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Cellular characterization of an in-vitro cell culture model of seal-induced cardiac ischaemia

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Abstract

The lack of a well-characterized in-vitro cell culture model of load-induced cardiac ischaemia has hampered investigations into the mechanism of ischemic injury. We therefore developed a new in-vitro model of cardiac ischaemia that mimics distinct features of ischaemic injury.

Neonatal rat heart cells were cultured in a sealed flask for 24–72 h. In this environment, the cells were exposed to stresses of hypoxia, acidosis and stagnant incubation medium. The pO_2 and pH of the medium gradually decreased during the ischaemic insult and ultimately fell to a level of 14 mmHg and pH 6.8, respectively. The model triggered severe cell injury, including morphological degeneration, CPK release, beating impairment and ATP depletion. Apoptosis occurred in some cardiomyocytes as early as 24 h after onset of seal-induced ischaemia. This was evidenced by positive nuclear staining using Hoechst 33258 and by the induction of caspase-3 mRNA. By 72 h, internucleosomal DNA fragmentation was observed in 45% of the myocytes; however, a non-myocyte preparation subjected to the same ischaemic insult exhibited no evidence of DNA fragmentation.

These results demonstrate that neonatal cardiomyocytes subjected to the new simulated ischaemia model exhibit several similarities to cardiac ischaemia, including the simultaneous appearance of necrosis, breakdown of cellular ATP, beating cessation and apoptosis. The new model should prove useful in unravelling the molecular alterations underlying ischaemic injury and myocardial apoptosis.

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Introduction

Infarct size is a major determinant of congestive heart failure, a complex clinical syndrome characterized by haemodynamic, neuroendocrine and peripheral alterations (Buja 1998; Swynghedauw 1999). Although initial studies examining the causes for enlarged infarct size have focused on the contribution of necrosis, several more recent studies have suggested that apoptosis may also contribute to ischaemia-induced cell death (Fliss & Gattinger 1996; Olivetti et al 1997; Haunstetter & Izumo 1998). It has been proposed that since cardiomyocytes are terminally differentiated, and as such lose their ability to duplicate soon after birth (Liu et al 1995; Mitcheson et al 1998), the regulation of apoptosis is of major importance in determining the cellular content of the adult heart. However, intact heart studies have yielded varying interpretations regarding the importance of necrosis and apoptosis on cell death. Since many of the techniques used to distinguish apoptosis from necrosis are more clearly delineated in isolated cell preparations, the lack of a well-characterized in-vitro model of stress-induced cardiac ischaemia has hampered further mechanistic investigation (Hasenfuss 1998).

Cultured cardiomyocytes provide an advantage over other preparations because they are separated anatomically and functionally from nerves, connective tissue and blood vessels, and can thus be used to investigate cellular and molecular mechanisms (Mitcheson et al 1998). Despite such advantages, the majority of ischaemic studies have been performed on whole animal or intact heart preparations (Hasenfuss 1998).

This study was designed to establish a new in-vitro model of cardiac ischaemia that mimics distinct features occurring in clinical ischaemic cardiomyopathy. To produce this model, neonatal rat heart cells were cultured in a sealed flask for 24–72 h. The myocardial response to this new simulated ischaemia model resembles that of the ischaemic condition.

Materials and Methods

Preparation of cardiac myocyte and non-myocyte culture

The preparation of primary cardiac myocyte and non-myocyte cultures from one-day-old Wistar rats is described by Sadoshima et al (1992). For selective enrichment of the cardiac myocytes, dissociated cells were pre-plated for 1 h, during which time the non-myocytes readily attached to the bottom of the culture dish (Polinger 1970). Non-adherent cells, consisting of mostly myocytes, were then plated at a density of $2–5 \times 10^6$ cells mL^{-1}/dish. Bromodeoxyuridine (0.1 mM) was added during the first two days to prevent proliferation of the non-myocytes. This procedure yielded cultures with 90–95 % myocytes, as assessed by microscopic observation of cell beating. The myocytes were maintained in serum-containing culture medium, Dulbecco's modified Eagle's medium/F-12 (1:1 v/v) (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan), supplemented with newborn calf serum (5 %; Dainippon), 3 mM pyruvic acid, 100 μM ascorbic acid, 5 μg mL^{-1} insulin, 5 μg mL^{-1} transferrin, and 5 ng mL^{-1} selenium (Boehringer Mannheim, Germany), for 48 h followed by serum-free medium.

