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Cultured neonate rat myocytes as a model for the study of myocardial ischaemic necrosis

*TREVOR J. C. HIGGINS, PETER J. BAILEY, DAVID ALLSOPP AND DERYL A. IMHOF

Bioscience II Department, ICI Pharmaceuticals Division Limited, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K.

The preparation of cultures of neonate rat heart muscle cells is described. These cultures, when subjected to anoxia, show enzyme release that can be directly related to the uptake of a vital dye such as trypan blue. Enzyme release is a valid method of estimating cell necrosis in this model. The survival of anoxic cultures is closely associated with glycolytic activity. Glycolysis rate falls and enzyme release increases as the medium glucose concentration is reduced. If glycolysis is inhibited by either 2-deoxyglucose or L-lactate, enzyme release under anoxic conditions is enhanced. Enzyme release correlates inversely with glycolytic activity and the intracellular ATP content of the cultures. Addition of ATP to anoxic cultures partially ameliorates the effect of the anoxia on enzyme release. Elevation of the calcium content of the culture medium exacerbates the damage caused to cardiac myocytes by anoxic insult. This effect can be obtunded by calcium-antagonist drugs such as verapamil or nifedipine and can be explained in terms of a reduction in utilization of intracellular ATP by the anoxic myocytes. These observations indicate that cultured myocytes may represent a useful model of hypoxic injury against which novel pharmacological agents, that may reduce hypoxic or ischaemic injury in vivo, could be evaluated.

There are many experimental models for the study of acute myocardial ischaemia and infarction in animals. In vivo models employing the temporary or permanent occlusion of one or more coronary arteries have the disadvantage that the degree of ischaemia obtained varies widely within and between animals due to fluctuations and variations in collateral flow (Reimer et al 1977). Such studies are also time-consuming and expensive. Several techniques using in vitro perfusion techniques of isolated cardiac muscle have been described (Neely et al 1973; Manning et al 1980). These generally employ hearts or heart tissue, usually papillary muscle, from small mammals. These techniques, although assuring a more easily controlled and reproducible level of ischaemia, produce cardiac preparations with limited functional stability (Kloner & Ingwall 1980).

A system employing cell or organ cultures of myocardial tissue facilitates the manipulation of the environment of the myocardial tissue without complicating factors derived from the existence of a variable and uncertain vascular supply system. Ingwall et al (1975, 1978) have demonstrated the use of foetal mouse hearts in organ culture to study the process of myocardial injury. Similarly, tissue cultures of isolated cells derived from neonate animals have been used by Allsopp et al (1980) and Van der Laarse et al (1979) to study the effects of withdrawal of oxygen from cultures under varying supplies of utilizable substrate. The outstanding advantages of these culture techniques are the high level of reproducibility obtainable and their low cost. The disadvantage of the technique is that the cultures are anoxic and not ischaemic. The differences in metabolism under these two conditions have been described by Neely & Morgan (1974).

Our aim has been to define the conditions necessary to facilitate the use of anoxic myocyte cultures as a model for studying the process of cell necrosis which occurs as a result of withdrawal of oxygen and to investigate the validity of enzyme release as a measure of cell necrosis in cardiac cell cultures.

METHODS AND MATERIALS

Preparation of neonatal myocyte cultures

Cultures of myocytes were prepared by serial digestion of chopped ventricles using collagenase as described by Allsopp et al (1980). Cultures so prepared appear to be stable as judged by beating activity for at least 3 weeks. Overgrowth of the myocytes by fibroblastoid cells appears to occur after this time. To minimize interference by these non-muscle cells, investigations were made 7–8 days after preparation of the cultures.

Preparation of anoxic myocyte cultures

The medium was identical to that used for culture maintenance except that the glucose and calcium

* Correspondence.

concentrations were altered or other substances added as necessary.

Cultures were made anoxic by replacing the culture medium with one that had been continuously gassed with oxygen-free nitrogen (Higgins et al 1980). Cultures were then sealed and incubated at 37 °C for fixed times. Each treatment group comprised five cultures.

