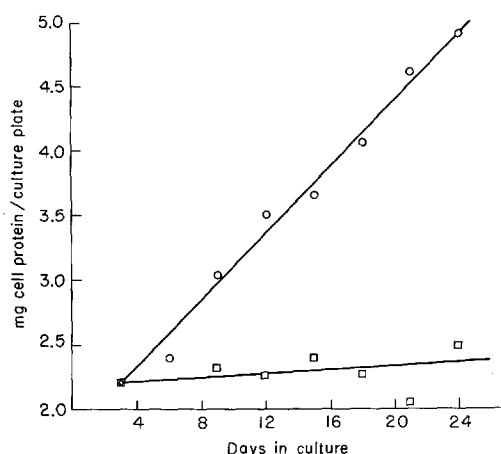


FIGURE 1 Cell protein in cultured heart cells as a function of length of culture. Each point represents the average of three culture plates from a single culture. Circles represent a normal heart cell culture; squares represent the same culture treated with growth medium containing 30 mM 2-deoxyglucose from day 3 onward.

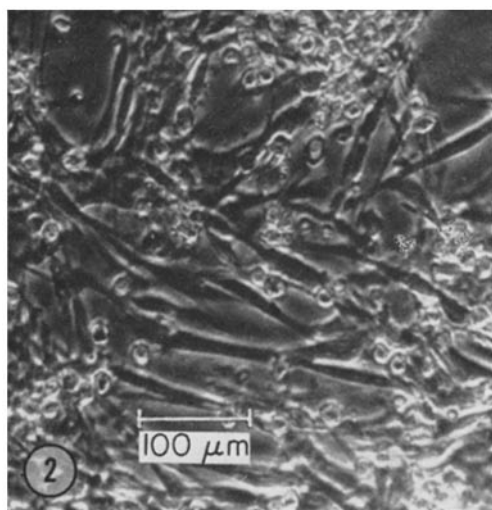


FIGURE 2 Phase contrast photomicrograph of a culture which had been treated with growth medium containing 2-deoxyglucose (30 mM) from day 3. The picture was taken after 1 wk of exposure to 2-deoxyglucose. $\times 150$.

layer. Throughout the remainder of the culture, there is a linear increase in cell protein (Fig. 1). In this study, it is assumed that an increase in all protein is indicative of cell growth and replication. This assumption is substantiated by the observa-

tion that an increase in cell protein is paralleled by an increase in cell number in cultured heart cells (18).

Cells which have been treated with growth medium containing 2-deoxyglucose showed little, if any, increase in cell protein (Fig. 1). Cell growth and replication in these cells is apparently arrested.

2-Deoxyglucose also affects the cellular morphology of the cultured cells. Cultured heart cells which are treated with medium containing 2-deoxyglucose appear as elongated, spindle-shaped cells (Fig. 2). Unlike the normal heart cell cultures, these 2-deoxyglucose-treated cells do not grow together to form a confluent monolayer.

In addition, 2-deoxyglucose profoundly increases the duration of beating of the cultured cells. Normal heart cells, as cultured in our laboratories, continue to beat for approximately 3–4 wk in culture. In contrast, 2-deoxyglucose (30 mM)-treated cells have continued to beat for up to 65 days in culture.

Beating and cell growth and replication (as indicated by protein increases) were affected by inhibitors of electron transport (antimycin, rotenone) and by inhibitors of oxidative phosphorylation (oligomycin). A 12-day old culture of heart cells, beating approximately 60 times per minute, was incubated at 37°C with growth medium containing these inhibitors (1×10^{-7} M). Within 0.5 h after the inhibitors had been added, a decrease in the beating rate was observed. The beating rate continued to decline; and within 6–8 h, there was a complete cessation of beating in the inhibitor-treated cells.

Other workers have examined the effect of oligomycin (0.5 μ g/ml) on the ATP level in cultured heart cells incubated at 22°C. In cells so incubated, there was a small, gradual decrease in the ATP level and in the beating rate over a 1 h incubation (19). Longer incubation at physiological temperatures (37°C) might further deplete the ATP reserves of the cells. This could result in the elimination of beating as was observed in the present study.

In addition to eliminating beating, these inhibitors also affected cell growth and replication. Table I shows that the inhibitor-treated cells had less cell protein synthesis than the control cells.

Of the three inhibitors, rotenone was the least effective inhibitor of cell growth. This might be explained by the fact that rotenone blocks only

TABLE I
The Effects of Metabolic Inhibitors on Cell Growth in Cultured Rat Heart Cells

	Days in culture		Increase in protein (μg)
	12 (μg Protein/plate)	18	
Control	2,650	4,050	1,400
Oligomycin (10^{-7} M)	2,650	2,700	50
Antimycin A (10^{-7} M)	2,650	3,200	550
Rotenone (10^{-7} M)	2,650	3,650	1,000

Each protein determination represents the average of three plates.

the transport of electrons derived from NAD^+ linked oxidations. Consequently, electrons derived from succinate or fatty acyl CoA oxidations would still be transported through the electron transport complex with the concomitant production of ATP.

The cultured heart cells took up large quantities of glucose from the growth medium (Fig. 3). As the cells aged, the glucose consumption per milligram of cell protein declined. By day 28 when the control cells stopped beating, glucose consumption had fallen to less than half of its maximum level.

The glucose consumption during the first three days of culture was significantly lower than the glucose consumption later in the culture (Fig. 3). This may suggest that the cells are relying on an energy source other than glucose during this period. Since lipids are the principal metabolic fuel in the adult heart (2), lipid oxidation may also play an important role in these cultured cells. Also during the first day or two of culture, the cells are undergoing an adjustment to their in vitro environment. During this period there is little cell division or beating; therefore, the energy requirements of the cells would be less.

