IN VITRO
Volume 14, No. 8, 1978

ISCHEMIC MYOCARDIAL INJURY IN CULTURED HEART CELLS: LEAKAGE OF CYTOPLASMIC ENZYMES FROM INJURED CELLS

DANIEL ACOSTA, MARIANNE PUCKETT, AND REAGAN McMILLIN

Department of Pharmacology, College of Pharmacy, University of Texas, Austin, Texas 78712

SUMMARY

An in vitro model of myocardial ischemia has been established with primary monolayer cultures of postnatal rat myocardial cells. Ischemic conditions were simulated in vitro by subjecting the myocardial cell cultures to various levels of oxygen and glucose deprivation. The experimental protocol consisted of treatment with 20% or 0% O_2 and 1000, 500 or 0 mg glucose per l of medium for 4 or 24 hr. Control cultures were treated with 20% O_2 and 1000 mg glucose. After the ischemic treatments, cultures of beating muscle (M) cells were evaluated for signs of injury, i.e. leakage of cytoplasmic enzymes into the culture medium. Differences were found in leakage of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) from the cultures that were exposed to partial ischemia of glucose deprivation and from those cultures that were exposed to total ischemia of oxygen and glucose deprivation. Glucose deprivation alone resulted in a slight-to-moderate loss of LDH and CPK from the cells, whereas total ischemia resulted in a significant release of the two cytoplasmic enzymes. When the cultures were allowed to recover after ischemic treatment in complete medium (1000 mg glucose) and a normal atmosphere of 20% O_2, they had levels of LDH leakage comparable to those of control cultures. Cell viability and total protein content of the ischemic cultures did not differ significantly from controls.

Key words: myocardial ischemia; cell injury; lactate dehydrogenase; creatine phosphokinase; cultured myocardial cells; glucose deprivation.

INTRODUCTION

In a previous report, we proposed that primary cultures of rat myocardial cells may serve as experimental cellular models to study ischemic injury of myocardium (1). Because it is not possible to reproduce true ischemia in cultured heart cells, we attempted to simulate conditions associated with ischemia. We demonstrated that oxygen and glucose deprivation in cultured heart cells resulted in a time-related inhibition of beating activity and a marked alteration in cellular morphology (1). Similar results on beating function were observed by DeLuca, Ingwall and Bittl (2) and Ingwall et al. (3) with fetal mouse heart in organ culture as an experimental model of ischemia. These investigators observed that total deprivation of oxygen and glucose resulted in marked inhibition of beating activity and cellular function, but that the deprivation of glucose in the presence of oxygen did not injure the cells. We similarly observed that deprivation of oxygen and glucose was more deleterious to the cultures than deprivation of glucose alone.

In this present report, we have evaluated further the injurious effects of oxygen and glucose deprivation in the cultures by measuring the leakage of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) from the cells into the culture medium (4).

MATERIALS AND METHODS

About 30 hearts from 2- to 5-day old Sprague-Dawley rats were minced into small fragments which then were repeatedly digested with 0.15% trypsin (Difco 1:250) to dissociate the cells. Beating myocardial cells were separated from nonmuscle cells by a pour-off technique based on the rate of attachment of the cells to the culture dishes (5). Culture dishes (35-mm, Falcon) were inoculated with 1×10^6 cells in 2 ml Eagle's minimum essential medium (MEM) with 5% fetal bovine serum. The medium and serum were obtained from Grand Island Biological Co. The cultures were grown to confluence for 4 days in a humidified environment (37° C) of 5% CO_2–95% air (approximately 20% O_2) to maintain a pH of 7.2 to 7.4.

TABLE 1

EFFECT OF OXYGEN AND GLUCOSE DEPRIVATION ON VIABILITY, TOTAL PROTEIN AND ENZYME LEVELS IN PRIMARY CULTURES OF RAT MYOCARDIAL CELLS