Simulated ischaemia model

Ischaemia experiments were performed 24 h after transferring the cells to serum-free medium. A diagram of the experimental protocol for the simulated ischaemia model is shown in Figure 1A. To produce the ischaemic condition, cells were sealed in a small flask-type culture vessel (12.5 cm^2; Falcon). Before the sealing step, incubation flasks were filled with 2.5 mL normal media and 47.5 mL phosphate-buffered saline and bubbled with 5 % CO_2/95 % N_2 for 2 min to fix the initial pH at 7.4 and to eliminate oxygen from the remaining air space. Then the lid was tightly sealed, preventing gas from entering the flask. In the control group, the cells were incubated in 2.5 mL medium which was equilibrated with a 5 % CO_2/95 % air atmosphere. Only the culture medium in the control group was changed daily. The simulated ischaemia model combined the stresses of hypoxia, acidosis and no flow.

Evaluation of morphology and beating status

The morphological and beating status of the cardiomyocytes were monitored with an inverted phase contrast microscope and video monitor at a magnification of 150–400, in a controlled chamber at 37°C (Takahashi et al 1997). The shape and location of each myocyte was recorded by a photograph before initiating the experiment and after 24–72 h of simulated ischaemia. The morphological changes, such as cell lysis, formation of blebs or ballooning of the cell membrane, were observed upon ischaemia. Beating status was quantified by counting the number of beating cells in the same area. The beating status in the same cells was introduced into an intensified charged couple device camera and videotaped by a VHS recorder. The beating cell number was estimated by the ratio of before and after ischaemic insult. The morphological changes and altered beating status induced by ischaemic insult were estimated for each cell and expressed as percentage of the total observed cells.

Determination of creatine phosphokinase (CPK) and ATP content

The CPK and ATP content of the myocytes was measured according to manufacture's instructions using the commercially available CPK-test Wako kit (Wako Chem., Osaka, Japan) and the ATP-assay kit (Toyobichetto, Toyo inc., Tokyo, Japan), respectively. The results are expressed as IU (μg protein)$^{-1}$ or pmol (μg protein)$^{-1}$. The protein concentration was determined by the method of Lowry et al (1951), using bovine serum albumin as a standard.

Apoptosis detection

Genomic DNA was isolated and detected as described by Cigola et al (1997). Each DNA sample was electrophoretically separated on 1.5 % agarose gels and stained with ethidium bromide. For visualizing apoptotic nuclei, cells were fixed with 1 % paraformaldehyde for 30 min