Investigation of the relation between loss of cell viability and enzyme release

Cultures of myocytes were incubated in anoxic or normoxic medium containing 1 mM glucose. After fixed incubation times at 37 °C each vial was quickly opened and 200 µl withdrawn within 10 s, and immediately assayed for lactate dehydrogenase (LDH) activity using a rapid continuous flow assay technique based on that of Brooks & Olken (1965). The cultured cell sheets were stained with trypan blue and fixed with glutaraldehyde as described by Van der Laarse et al (1979). The cultures were then photographed under fixed conditions of lighting and photographic exposure time. Several representative fields of each culture were photographed and the stained area determined by planimetry. The results were expressed as the % of the optical field stained with trypan blue, and LDH in mU ml^{-1}. The reintroduction of oxygen to anoxic cultures does not cause enzyme release when the time between opening the vial and sampling is short (Higgins et al 1980).

Determination of metabolic activity of myocyte cultures

Batches of medium containing [5-^{3}H]D-glucose to give 0·05 µCi µmol^{-1} glucose ml^{-1} of medium, to which had been added various concentrations of glucose and either 2-deoxyglucose, L-lactate or ATP, were gassed with nitrogen or equilibrated with room air. After incubation of the cultures glycolytic activity was determined on 200 µl of the remaining medium. Glycolytic glucose consumption was measured using the conversion of [5-^{3}H] glucose to ^3H_2O by the method of Rovetto (1977).

The extraction and assay of ATP from myocyte cultures was as described by Higgins et al (1980).

Investigation of the role of calcium concentration in the medium in the process of anoxia-induced cell damage

The effect of calcium concentration in the medium was studied using medium with glucose concentration 5 mM whilst the calcium concentration was varied by adding weighed amounts of $CaCl_2$ to medium that initially contained only 0·15 mM $CaCl_2$ derived from the added serum and Hams F-10 (× 10 strength) medium concentrate (Higgins et al 1980).

RESULTS AND DISCUSSION

Heart cell cultures are a convenient and reproducible system for studying the effect of oxygen deprivation, on cardiac tissue. Care must be taken in extrapolating observations made on anoxic tissue to tissue or organ ischaemia, for, under ischaemic conditions, nutrient flow to the affected tissue may be severely restricted but there may still be residual uptake and utilization of oxygen, unlike in anoxia where there is no oxygen. Thus conditions used in the in vitro model may deviate from clinical ischaemia. However, under anoxic conditions, with the heart cell cultures bathed in a fixed volume of medium, toxic metabolites accumulate and substrate concentrations fall leading to tissue necrosis (Allsopp et al 1980), indicated by the appearance of intracellular enzymes in extracellular spaces and fluid (Karmen et al 1955; Kibe & Nilsson 1967; Van der Laarse et al 1979). The appearance of heart-specific enzymes in the blood has been investigated as a possible index of myocardial infarct size in animals and man (Roe & Starmer 1975; Shell et al 1971, 1973).

Previous workers have demonstrated that anoxic and glucose deficient heart cells will cease to beat spontaneously and will release enzyme into the medium (DeLuca et al 1974; Acosta & Puckett 1977; Roeske et al 1978). The incubation time under these conditions has varied between 4 h (De Luca et al 1974) and 23 h (Acosta & Puckett 1977); our model employs periods of up to 24 h and differs from those of other workers in that a substrate utilizable under anoxic conditions is supplied.

The enzyme marker for these studies should have sufficient stability in the culture medium under experimental conditions to reflect quantitatively the extent of cell necrosis. Lactate dehydrogenase (LDH) has been shown to have a half-life of 52·5 ± 5·8 h in the medium used here compared with 6·8 ± 0·7 h for creatine kinase (CPK) (Higgins et al 1980), and was preferred to CPK as the marker of cell damage although CPK is a more muscle-specific marker.

Relations between enzyme release and loss of cell viability

Fig. 1 shows the time course of LDH release from and uptake of trypan blue stain by heart cells incubated under normoxic and anoxic conditions.