Treatments	Viable Cells[a]	Protein Plate[b]	Total CPK[c]	LDH Release[d]
	% of Total	*mg*	*mU*	*U per ml*
Glucose deprivation[e]				
4-hr Treatment				
1000 mg Glucose (control)	93.4	—	83.6 ± 8.0	92.8 ± 8.2
500 mg Glucose	92.5	—	72.0 ± 6.0	102.1 ± 5.2
0 mg Glucose	93.0	—	70.6 ± 7.0	97.4 ± 6.4
24-hr Treatment				
1000 mg Glucose (control)	93.3	1.50 ± 0.13	76.5 ± 4.7	88.2 ± 6.4
500 mg Glucose	93.7	1.73 ± 0.10	58.3 ± 3.8[f]	95.1 ± 9.7
0 mg Glucose	93.9	1.45 ± 0.18	50.0 ± 10.1[f]	97.4 ± 6.0
Oxygen and glucose deprivation[g]				
4-hr Treatment				
Control	93.4	—	83.6 ± 8.0	92.8 ± 8.2
Oxygen-derived cultures				
1000 mg Glucose	94.5	—	50.0 ± 4.0[f]	123.0 ± 13.2[f]
500 mg Glucose	92.1	—	49.6 ± 6.0[f]	141.5 ± 12.7[f]
0 mg Glucose	92.5	—	29.6 ± 3.0[f]	150.8 ± 14.2[f]
24-hr treatment				
Control	93.3	1.50 ± 0.13	76.5 ± 4.7	88.2 ± 6.4
Oxygen-deprived cultures				
1000 mg Glucose	96.6	1.48 ± 0.12	46.3 ± 8.0[f]	155.4 ± 21.1[f]
500 mg Glucose	96.6	1.57 ± 0.11	47.0 ± 5.6[f]	248.2 ± 17.6[f]
0 mg Glucose	88.9	1.16 ± 0.08[f]	30.3 ± 3.9[f]	292.3 ± 32.2[f]
Recovery[h]				
Control	—	—	—	85.8 ± 6.4
Oxygen-deprived cultures				
1000 mg Glucose	—	—	—	87.0 ± 6.7
500 mg Glucose	—	—	—	83.5 ± 5.2
0 mg Glucose	—	—	—	89.9 ± 5.8

[a] Viability was determined by the trypan-blue-dye-exclusion test. Each value represents the average of 10 observations on each of five cultures.

[b] Each protein determination represents the mean ± S.D. of five cultures.

[c] Total creatine phosphokinase (CPK) was determined by disrupting the cells with alternate cycles of freezing and thawing and collecting the cell lysate for the measurements as conventional mU per plate. Each value represents the mean ± S.D. of five cultures.

[d] The amount of LDH released from the cells into the culture medium was calculated as conventional units per ml of medium. Each value represents the mean ± S.D. of five cultures.

[e] The cultures were exposed to an environment of 95% air-5% CO_2 (approximately 20% O_2) and the indicated concentrations of glucose per l of medium for 4 and 24 hr.

[f] $p < 0.05$ when compared to controls.

[g] The cultures were exposed to an environment of 95% N_2-5% CO_2 (approximately 0% O_2) and the indicated concentrations of glucose per l of medium for 4 and 24 hr.

[h] After depriving the cultures of oxygen and the indicated concentrations of glucose for 24 hr, both control and treated cultures were allowed to recover in complete medium (1000 mg glucose per l) and an atmosphere of 95% air-5% CO_2 for 24 hr.

On day 4, fresh medium was added to the cultures and the experimental treatments were begun. The experimental protocol consisted of five replicate cultures exposed to either 0% or 20% O_2 and to various concentrations of glucose in MEM: 0, 500 or 1000 per l. Control cultures were treated with 20% O_2 and 1000 mg glucose per l of medium. Each experimental group of cultures was exposed to its respective levels of glucose and oxygen for 4 or 24 hr. Some of the cultures were allowed to recover after ischemic treatment for 24 hr in complete medium (1000 mg glucose per l) and 20% O_2. The cultures were gassed according to the procedure reported elsewhere (1). In order to determine the actual oxygen tension in the cultures, pO_2 was measured in the medium of treated and control cultures with a modified Basic In-Vivox Monitoring Kit (Critikon). Cultures that were exposed to an atmosphere of 95% air-5% CO_2 (approximately 20% O_2) had a pO_2 reading of 120 mm Hg. An atmosphere of 0% O_2, 95% N_2 and 5% CO_2 gave a pO_2 reading of <5 mm Hg.

After the various treatments, the cultures were immediately evaluated for changes in cell viabil-

ity, protein content and leakage of cytoplasmic enzymes. The viability of control and treated cultures was measured by the trypan-blue-dye-exclusion method in which the dye is absorbed by nonviable cells but excluded by viable cells (6). Total protein was measured by the method of Oyama and Eagle (7) using crystalline bovine serum albumin as a standard. LDH was assayed by the procedure described by Dujovne et al. (8) for cultured cells. The activity of LDH was expressed in conventional units per ml of culture medium. Because CPK is not stable in culture medium, we measured total CPK activity in the cultures by disrupting the cells with alternate cycles of freezing and thawing and collecting the cell lysate for measurements as mU per plate. In this manner, we obtained an indirect measure of CPK loss from ischemic-treated cultures. CPK was assayed with Calbiochem Stat-Packs based on the method of Rosalki (9).