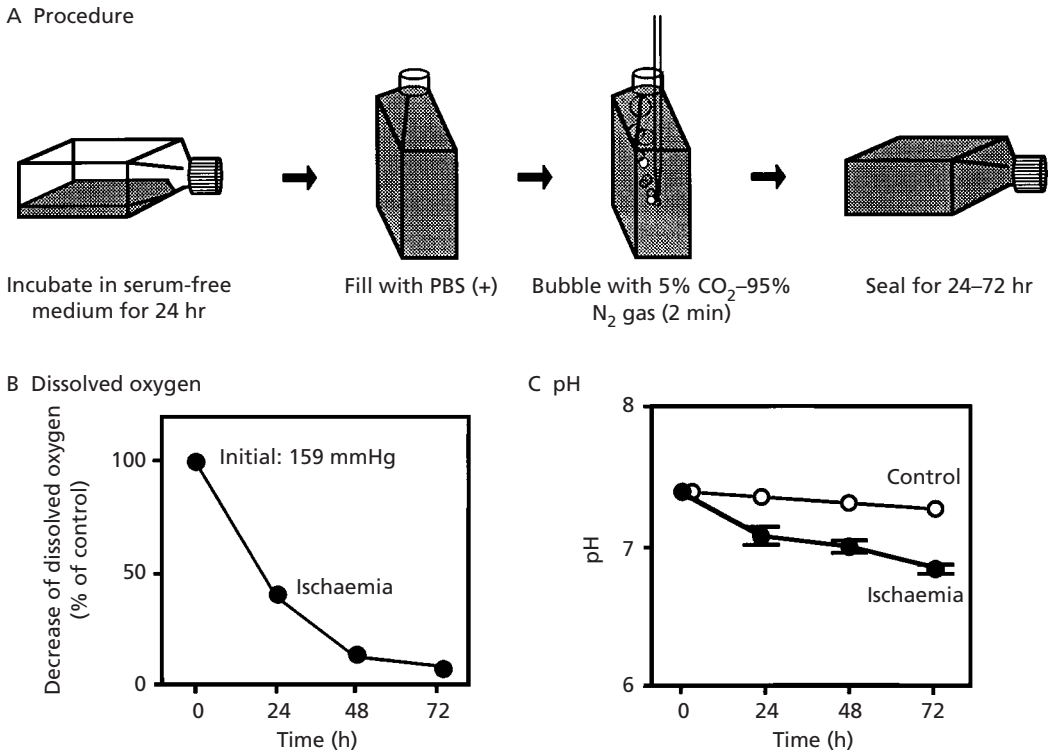

Figure 1 Protocol for the new simulated ischaemia model. A. Isolated neonatal cardiomyocytes were placed in flasks containing normoxic medium for 24 h. The flasks were filled with PBS and the new medium equilibrated with 5% CO_2/95% N_2. The flasks were then sealed for the appropriate period of simulated ischaemia. B. Oxygen content of the culture medium before and after onset of simulated ischaemia. The time shown represents the time of ischaemia. Oxygen content was measured with an oxygen meter and the data shown represent the means of two experiments. The initial O_2 pressure was 159 mmHg when the air pressure was 760 mmHg. C. pH of the culture medium before and after onset of the ischaemic insult. The data represent the means ± s.e.m. of three samples.

at room temperature and then stained with the fluorescent dye Hoechst 33258 (Sigma, MO) for 15 min. The analysis was performed with the Olympus fluorescent microscopy systems (Tokyo, Japan).

Semi-quantitative RT-PCR

cDNA was synthesized in a total volume of 20 μL reaction mixture containing 1 μg of RNA isolated from ischaemic myocytes. The first strand cDNA (1 μL) was used as a template in the 20-μL PCR reaction mix containing 1 μmol L^{-1} of each primer set and 1 U Ampli Taq Gold (Perkin-Elmer, Branchburg, NJ). To enable cross-comparison among the different samples, the housekeeping gene β-actin mRNA was used for normalization of expression of caspase-1 and caspase-3 mRNA. The primer sets (Keane et al 1995; Ni et al 1997) used were as follows: sense primer for caspase-1, 5′-CAC ATT GAA GTG CCC AAG CT-3′; and anti-sense primer for caspase-1, 5′-TCC AAG TCA CAA GAC CAG GC-3′; sense primer for caspase-3, 5′-GGT ATT GAG ACA GAC AGT GG-3′; and anti-sense primer for caspase-3, 5′-CAT GGG ATC TGT TTC TTT GC-3′; sense primer for β-actin, 5′-CAA GAG ATG GCC ACG GCT GCT-3′; and anti-sense primer for β-actin, 5′-TCC TTC TGC ATC CTG TCG GCA-3′. PCR was simultaneously carried out for caspase-1 and caspase-3 in a Perkin-Elmer 2400 thermal cycler for 36 cycles of melting, annealing, and extension at 94°C for 30 s, 64°C for 45 s, and 72°C for 1 min, respectively. Under these conditions, amplification of all cDNA fragments produced a single product within a linear range of 33–42 cycles (data not shown). PCR for β-actin was carried out in a Perkin-Elmer 2400 thermal cycler for 25 cycles of melting, annealing, and extension at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s respectively. The sizes of PCR products were expected to be 299, 280 and 340 bp for mRNA encoding caspase-1, caspase-3 and β-actin, respectively. PCR products were separated by electrophoresis using 3% agarose gels. After staining with Vistra Green (Molecular Dynamics), sample fluorescence was measured by FluorImager 595