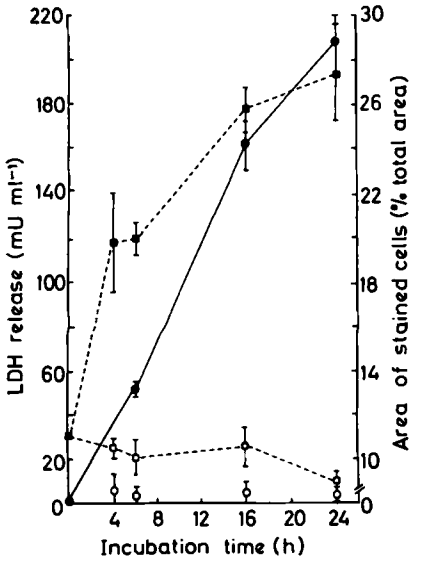

FIG. 1. The relation between release of lactate dehydrogenase (LDH) release, trypan blue uptake and time in either anoxic or normoxic incubation conditions. Trypan blue uptake expressed as the % of coloured cells. LDH release, is expressed as mU ml^{-1}. ■, trypan blue uptake under anoxic conditions; □, trypan blue uptake under normoxic conditions; ●, LDH release under anoxic conditions; ○, LDH release under normoxic conditions. Data presented as mean ± s.e.m. of five cultures.

Dye uptake and LDH release showed good correlation ($r = 0{\cdot}990$ $P < 0{\cdot}01$), suggesting that LDH release is a sensitive marker of myocardial cell viability loss as defined by uptake of trypan blue in accordance with Van der Laarse et al 1979 who did not quantify the relation between enzyme release and dye uptake.

Effect of glucose concentration in the medium on intracellular enzyme release

Van der Laarse et al (1979) suggested that the provision of energy via appropriate substrates is important for the integrity and survival of the anoxic cardiac cell. Fig. 2 shows the effect of the glucose concentration of the medium on enzyme release from anoxic and normoxic cell cultures. Cultures were incubated for 16 h. Glucose concentrations above 3 mM satisfy the metabolic requirements and maintain viability of the anoxic cell, below 3 mM however, the anoxic cell is not adequately protected against necrosis. The glucose concentration at which this sharp change in benefit occurs may be in the region of the K_m for the cellular glucose carrier. The effect of a critical glucose concentration has been observed by Hearse et al (1978) using a perfused ischaemic heart model.

The effect of inhibition of glycolysis on intracellular enzyme release

The dependence of anoxic cell survival on glycolytic activity was further investigated by using the inhibitors of glycolysis, L-lactate, a stereo-specific inhibitor of glycolysis and direct inhibitor of glyceraldehyde 3-phosphate dehydrogenase (Mochizuki & Neely 1979) and 2-deoxyglucose, an analogue of glucose which is taken up into cardiac cells and phosphorylated with an efficiency close to that of the natural substrate (Flohr & Breull 1979). Deoxyglucose 6-phosphate cannot be used for glycolysis because it is not a substrate for the enzyme hexose phosphate isomerase. Thus 2-deoxyglucose inhibits by a mechanism at the hexose end of the glycolytic pathway, while lactate probably inhibits elsewhere in the pathway. Fig. 3 shows the effect of increasing concentrations of 2-deoxyglucose or L-lactate on enzyme release from anoxic cell cultures after 16 h incubation in medium containing a range of glucose concentrations, and that inhibition of glycolysis by either mechanism severely jeopardizes the anoxic myocyte.