Data were expressed as the standard deviation of their mean (±S.D.). The statistical significance of the results was evaluated by Student's *t*-test ($p < 0.05$).

Results

Experimental treatments were begun on day 4 after the cultures had formed confluent monolayers. In order to determine whether the "ischemic" treatments had a lethal effect on the cells, viability and total protein of the cultures were first measured (Table 1). Neither glucose deprivation alone nor oxygen and glucose deprivation in combination had a deleterious effect on cell viability. The duration of treatment (4 or 24 hr) also had no adverse effect. It should be noted that the most severe treatment of oxygen and glucose deprivation (24 hr) did result in a slight decrease in cell viability from 93.3% (control) to 88.9%. The viability study correlated well with the effects of ischemia on cell growth over a 24-hr period. Total protein was not significantly reduced in the cultures deprived of oxygen and glucose except for the most severe treatment of the 24-hr period. Because viability was only slightly affected at this time period, it is likely that cell growth was retarded during total deprivation and that the drop in total protein was not due to cell death.

The next aspect of our study was to investigate the actions of in vitro ischemia on cytoplasmic enzyme leakage form the cells. Glucose deprivation alone resulted in greater loss of CPK (Table 1) from the cultures than LDH, especially at the 24-hr treatment period. There was a concentration-dependent loss of CPK as the glucose levels in the medium were reduced. When the cultures were deprived of oxygen and glucose, both the 4- and 24-hr treatments produced significant losses of CPK and LDH from the cultures. A comparison of the two cytoplasmic enzymes shows that CPK loss was similar at both 4 and 24 hr of treatment, whereas there was much greater leakage of LDH at 24 hr than at 4 hr. In all cases, there was a concentration-dependent loss of the two enzymes as the glucose levels in the medium were reduced. If the ischemic cultures were replenished with complete medium (1000 mg glucose per l) and 20% O_2 and were allowed to recover for 24 hr, LDH values in the medium returned to normal when compared to controls.

Discussion

In vivo ischemia is associated with at least four potentially deleterious conditions: (a) deprivation or deficiency of oxygen; (b) deprivation of energy sources (in this regard, glucose is relatively less important for normal cardiac tissue than lipids or fatty acids, but for ischemic heart tissue glucose becomes an important energy substrate); (c) decreased ability to remove end products of cellular metabolism (in vivo this condition probably leads to the accumulation of high concentrations of acid metabolites in the intercellular tissue spaces, but this is presumably not the case in a monolayer culture exposed to a relatively large volume of medium); and (c) severe tissue acidosis (*not reproduced here*). The proposed cell-culture model obviously cannot duplicate the processes that occur during ischemia, but two of these conditions have been simulated in the present work by depriving the cultures of oxygen and glucose. The myocardial cell cultures may then be studied for biochemical and morphological alterations. Because the ultimate effect of ischemia on the heart is injury and possible destruction of individual myocardial cells, a cell-culture model may contribute to a better understanding of the cellular and subcellular responses to ischemia.

The cellular injury produced to myocardium by ischemia has been the subject of many investigations (10–12). As models for ischemic injury, various experimental techniques have been utilized ranging from occlusion of coronary arteries of intact hearts in animals to perfused hearts and isolated tissue preparations. In vitro models of myocardial ischemia utilizing cell or tissue culture recently have been proposed as alternate methods to study ischemic injury (1–3). Cell-culture systems

offer the following advantages: (a) ease of observation and biochemical measurement of treatments applied to the cultures; (b) maintenance of a uniform chemical and physical environment; and (c) larger sample size to enhance statistical evaluation of treatments. Finally, myocardial cell injury may be evaluated in primary cultures immediately derived from the animal, and thus the use of cell lines that may undergo functional changes in culture is avoided.

It has been known for many years that injured myocardial cells release enzymes and that the measurement of specific cardiac enzymes in the serum has been used in the assessment of myocardial infarction (11). Sobel (11) and Shell, Kjekshus and Sobel (13) have suggested that quantitative measurements of CPK release from the myocardium may reflect the extent and severity of ischemic cell injury. In this present study with myocardial cell cultures, the release of two cytoplasmic enzymes, CPK and LDH, after ischemic insult were used as indices of cell injury. As might be expected, ischemic injury resulted in significant leakage of the two enzymes from the cells into the culture medium. However, the release of these enzymes was not the result of loss of cell viability, as indicated in Table 1. Therefore the cells were not irreversibly injured.