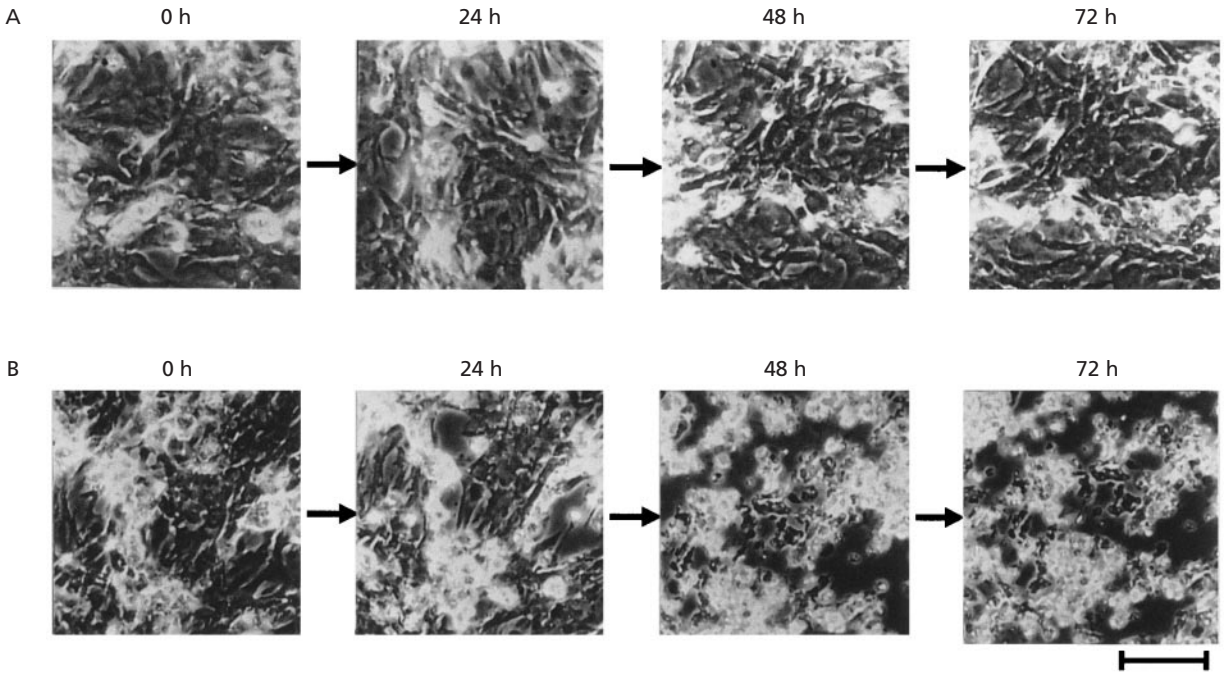

Figure 2 Characteristic morphological changes of cardiomyocytes induced by an ischaemic insult. Cardiomyocytes were exposed to an ischaemic insult for the indicated period of time. The photographs show the morphology of a representative cell exposed to various periods of ischaemia up to 72 h. The bar represents 100 μm.

(Molecular Dynamics) for semi-quantitative analysis of caspase-1, caspase-3 and β-actin PCR products.

Statistics

Depending on the design of the experiment, statistical significance was determined by the Student's *t*-test, χ^2-test, or analysis of variance with Bonferroni's method used to compare individual data points for a significant F value. Each value is expressed as the mean $\pm$ s.e.m. Differences were considered significant when the calculated *P* value was less than 0.05.

Results

Morphological and functional damage to cultured cardiomyocytes induced by ischaemia

The method of inducing simulated ischaemia by placing cultured neonatal rat heart cells in a sealed flask for 24–72 h is summarized in Figure 1A. In the sealed flasks, the cells were exposed to stresses of hypoxia, acidosis and stagnant incubation medium, the latter mimicking the no reflow phenomenon. The pO^2 (Figure 1B) and pH (Figure 1C) of the media gradually decreased during the ischaemic insult, with 72-h values being 14 mmHg and pH 6.8, respectively. By comparison, the media in a control flask was maintained at the initial pH (7.3–7.4) and oxygen tension (159 mmHg) throughout the 72-h control incubated period.

Figure 2 and Table 1 show the time course of morphological degeneration in myocytes after 24–72 h of ischaemia. The 72-h ischaemic cells exhibited pronounced injury, including ballooning and cellular lysis. Table 1 shows the estimated degree of morphological degeneration based on a scoring method. Simulated ischaemia increased the amount of morphological degeneration of the myocyte preparation by 57 % after 24 h, 78 % after 48 h and 87 % after 72 h of ischaemia. However, the same insult did not cause morphological degeneration of non-myocytes (data not shown).