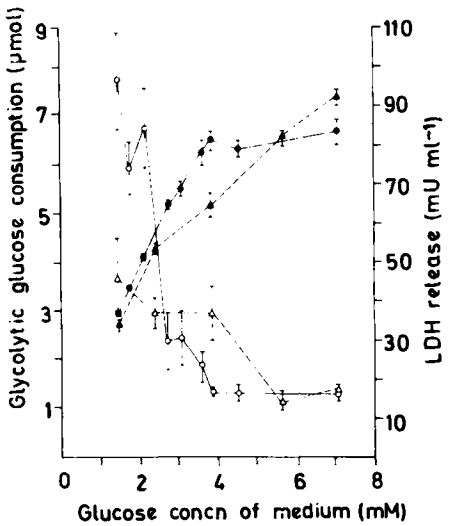

FIG. 2. The relation between glycolytic rate and lactate dehydrogenase release and medium's glucose concentration; Enzyme release from normoxic (△) and anoxic (○) cultures, are expressed as mU ml^{-1} of culture medium. Glycolytic glucose consumption for normoxic (▲) and anoxic (●) cultures are expressed as μmol 16 h incubation. Data are presented as mean ± s.e.m. for five cultures.

The ATP content of the cultures was also determined. Release of LDH correlated well with intracellular ATP content for both 2-deoxyglucose (Fig. 4B) and L-lactate (Table 1) as with glycolytic activity (Fig. 4A & Table 1). These observations

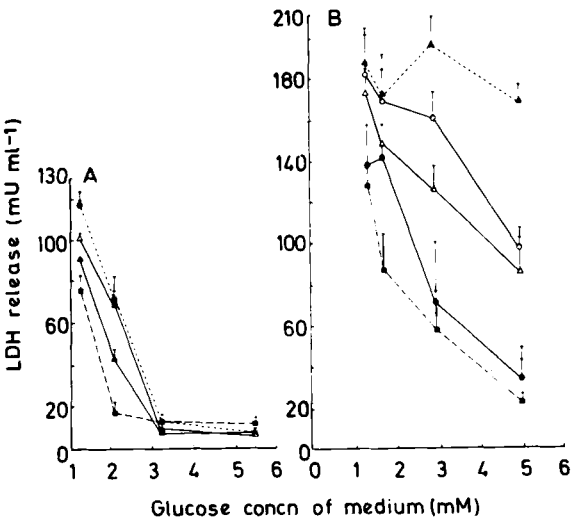

FIG. 3. A. The relation between enzyme release and medium glucose concentration in the presence of nil (■), 10 mM (▲), 20 mM (△) and 40 mM (●) L-lactate in the culture medium under anoxic incubation conditions. Data are presented as mean ± s.e.m. for five cultures. B. The relation between enzyme release and medium glucose concentration in the presence of nil (■), 1·5 mM (●), 2·5 mM (△), 5 mM (○) and 10 mM (▲) 2-deoxyglucose in the culture medium under anoxic conditions.

clearly point to a relationship between the supply of glycolytically derived ATP and cellular integrity in anoxia, consonant with the observations of Gebhard et al (1977).

Effect of exogenous ATP on intracellular enzyme release

To test the hypothesis that the energetic state of the anoxic myocardial cell is important to cell survival, cell cultures were incubated for 16 h in an anoxic medium containing 1·5 or 5 mM glucose, supplemented by the addition of ATP (10^{-7} to 10^{-2} M). The results obtained for LDH release are shown in Fig. 5.

Table 1. Correlation of enzyme release, glycolytic activity and cellular ATP for cultures incubated under anoxic conditions. Data are presented as the product moment coefficients of correlation.

		Correlation coefficients	
Conditions	No. of observations	Enzyme release vs glycolysis	Enzyme release vs intracellular ATP content
Varied glucose concentration (Fig. 2)	10	−0·955 ($P < 0·001$)	N.D.
Varied glucose + L-lactate conc.	16	−0·845 ($P < 0·002$)	−0·743 ($P < 0·005$)
Varied glucose + 2-deoxyglucose concentrations	20	−0·908 ($P < 0·001$)	−0·941 ($P < 0·001$)

N.D.: In this experiment the intracellular ATP content was not determined.

The presence of high glucose in the medium caused a reduction of enzyme leakage, and ATP concentrations of 10^{-5} M and above were able to decrease cell damage as was ATP (> 10^{-4} M) in low glucose concentrations in the medium; data support the idea that intracellular ATP may be a determinant of cell survival under anoxic conditions.

