# BIOCHEMICAL RESPONSES OF MYOCARDIAL CELLS IN CULTURE TO OXYGEN AND GLUCOSE DEPRIVATION

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SUMMARY. The biochemical responses of myocardial cells to oxygen and glucose deprivation, i.e., "ischemia", have been measured in two tissue culture models: beating intact fetal mouse hearts maintained in organ culture and beating chick heart cells in monolayer culture. The responses to 1-4 hours of ischemia include loss of beating function, depletion of ATP content and release of cytoplasmic enzymes. It is suggested that these culture models are useful in the study of the biochemical responses of the myocardium to ischemia. Culture models offer advantages over adult in vivo and in vitro models of ease and reproducibility of preparation and the ability to control experimental conditions.

Definition of those biochemical parameters which characterize reversible and irreversible cell damage is an important problem in cell biology and has clinical significance for example, in ischemic injury (1,2). The experiments described in this report have used cell and organ cultures of embryonic heart as models for studying responses of myocardial cells to "ischemia", i.e., oxygen and glucose deprivation. Responses measured include loss of beating function, ATP depletion and the time-dependent profiles of enzyme release (specifically lactic dehydrogenase (LDH) and creatine kinase (CPK) ). These cultures are representative of whole heart and contain myoblasts, myocytes and fibroblasts; biochemical changes represent the average response of all cells.

## MATERIALS AND METHODS

Preparation of cultures. Intact beating hearts from 17 to 20-day fetal mice (approximately 2-5 mg wet weight) were maintained on stainless steel grids at an air-medium interface in organ culture dishes as previously described (3). Hearts were cultured for 1 to 4 days in Medium 199 (Grand Island Biologicals). Each experiment was performed using hearts from matched litter mates.

Synchronously beating heart cells in culture were obtained from 11-day

chick embryos using the repeated trypsinization procedures of DeHaan (4) and Harary (5). Cells were plated in culture dishes (Falcon) at a density of $3 \times 10^6$ cells/35 mm plate in Minimum Essential Medium (MEM) (Grand Island Biologicals) supplemented with 5% select calf serum. After 24 hours, and daily thereafter, cultures were supplied MEM only.

Imposition of ischemia. Cell and organ cultures were made "ischemic" by replacing the culture medium with argon-saturated glucose-free MEM and incubating them in a sealed chamber continually flushed with nitrogen. Oxygen content of the medium was measured using Model 213 Gas Analyzer (Instrumentation Laboratories, Inc.) and was found to be <5 mmHg. Controls were maintained in complete MEM in the presence of oxygen. At the end of the ischemic period, control and experimental cultures were supplied complete oxygenated medium 199 or MEM.

ATP assays. Hearts or cells collected by scraping were homogenized in cold 0.4 N perchloric acid. After neutralizing with KOH, the suspension was centrifuged at 800 x g for 5 minutes. An aliquot of the supernatant was diluted ten times and ATP content was measured with crystalline firefly luciferase as described (6). Known amounts of ATP added to the sample prior to homogenization were completely recovered, demonstrating that there is no loss of ATP during handling of the sample.

LDH and CPK assays. Samples were homogenized in cold 0.05 M TRIS buffer, pH 7.8, containing 1 mM EGTA and 1 mM β-mercaptoethanol, and aliquots were assayed for enzyme activity and protein content. CPK activity was assayed according to the method of Rosalki (7). LDH was assayed by the method of Bernstein and Everse (8). Enzyme levels in cultured cells or hearts were calculated as International Units/mg protein. Total protein was obtained after solubilization in 1 N NaOH for 24 hours at room temperature. Assays were performed in duplicate according to the method of Lowry (9) using crystalline bovine serum albumin as a standard.

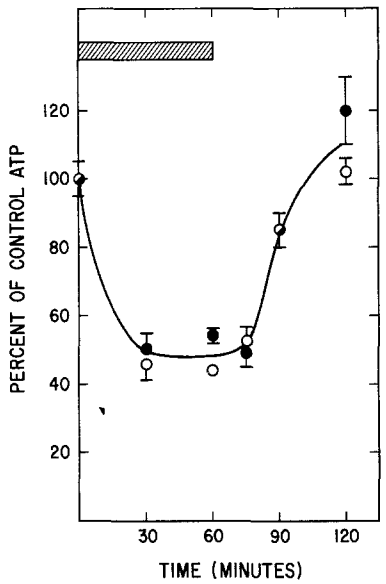


FIGURE 1: The effect of 1 hour of ischemia on the ATP content of fetal mouse hearts in organ culture. Data represent the average ATP content of fetal mouse hearts from matched litter mates and is expressed as the percent of the ATP content in hearts subjected to ischemia compared to control hearts. Control levels of ATP were 41 $\pm$ 4 mμmoles ATP/mg protein. Beating was lost by 30 minutes. At 60 minutes oxygen and glucose were resupplied and beating function was immediately restored.