Table 2 shows the effect of ischaemia on CPK loss, beating cessation and ATP depletion. The CPK activity of the control cardiomyocytes was maintained over the range 4–6 IU (μg protein)$^{-1}$, with little CPK leaking into the culture medium during the 24–72-h normoxic incubation. By contrast, CPK activity of the ischaemic cardiomyocyte decreased between 50 and 89 % over the ischaemic period (24–72 h). Similarly, whereas greater than 90 % of control cells maintained a normal beating pattern with a mean rate of 135 beats min^{-1} after a

Table 1 Ischaemia-induced morphological degeneration and apoptosis of cultured cardiomyocytes.

Time (h)	Morphological degeneration (degenerated cells/total cells)		Apoptosis (apoptotic cells/total cells)	
	Control	Ischaemia	Control	Ischaemia
0	0/71 (0%)	0/110 (0%)	–	–
24	0/71 (0%)	63/110 (57%)*	21/628 (3%)	89/689 (13%)*
48	0/71 (0%)	86/110 (78%)*	43/792 (5%)	160 /751 (21%)*
72	0/71 (0%)	96/110 (87%)*	29/315 (9%)	189/420 (45%)*

Cardiomyocytes were exposed to an ischaemic insult for the indicated time. Samples (n = 71–110 cells for morphological degeneration; n = 315–792 cells for apoptosis) were obtained from three different primary culture preparations. $^*P < 0.01$ vs control group at each time point (χ^2-test).

Table 2 Ischaemia-induced dysfunction of cultured cardiomyocytes.

Time (h)	CPK activity (IU (μg protein)$^{-1}$)		Beating cell number (beating cells/observed cells)		ATP content (μmol (μg protein)$^{-1}$)	
	Control	Ischaemia	Control	Ischaemia	Control	Ischaemia
24	4.13 ± 0.44	2.21 ± 0.19**	36/40 (90%)	29/40 (73%)*	138.4 ± 6.9	147.1 ± 12.0
48	5.52 ± 0.16	2.02 ± 0.04**	28/30 (93%)	5/30 (17%)**	156.2 ± 5.7	106.3 ± 9.7*
72	5.77 ± 0.87	0.62 ± 0.36**	34/37 (92%)	4/40 (10%)**	184.3 ± 4.8	120.9 ± 23.8*

Cardiomyocytes were exposed to an ischaemic insult for the indicated period of time. For ischaemia-induced CPK loss, the data represent mean ± s.e.m. of three to six samples obtained from three different culture preparations. $^{**}P < 0.01$ vs the control group at each time point (Student's *t*-test). For beating inhibition, the samples (n = 30–40 cells) were obtained from three different culture preparations. $^*P < 0.05$ and $^{**}P < 0.01$ vs the control group at each time point (χ^2-test). For ischaemia-induced intracellular ATP depletion, the data represent mean ± s.e.m. of six samples obtained from three different culture preparations. $^*P < 0.05$ vs the control group at each time point (Student's *t*-test).

72-h normoxic incubation, approximately 90% of the ischaemic cells lost their ability to beat after 72 h of ischaemia. As predicted, simulated ischaemia led to a significant reduction in intracellular ATP content. After a 48- and 72-h ischaemic insult, intracellular ATP content decreased from an average of 156.2 to 106.3 nmol (μg protein)$^{-1}$ and from an average of 184.3 to 120.9 nmol (μg protein)$^{-1}$, respectively.