Effect of calcium concentration of the medium on intracellular enzyme release

After 16 h incubation under anoxic conditions in the presence of 5 mM glucose, enzyme leakage was related to the increase in calcium concentration in the medium (Fig. 6). Under normoxic conditions increased calcium concentration did not affect LDH release.

The cardiac cell may have a metabolic mechanism for controlling calcium influx to protect the ventricular myocardial cell in anoxia and ischaemia (Sperelakis & Schneider 1976). However, if the extracellular calcium concentration is artificially elevated, then intracellular calcium concentrations may rise and the toxic effect of calcium become apparent (Fig. 6). Winegrad & Shanes (1962) demonstrated that increasing the extracellular concentration of calcium increases the intracellular level of calcium in the myocardial cell.

Further studies were done by incubating cultures under anoxic or normoxic conditions in the presence

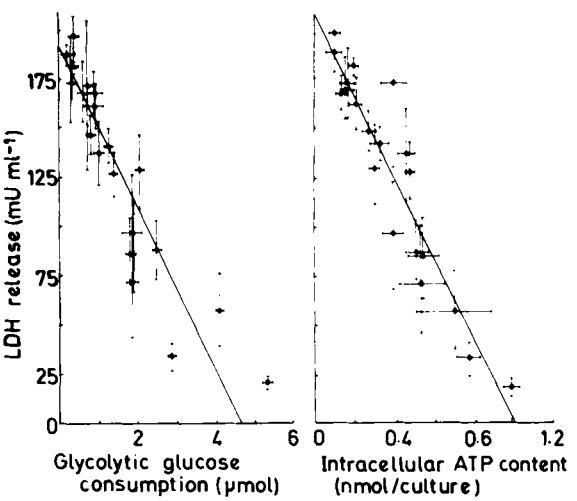

FIG. 4. A. The relation between enzyme release (mU ml⁻¹) from and glycolytic glucose consumption by anoxic myocytes incubated in the presence of various glucose and 2-deoxyglucose concentrations. B. The relation between enzyme release (mU ml⁻¹) from and ATP content of anoxic myocytes incubated in the presence of various glucose and 2-deoxyglucose concentrations. Data are expressed as mean ± s.e.m. for five cultures.

Table 2. Effect of 10^{-7} M verapamil or nifedipine on LDH release, lactate accumulation and ATP content of or anoxic myocytes in the presence of a low (1·9 mM) or raised (4 mM) medium calcium concentration.

Conditions	LDH release (mU ml^{-1})	ATP content (nmol culture^{-1})
Normoxia:		
1·9 mM Ca^{2+} (control)	7·985 ± 1·563	2·011 ± 0·069
+10^{-7} M verapamil	6·583 ± 0·833	1·916 ± 0·083
+10^{-7} M nifedipine	5·602 ± 1·027	2·113 ± 0·096
4·0 mM Ca^{2+} (control)	10·040 ± 2·134	2·104 ± 0·102
+10^{-7} M verapamil	8 132 ± 1·157	1·909 ± 0·086
+10^{-7} M nifedipine	9·158 ± 0·921	2·375 ± 0·082*
Anoxia:		
1·9 mM Ca^{2+} (control)	15·599 ± 1·131	1·884 ± 0·083
+10^{-7} M verapamil	12·002 ± 1·513*	1·863 ± 0·093
+10^{-7} M nifedipine	9·526 ± 2·001**	1·932 ± 0·075
4·0 mM Ca^{2+} (control)	37·943 ± 5·398††	1·625 ± 0·056†
+10^{-7} M verapamil	20·971 ± 5·317*	1·841 ± 0·135*
+10^{-7} M nifedipine	24·243 ± 3·821*	1·820 ± 0·032**

Data are presented as mean ± s.e.m. for 5 cultures.
Unless otherwise shown all other differences are not statistically significant.
* $P<0.05$
** $P<0.01$ versus corresponding control value.
† Significance of difference between high and low Ca^{2+} controls: $P < 0.025$.
†† Significance of difference between high and low Ca^{2+} controls: $P < 0.005$.

of 5 mM glucose with calcium concentration normal (1·9 mM) or raised (4 mM). Cultures were incubated in the presence of 10^{-7} M nifedipine or verapamil. Under normoxic conditions increased calcium had no significant effect on enzyme leakage (Table 2) and neither calcium antagonist affected enzyme release. No significant changes were observed in the ATP content of the cultures except those with an elevated calcium concentration and 10^{-7} M nifedipine.