## RESULTS AND DISCUSSION

Figure 1 shows the effect of 1 hour of oxygen and glucose-deprivation, i.e., "ischemia", on the ATP content of beating fetal mouse hearts maintained 2 days in organ culture. By 30 minutes of ischemia, beating function ceased and the ATP content was decreased by 50%. At 60 minutes, cultures were re-supplied oxygen and glucose. Beating resumed immediately even though ATP levels were still depressed. By 60 minutes post-ischemia, ATP levels in the experimental hearts were equal to or slightly greater than control values. This suggests that most if not all of the cells recovered normal oxidative capacity and were not irreversibly injured. This conclusion is further supported by the observation that no enzyme loss from the hearts could be detected even 24 hours after the period of ischemia.

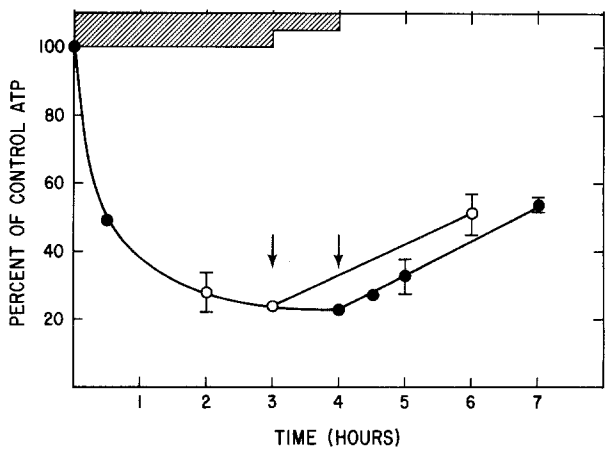


FIGURE 2: The effect of 3 (○) and 4 (●) hours of ischemia on ATP levels in fetal mouse hearts in organ culture. Arrows indicate the time of resupply of oxygen and glucose. All points are average values obtained from four or more hearts. Beating had ceased after 30 minutes of ischemia; sluggish beating resumed within 1 to 2 hours after resupply of oxygen and glucose.

Figure 2 shows that increasing the ischemic period to 3 or 4 hours results in depleting ATP levels in fetal mouse hearts approximately 75%. Resupply of oxygen and glucose after this period of deprivation did not lead to rapid restoration of beating function or of ATP levels. Beating function was not restored until 1 hour after resupply and was sluggish. ATP levels were only partially restored even by 4 hours after resupply. No significant enzyme loss could be detected at the end of the four hour period of ischemia, but by two hours after resupply of oxygen and glucose, hearts which had been made ischemic contained 30% less creatine kinase activity as control hearts. These responses suggest that some irreversible cell damage and/or depression of repair processes have occurred.

In a single experiment, a four hour period of ischemia depressed ATP levels in fetal mouse hearts to 5% of control levels. Forty percent of the LDH content was lost from these hearts after 1 hour of resupply of oxygen and glucose; 80% of LDH activity was lost by 14 hours after resupply. LDH activity lost from the hearts was fully recovered in the medium. Beating

function was never restored and by 14 hours after resupply of oxygen and glucose, the hearts were opaque. Depression of ATP levels to this extent clearly indicates significant cell necrosis.

The response of intact beating hearts in culture to anoxia and glucose deprivation can be summarized as follows: no enzyme release can be detected from hearts in which the ATP levels are depleted 50% and such hearts can fully recover following resupply of oxygen and glucose. If ATP levels fall below 80% however, enzyme release is significant and full recovery is not possible.

Figure 3 shows the results of several experiments in which CPK and LDH

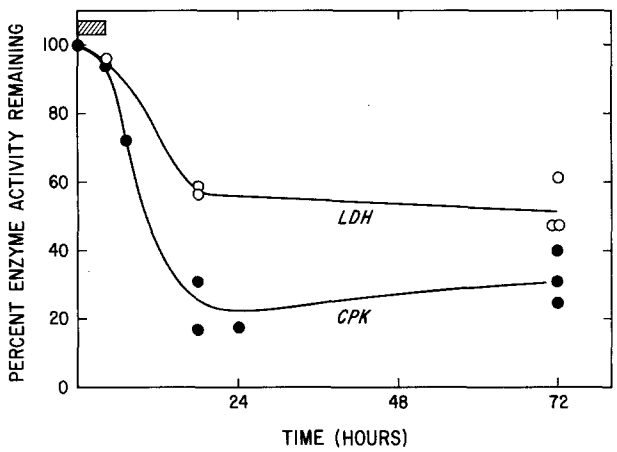


FIGURE 3: Effect of ischemia on the long term release of CPK and LDH from fetal mouse hearts obtained from several litters. Oxygen and glucose were resupplied at 4 hours. Data are expressed as percent of enzyme activity remaining in hearts subjected to ischemia compared to control hearts from matched litter mates at the same time in culture. Values for the CPK and LDH content of control hearts were 2-3 IU/mg protein.

loss were followed for several days after 4 hours of ischemia. The profiles of enzyme loss with time are essentially the same for both enzymes. This observation is in general agreement with Hearse et al (10) and Schulze et al (11) using isolated perfused rat heart but in disagreement with the profile of LDH and CPK activities released into the serum of patients following myocardial infarction. In such patients, maximum elevated LDH activity detected in serum