Ischaemia-induced apoptosis of cultured cardiomyocytes

Figure 3 and Table 1 show ischaemia-induced apoptosis of cultured neonatal rat heart cells. In myocytes, a typical DNA ladder was identified by agarose gel electrophoresis (Figure 3A). Interestingly, non-myocytes did not show fragmentation of DNA, even after 72 h of ischaemia (Figure 3A). When the myocytes were stained with Hoechst 33258, a specific DNA stain, and assessed by fluorescence microscopy, cells with fragmented nuclei were more clearly visualized in ischaemic cells compared with control cells (Figure 3B). The number of cells undergoing apoptosis was quantified by fluorescence microscopy after staining (Table 1). The histochemical analysis is more sensitive than DNA electrophoresis. Many of the control myocytes were alive, as only approximately 9% of the cells underwent nuclear fragmentation with Hoechst dye during the 72-h period. By comparison, a significant number of ischaemic myocytes (45%) became apoptotic after 72 h. Furthermore, the simulated ischaemia model caused a 1.6-fold induction of caspase-3 mRNA after 24 h (Figure 3C). However, we failed to detect an alteration in caspase-1 mRNA levels during 24 h (not data shown).

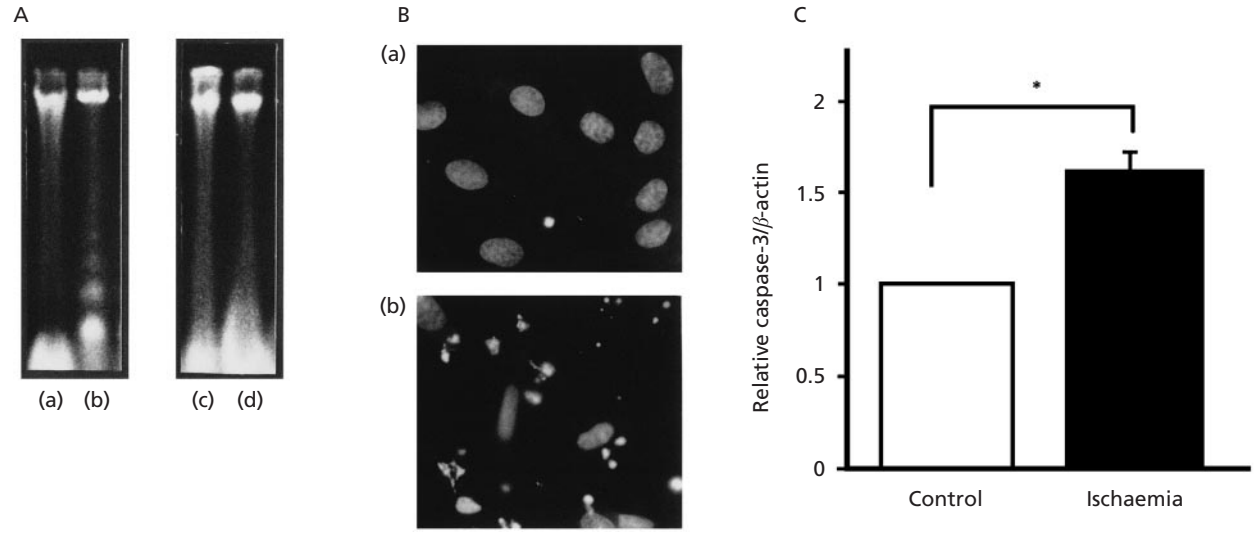

Figure 3 Ischaemia-induced apoptosis of cultured cardiomyocytes. A. ischaemia-induced DNA fragmentation of cardiomyocytes subjected to an ischaemic insult. DNA was isolated from myocytes (left panel) and non-myocytes (right panel) which were incubated for 72 h under either normoxic (a, c) or ischaemic (b, d) conditions. After agarose gel electrophoresis the DNA ladder pattern was visualized after ethidium bromide treatment. B. The Hoechst 33258 staining pattern of myocytes after a 48-h ischaemic insult under normoxic (a), or ischaemic (b) conditions. Original magnification $\times 600$. C. ischaemia-induced caspase-3 mRNA expression. The bar graph depicts the quantitative analysis of caspase-3 gene expression in myocytes after a 24-h ischaemic insult. Fluorescence of each RT-PCR product signal was quantified by an image analyser and normalized relative to β-actin. Gene expression was expressed as a ratio relative to the control. Data represent the mean $\pm$ s.e.m. of seven samples prepared from three different culture preparations. $^*P < 0.01$ compared with control.