Under anoxic conditions depressed ATP levels were further depressed by increased calcium concentration. The amelioration of enzyme leakage and protection of ATP levels by the calcium antagonists suggest that anoxic cells may be caused to develop increased tension by the high extracellular calcium concentration. This elevated inotropic state in the absence of oxygen may cause intracellular ATP levels to fall and intracellular calcium homeostasis to be lost, as suggested by Nayler et al (1976) and Bricknell & Opie (1978). Negative inotropic agents such as verapamil or nifedipine, by partially antagonizing calcium uptake may reduce the inotropic state of the anoxic cells and thereby reduction in ATP utilization thus salvaging ATP for use in protecting the cell against the anoxic insult.

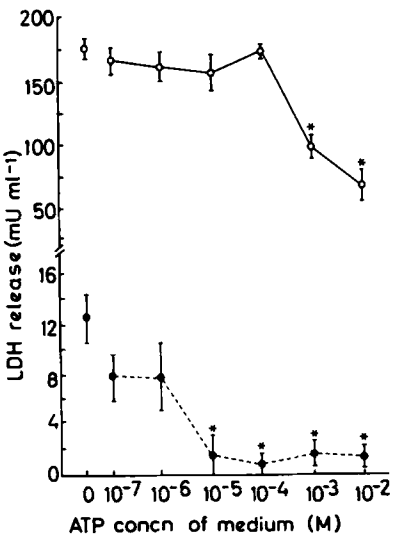

FIG. 5. The effect of increasing medium ATP concentration on enzyme release from anoxic myocytes incubated in the presence of 2·5 mM (○) or 5 mM (●) glucose. Data are expressed as mean ± s.e.m. for five cultures, * $P < 0.005$ versus untreated control (zero ATP).

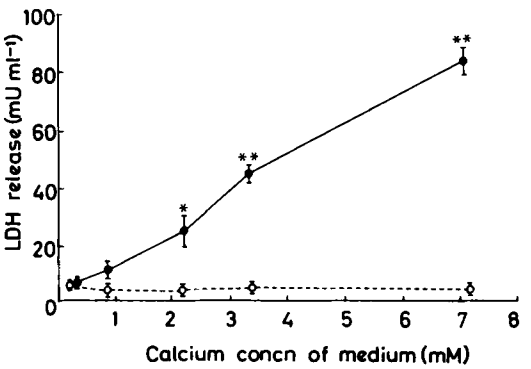

FIG. 6. The effect of medium calcium concentration on LDH release from myocyte cultures under anoxic (●) and normoxic (○) conditions. Data are presented as the mean ± s.e.m. for five cultures. * $P < 0.01$; ** $P < 0.001$ for difference between anoxic and normoxic cultures.

This study shows that the survival of the anoxic myocardial cell is dependent upon both its capacity to produce ATP by glycolytic activity and its ability to reduce ATP utilization by activities not essential for survival. Reduction of glycolytic activity can result in exacerbated damage to the anoxic myocyte. A similar effect can be produced by stimulation of ATP utilization in the anoxic cell.

The ischaemic state and the anoxic state are similar in that both are characterized by a sharp fall in intracellular ATP and creatine phosphate levels so it is reasonable to suggest that pharmacological agents which can promote a reduction in ATP utilization by anoxic heart cell cultures and concommitantly reduce enzyme leakage may prove to be effective in vitro or in vivo models of ischaemia. Anoxic myocyte cultures represent a promising model for the in vitro identification of compounds capable of reducing the extent of myocardial necrosis consequent on ischaemia.

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