The concept of reversible and irreversible ischemic injury to the myocardium as first defined by Jennings, Kaltenbach and Smelters (14) and Jennings, Ganote and Reimer (15) may apply to our cell-culture model. Irreversible cell injury results when the injured cells are incapable of recovery when resupplied with oxygen and energy substrates and thus continue to degenerate and become necrotic. Reversibly injured cells will recover upon restoration of oxygen and essential substrates. We have demonstrated previously that "ischemic" myocardial cell cultures that had lost their ability to beat regained their beating activity when replenished with oxygen and glucose. In this present report, these same "ischemic" cultures that showed evidence of extensive enzyme leakage had levels of cytoplasmic enzyme release comparable to those of the controls after recovery for 24 hr with oxygen and glucose. Thus the responses of cultured myocardial cells to in vitro ischemia are similar to other experimental models of ischemic heart injury in which reversibly injured cells have been documented to recover after reoxygenation (16-18).

This present study is also in agreement with other investigations on the protective role of glucose in jeopardized myocardium during a period of oxygen deprivation (16-20). If the cultures were completely deprived of glucose and oxygen, there was a greater release of cytoplasmic enzymes than from cultures that had a deficiency of oxygen but normal glucose levels. Because ATP levels decline during myocardial ischemia, the presence of glucose during periods of hypoxia or anoxia will stimulate glycolytic ATP production which will temporarily help to maintain cellular function and integrity (16, 17). In addition, the recovery of reversibly injured ischemic myocardium is promoted when the tissue is reoxygenated in the presence of glucose (17), but irreversibly injured cells will continue to degenerate even in the presence of oxygen and glucose (16). Our study with cultured myocardial cells reinforces the beneficial effect of glucose during oxygen deprivation as well as during a recovery period with reoxygenation.

Another response of cultured myocardial cells to ischemia, which is comparable to other models of jeopardized myocardium, is a decrease in protein synthesis (21, 22). After exposure to total ischemia for 24 hr, the myocardial cell culture model had a significant drop in total cell protein, which most likely reflected a decrease in protein synthesis because cell viability was only slightly affected. The mechanism of inhibition of myocardial protein synthesis by ischemia is not clear, but it may relate to an inhibition of peptide chain elongation (22).

An important element of in vivo ischemia that was not simulated in this present study is the production of cellular acidosis that may develop as a consequence of enhanced production and accumulation of lactic acid and other acidic metabolites (23, 24). According to several investigators, the production of acidosis may have a significant role in ischemic myocardial injury. One prominent effect of acidosis is depression of cardiac contractility (23, 24) which is thought to arise from an alteration of calcium kinetics in the heart. Another deleterious effect associated with acidosis is the activation and intracellular release of lysosomal hydrolases which may initiate intracellular lysis and lead to irreversible cell injury (25-27). Because of the obvious importance of acidosis in ischemic myocardial injury, we are presently investigating the cytotoxic interactions of oxygen and glucose deprivation with acidosis in our cell-culture model. We have demonstrated previously that treatment of heart-cell cultures with acidosis and free fatty acids was injurious to the cells (28).

In summary, our studies demonstrate that an in vitro model of myocardial ischemia may simulate qualitatively some of the responses observed in vivo, especially loss of contractile activity and enzyme leakage. We are presently investigating the responses of such cellular organelles as mitochondria and lysosomes to oxygen and glucose deprivation.