Discussion

Our understanding of the pathophysiology and treatment of myocardial ischaemic injury would not have been possible without a number of animal models of ischaemic heart disease (Hasenfuss 1998). To study pathophysiological processes, such as remodelling, or the function of subcellular systems, such as excitation–contraction coupling processes, contractile protein function or energetics, the model of myocardial ischaemia should mimic the clinical setting as closely as possible. Cultured myocytes are a useful experimental preparation, and complement other models of the in-vivo myocardium available in the field of cardiac research. The recent use of cultured myocytes for molecular biological experiments is a prime example of their value (Mitcheson et al 1998). Our results demonstrate that the new simulated ischaemia model has unique advantages. Cardiomyocytes cultured in a sealed flask for 24–72 h were exposed to stresses of hypoxia, acidosis and stagnant incubation medium. The pO_2 and pH of the medium gradually decreased during the ischaemic insult and ultimately fell to a level of 14 mmHg and pH 6.8, respectively. Our model triggered severe cell injury, including morphological degeneration, CPK release, beating impairment and ATP depletion. These data reveal that simulated ischaemia causes cell necrosis and severe myocardial dysfunction.

Although cardiomyocyte apoptosis has been implicated in cardiac remodelling and the progression of myocardial dysfunction (Haunstetter & Izumo 1998), the significance of apoptosis in ischaemic injury is an area of debate. This study supports the notion that apoptosis can contribute to ischaemic injury. As seen in Figure 3 and Table 1, a substantial number of the cultured neonatal rat cardiomyocytes underwent apoptosis during ischaemia. In accordance with the properties of cells undergoing apoptosis, the DNA isolated from the ischemic myocytes exhibited a typical DNA ladder after agarose gel electrophoresis. By contrast, non-myocytes failed to show similar DNA fragmentation, even after 72 h of ischaemia (Figure 3A).

Many of the ischaemic myocytes, but not the ischaemic non-myocytes, stained positively with Hoechst 33258, a specific DNA stain used to detect fragmented nuclei. Whereas very few control myocytes exhibited nuclear fragmentation with the Hoechst dye, ischaemia (72 h) was associated with an increase in the number of apoptotic (Hoechst positive) cells from 13 to 45%.

The mechanism underlying ischaemia-induced damage is an area of considerable interest. Very high concentrations of Ca^{2+} can lead to necrosis through the activity

of Ca^{2+}-sensitive hydrolases (Berridge et al 1998). Calcium overload has also been implicated in the onset of apoptosis (McConkey & Orrenius 1997; Berridge et al 1998). In some cells, the release of Ca^{2+}, or the inhibition of Ca^{2+} uptake by the endoplasmic reticulum, leads to the activation of stress signals, which switch on the genes associated with cell death. Similarly, excessive accumulation of Ca^{2+} by the mitochondria initiates a programme of events leading to cell death (Berridge et al 1998). However, many other factors are also involved in ischaemia-induced cell death. Myocardial ischaemia is known to up-regulate the renin–angiotensin II system, which not only influences myocardial ischaemia by affecting haemodynamics and haemostatic activity (Hasenfuss 1998; Swynghedauw 1999), but also promotes apoptosis (Kajstura et al 1997).

A key phenomenon of apoptotic cell death is the activation of caspases (Haunstetter & Izumo 1998). Our simulated ischaemia model caused the induction of caspase-3 mRNA after 24 h (Figure 3C). It has been proposed that ischaemia promotes a permeability transition in the mitochondria, leading to the release of cytochrome c and the activation of caspase-3. Indeed, Narula et al (1999) have provided evidence that cytochrome c-dependent activation of caspase-3 occurs in human cardiomyopathy. However, a caspase cascade is also activated by death receptor complexes (Cohen 1997; Haunstetter & Izumo 1998; Schwartz 1998; Reed & Paternostro 1999). Once downstream caspases that execute the lethal cuts to vital cellular components are activated, cell death appears to be inevitable (Haunstetter & Izumo 1998). Therefore, preventing the activation of the caspase cascade is crucial in modulating ischaemia-induced apoptosis.

In summary, this study describes a new model of ischaemia, in which the exposed cell experiences morphological degeneration, CPK release, beating cessation and ATP depletion. The affected cell also exhibits hallmarks of apoptosis, such as nucleosomal DNA fragmentation and nuclear staining with Hoechst dye 33258. It is proposed that the new model may be useful in unravelling the molecular alterations underlying ischaemic injury and myocardial apoptosis.

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