Cultured cells which had been treated with 2-deoxyglucose showed reduced glucose consumption (Fig. 3). However, the level of glucose consumption in these cells remained constant throughout the culture period. Only trace amounts of 2-deoxyglucose were taken up from the growth medium by the cultured cells.

The cultured heart cells produced large amounts of lactate and released it to the growth medium (Fig. 4). Lactate production was greatest between the third and sixth days of culture; thereafter lactate production decreased in parallel to the decrease in glucose consumption (Fig. 3).

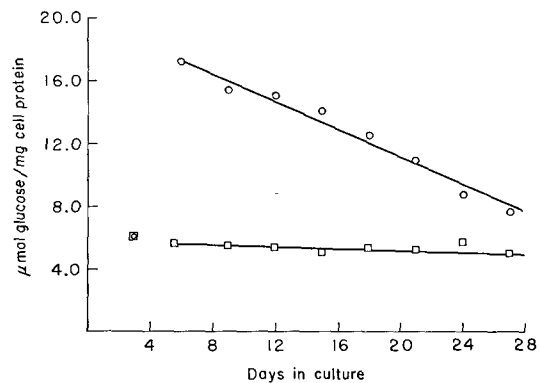


FIGURE 3 Glucose uptake from the growth medium in normal (circles) and in 2-deoxyglucose-treated (squares) heart cell cultures. Each point represents the glucose uptake during the 3-day interval.

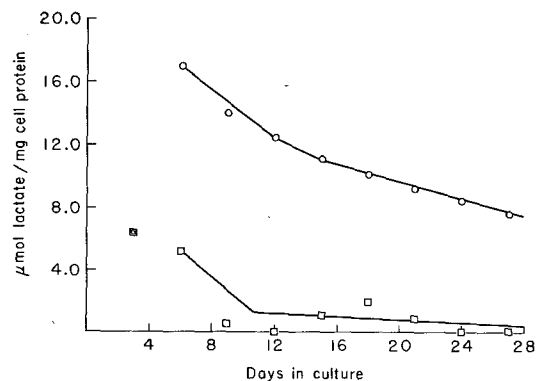


FIGURE 4 Lactate production in normal (circles) and in 2-deoxyglucose-treated (squares) heart cell cultures. Each point represents the lactate production during the 3-day interval. The lactate contributed by the serum component of the growth medium was subtracted from the total lactate content of the used growth medium to yield the lactate production due to the cultured cells.

The 2-deoxyglucose-treated cells produced relatively smaller amounts of lactate (Fig. 4). As these cells aged, the lactate production decreased to near zero. It appears that glucose catabolism in these cells proceeds to end products other than lactate. Perhaps the citric acid cycle is a more important pathway for glucose catabolism in the 2-deoxyglucose-treated cells than in normal cultured heart cells.

DISCUSSION

Beating in cultures of rat heart cells is dependent on a functional electron transport complex and associated oxidative phosphorylation. If these processes are interrupted with metabolic inhibitors, beating stops. Cell growth and replication is also interrupted by the presence of these inhibitors (Table I).

The presence of 2-deoxyglucose in the growth medium profoundly affects the growth, morphology, and function of cultured heart cells. 2-Deoxyglucose at a concentration of 30 mM (twice the glucose concentration of the medium) sharply reduces the glucose consumption of the cultured cells (Fig. 3). This inhibition of glucose metabolism results in lower production of toxic end products of glucose catabolism (e.g. lactate, Fig. 4). Therefore, any deleterious effects of these catabolites on the cells would be reduced. This could be partially responsible for the prolongation of beating in the 2-deoxyglucose-treated cells.

Cultured cells treated with growth medium containing 2-deoxyglucose show inhibited cell growth and replication (Fig. 1). This could be related to lower energy availability as a result of the 2-deoxyglucose inhibition of glucose metabolism. In addition, 2-deoxyglucose might also affect the activity of the pentose phosphate pathway by inhibiting glucose-6-phosphate dehydrogenase, the enzyme catalyzing the first step of this pathway (8). Inhibition of this pathway would reduce the availability of ribose, which in turn would inhibit the synthesis of nucleic acids required for cell growth and replication. Inhibition of the pentose phosphate pathway would also decrease the availability of NADPH, a required reductant in many biosynthetic reactions.

Cultured heart cells treated with 2-deoxyglucose exhibit a prolongation of beating and an inhibition of net protein synthesis. This suggests that cell function (beating) and cell replication may be antagonistic processes in these cultured cells.

It might be that the cultured heart cells are unable to simultaneously fulfill the biosynthetic and energetic demands of cell replication and cell function. Inhibition of cell replication by 2-deoxyglucose would free the metabolic machinery and energy production of the cell for processes required for beating.

Alternatively, it is possible that 2-deoxyglucose treatment of cultured heart cells selects out for cardiac muscle cells. Long-term treatment of cultures with the compound may be detrimental to cells which are dependent on a smoothly functioning glycolytic pathway. 2-Deoxyglucose treatment might prevent any such cells from crowding out the functional muscle cells by selective proliferation. If cardiac muscle cells can utilize fatty acids as a source of energy for contraction they could continue to contract but not replicate. This suggests that cardiac muscle cells have two pools of ATP, one for contraction and one for cell growth and replication.

SUMMARY

Cell growth and beating in cultures of rat heart cells are inhibited by inhibitors of electron transport and oxidative phosphorylation. Treatment of the heart cells with growth medium containing the glucose analog, 2-deoxyglucose, results in decreased glucose consumption and lactate production. In addition, 2-deoxyglucose treatment of the cultured cells causes an elimination of cell growth and a prolongation of beating.

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