REFERENCES

1. Acosta, D., and M. Puckett. 1977. Ischemic myocardial injury in cultured heart cells: Preliminary observations on morphology and beating activity. In Vitro 13: 818-823.
2. DeLuca, M. A., J. S. Ingwall, and J. A. Bittl. 1974. Biochemical responses of myocardial cells in culture to oxygen and glucose deprivation. Biochem. Biophys. Res. Commun. 59: 749-756.
3. Ingwall, J. S., M. DeLuca, H. D. Sybers, and K. Wildenthal. 1975. Fetal mouse hearts: A model for studying ischemia. Proc. Nat. Acad. Sci. U.S.A. 72: 2809-2813.
4. Shell, W. E., and B. E. Sobel. 1976. Biochemical markers of ischemic injury. Circ. 53 (Suppl. 1): 98-106.
5. Wenzel, D. G., J. W. Wheatley, and G. D. Byrd. 1970. Effects of nicotine on cultured rat heart cells. Toxicol. Appl. Pharmacol. 17: 774-785.
6. Tolnai, S. 1975. A method for viable cell count. Procedure 70111. T.C.A. Manual 1: 37-38.
7. Oyama, V. I., and H. Eagle. 1956. Measurement of cell growth in tissue culture with a phenol reagent. Proc. Soc. Exp. Biol. Med. 91: 305-307.
8. Dujovne, C. A., D. Shoeman, J. Bianchine, and L. Lasagna. 1972. Experimental bases for the different hepatoxicity of erythromycin preparations in man. J. Lab. Clin. Med. 79: 832-844.
9. Rosalki, S. B. 1967. An improved procedure for serum creatine phosphokinase determination. J. Lab. Clin. Med. 69: 696-705.
10. Jennings, R. B., J. H. Baum, and P. B. Herdson. 1965. Fine structural changes in myocardial ischemic injury. Arch. Pathol. 79: 135-143.
11. Sobel, B. E. 1974. Salient biochemical features in ischemic myocardium. Circ. Res. 34-35 (Suppl. 3): 173-181.
12. Trump, B. F., W. J. Mergner, M. W. Kahng, and A. J. Saladino. 1976. Studies on the subcellular pathophysiology of ischemia. Circ. 53 (Suppl. 1): 17-26.
13. Shell, W. D., J. K. Kjekshus, and B. E. Sobel. 1971. Quantitative assessment of the extent of myocardial infarction in the conscious dog by means of analysis of serial changes in serum creatine phosphokinase activity. J. Clin. Invest. 50: 2614-2625.
14. Jennings, R. B., J. P. Kaltenbach, and G. W. Smetters. 1957. Enzymatic changes in acute myocardial ischemic injury: Glutamic oxaloacetic transaminase, lactic dehydrogenase and succinic dehydrogenase. AMA Arch. Pathol. 64: 10-16.
15. Jennings, R. B., C. E. Ganote, and K. A. Reimer. 1975. Ischemic tissue injury. Am. J. Pathol. 81: 179-198.
16. Hearse, D. J., and S. M. Humphrey. 1975. Enzyme release during myocardial anoxia: A study of metabolic protection. J. Mol. Cell. Cardiol. 7: 463-482.
17. Apstein, C. S., O. H. L. Bing, and H. J. Levine. 1976. Cardiac muscle function during and after hypoxia: Effects of glucose concentration, mannitol and isoproterenol. J. Mol. Cell. Cardiol. 8: 627-640.
18. Schelbert, H. R., J. S. Ingwall, H. D. Sybers, and W. L. Ashburn. 1976. Uptake of infarct-imaging agents in reversibly and irreversibly injured myocardium in cultured fetal mouse heart. Circ. Res. 39: 860-868.
19. Hearse, D. J., and E. B. Chain. 1972. The role of glucose in the survival and recovery of the anoxic isolated perfused rat heart. Biochem. J. 128: 1125-1133.
20. Henry, P. D., B. E. Sobel, and E. Braunwald. 1974. Protection of hypoxic guinea pig hearts with glucose and insulin. Am. J. Physiol. 226: 309-313.
21. Lesch, M., H. Taegtmeyer, M. B. Peterson, and R. Vernick. 1976. Mechanism of the inhibition of myocardial protein synthesis during oxygen deprivation. Am. J. Physiol. 230: 120-126.
22. Kao, R., D. E. Rannels, and H. E. Morgan. 1976. Effects of anoxia and ischemia on protein synthesis in perfused rat hearts. Circ. Res. 38 (Suppl. 1): 124-130.
23. Katz, A. M. 1973. Effects of ischemia on the contractile processes of heart muscle. Am. J. Cardiol. 32: 456-460.
24. William, J. R., S. W. Schaffer, C. Ford, and B. Safer. 1976. Contribution of tissue acidosis to ischemic injury in the perfused rat heart. Circ. 53 (Suppl. 1): 3-14.
25. Ricciutti, M. A. 1972. Lysosomes and myocardial cellular injury. Am. J. Cardiol. 30: 498-502.
26. Hoffstein, S., G. Weissmann, and A. C. Fox. 1976. Lysosomes in myocardial infarction: Studies by means of cytochemistry and subcellular fractionation, with observations on the effects of methylprednisolone. Circ. 53 (Suppl. 1): 34-40.
27. Decker, R. S., A. R. Poole, E. E. Griffin, J. T. Dingle, and K. Wildenthal. 1977. Altered distribution of lysosomal cathepsin D in ischemic myocardium. J. Clin. Invest. 59: 911-921.
28. Wenzel, D. G., and D. Acosta. 1975. Labilization of lysosomes and mitochondria *in situ* by hypoxia and hypoxia-related factors. Res. Commun. Chem. Pathol. Pharmacol. 12: 173-176.

The technical assistance of L. Arthur and C. Zamora is gratefully appreciated. The authors also express their appreciation to L. Hall for her accurate typing of the manuscript. This study was supported by Research Grant HL 18647 from the National Heart, Lung, and Blood Institute.