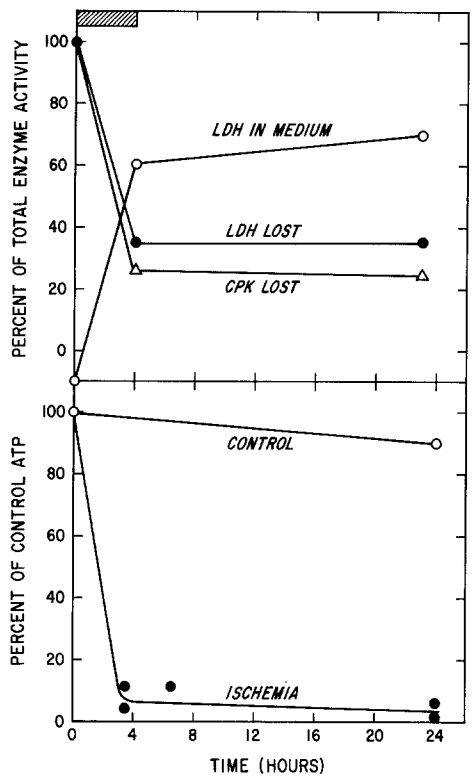


FIGURE 4: The effect of 4 hours of ischemia on ATP content and enzyme depletion in chick heart cell cultures. The lower half of the figure represents the depletion of ATP expressed as percent of control. Average control values for the cell cultures was 49 $\pm$ 4 mμmoles ATP/mg protein. Each ATP value represents an average obtained from four or more cultures assayed at various times. The upper half of the figure shows the percent of the total LDH (●) activity and CPK activity (Δ) remaining in the cells with time after the onset of ischemia. The precent of the total LDH activity detected in the medium is also shown (0). Control values for CPK and LDH activities are 1-1.5 IU/mg protein.

considerably lags detection of maximum elevated CPK activity (12).

Figure 4 shows the results of a representative experiment in which ATP and enzyme depletion were measured in beating heart cell cultures exposed to 4 hours of ischemia. At the end of the ischemic period, ATP levels were depressed 90% and 60-70% of LDH and CPK activities were lost from the cells. Even by 20 hours after resupply of oxygen and glucose, no change in ATP or enzyme levels could be detected. While most of the cells remained attached

to the plate during the experiment, the cells had ceased beating and were highly vacuolated. In a separate experiment, the rates of ATP depletion and enzyme release during the period of ischemia were measured. No LDH activity was detected in the medium until 2 hours after the onset of ischemia, when ATP levels were depleted over 60%. By the end of the four hour period of ischemia, cultures made ischemic contained only 20% of control LDH levels. The patterns of enzyme release from cell and organ cultures are very different: in heart cell cultures, enzyme release occurs rapidly beginning 2 hours after the onset of ischemia while in heart organ cultures, release occurs gradually between 4 and 20 hours after the onset of ischemia. This difference may reflect the time required for enzymes to diffuse through the organ. Alternatively, cells in an intact organ may be more protected than cells in monolayer culture.

These experiments suggest that heart cell and organ cultures are models useful in the study of the responses of the myocardium to ischemia. Using such models, it is possible to create a cellular environment simulating anoxia (or hypoxia if desired) and substrate deprivation characteristic of ischemia. In monolayer cell cultures, where each cell is exposed to the same environment, it may be possible to quantitate cell viability and membrane integrity. The organ culture model permits reproducible biochemical studies using a large number of intact beating hearts. Such models will be used to assess biochemical interventions designed to stimulate repair processes and to salvage damaged cells.

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REFERENCES

1. Zak, R. and Rabinowitz, M. (1973) Med. Clin. North Am. 57, 93-103.
2. Rovetto, M.J., Whitner, J.T. and Neely, J.R. (1973) Circ. Res. 32, 699-711.
3. Wildenthal, K. (1971) J. Appl. Physiol. 30, 153-157.
4. DeHaan, R.L. (1967) Develop. Biol. 16, 216-249.
5. Harary, I. and Farley, B. (1963) Exp. Cell Res. 29, 451-474.
6. Seliger, H.H. and McElroy, W.D. (Eds.) (1965) Light: Physical and Biological Action (U.S. Atomic Energy Commission Monograph) pp. 367-370, Academic Press, New York.
7. Rosalki, S.B. (1967) J. Lab. Clin. Med. 69, 696-705.
8. Bernstein, L.H. and Everse, J. (In press) Methods Enzymol. 41.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
10. Hearse, D.J., Humphrey, S.M. and Chain, E.B. (1973) J. Mol. Cell Cardiol. 5, 395-407.
11. Schulze, W., Krause, E.G. and Wollenberger, A. (1971) J. Mol. Cell Cardiol. 2, 241-251.
12. Coodley, E.L. (1970) Diagnostic Enzymology, pp. 39-71, Lea and Febriger, Philadelphia.