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Intracellular ATP is required for mitochondrial apoptotic pathways in isolated hypoxic rat cardiac myocytes

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Abstract

Objectives: The present study examined the possibility that intracellular ATP levels dictate whether hypoxic cardiac myocytes die by apoptosis or necrosis. **Background:** Although apoptosis and necrosis may appear to be distinct forms of cell death, recent studies suggest that the two may represent different outcomes of a common pathway. In ischemic myocardium, apoptosis appears early, while energy stores are presumably still available, followed only later by necrosis. **Methods:** Neonatal rat cardiac myocytes were exposed to continuous hypoxia, during which the intracellular ATP concentration was modulated by varying the glucose content in the medium. The form of cell death was determined at the end of the hypoxic exposure. **Results:** Under total glucose deprivation, ATP dropped precipitously and cell death occurred exclusively by necrosis as determined by nuclear staining with ethidium homodimer-1 and smearing on DNA agarose gels. However, with increasing glucose concentrations (10, 20, 50, 100 mg/dl) cellular ATP increased correspondingly, and apoptosis progressively replaced necrosis until it became the sole form of cell death, as determined by nuclear morphology, DNA fragmentation on agarose gels, and caspase-3 activation. The data showed a significantly positive correlation between myocyte ATP content and the percentage of apoptotic cells. Hypoxia resulted in lactate production and cellular acidification which stimulates apoptosis. However, acidification-induced apoptosis was also increased in an ATP-dependent fashion. Loss of mitochondrial membrane potential and cytochrome c release from the mitochondria was observed in both the apoptotic and necrotic cells. Furthermore, translocation of Bax from cytosol into mitochondria preceded these events associated with mitochondrial permeability transition. Increased lactate production and a lack of effect by the mitochondrial inhibitor oligomycin indicated that ATP was generated exclusively through glycolysis. **Conclusions:** We demonstrate that ATP, generated through glycolysis, is a critical determinant of the form of cell death in hypoxic myocytes, independently of cellular acidification. Our data suggest that necrosis and apoptosis represent different outcomes of the same pathway. In the absence of ATP, necrosis prevails. However, the presence of ATP favors and promotes apoptosis.
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Keywords: Apoptosis; Energy metabolism; Hypoxia/anoxia; Myocytes; Necrosis

1. Introduction

Ischemic injury in the myocardium causes myocyte cell death which displays features of apoptosis, necrosis, or both [1–4]. In view of the significant differences between these two forms of cell death, it is increasingly being

Abbreviations: CK, creatine kinase; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; Bax, Bcl-2-associated X protein; PMSF, phenylmethanesulfonyl fluoride; CPP32, cystein protease p32; DEVD, Asp–Glu–Val–Asp

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recognized that the clinical ramifications of ischemic injury can vary with the specific form of cell loss. For example, in contrast to apoptosis, necrotic cell death, with its attendant membrane disruption and release of cellular contents, can cause injurious inflammatory effects [5]. A great deal of effort is therefore being expended currently to elucidate the causes and consequences of myocardial apoptosis and necrosis. Such information will undoubtedly have a profound impact on the design and implementation of novel therapeutic approaches to myocardial ischemic injury.

Although apoptosis and necrosis have been considered until recently to be fundamentally different processes, recent data suggest that they are, in fact, initiated in identical fashion but have different outcomes [6]. The accumulated data strongly suggest that when cardiac myocytes sustain severe hypoxic injury they immediately initiate a programmed sequence of events designed to effect apoptosis. Several studies from our laboratory and others have clearly demonstrated that apoptosis is the earliest form of cell death in ischemic myocardium in both animals and humans [1–4]. It is only substantially later that necrosis appears in significant quantity in animal models and in humans [2–4]. In view of the apparent advantage of an apoptotic, non-inflammatory, elimination of injured cardiac myocytes, the frequent appearance of necrosis was puzzling at first. However, it now appears very likely that necrosis occurs only when the injured cardiac myocytes are unable to successfully complete the process of apoptosis [7,8]. One particularly likely cause of this interruption of apoptosis is the depletion of cellular ATP.

Since the execution of apoptosis requires energy, the depletion of cellular ATP will likely interrupt or suspend the apoptotic process. This conclusion is based on the findings of several studies which have demonstrated that, in the absence of cellular ATP, injured cells tend to die by necrosis, whereas in the presence of ATP the same cells are able to complete the apoptotic process in mitotic cells [9,10]. Therefore, as we have proposed previously, it seems very likely that, in the ischemic myocardium, injured myocytes ‘prefer’ to die by apoptosis, but are forced to abandon this mode of cell death when persistent severe ischemia causes these cells to be depleted of ATP [7,8]. We have further proposed that, upon restoration of sufficient ATP levels in the injured cardiac myocytes, the cells will resume their suspended apoptotic process [7,8]. We base these assumptions on our previous findings which demonstrate that reperfusion of ischemic myocardium causes the ‘acceleration’ of apoptosis, in both rat and human hearts, presumably as a result of the restoration of cellular ATP production [3,4]. Consequently, the severity and duration of the ischemia, as well as the timing of the reperfusion, will dictate the degree to which dying cells demonstrate the characteristics of either apoptosis, necrosis, or both [6]. The objective of the present study was therefore to test the hypothesis that, in cardiac myocytes subjected to hypoxic injury, the intracellular ATP content determines whether the cell will die by necrosis or apoptosis.

2. Methods

2.1. Cultured neonatal rat heart myocytes

Primary cultures of neonatal rat cardiac myocytes were prepared as described previously, with some modifications [11,12]. Briefly, hearts from ether-anesthetized 1–2-day-old Wistar rats were washed with phosphate-buffered saline (PBS), the ventricles were minced with scissors into 1–3 mm^3 fragments, and were then enzymatically digested four times for 10–15 min each in PBS containing 0.2% collagenase (Sigma type I). The liberated cells from each digestion were collected by centrifugation, were resuspended in HEPES buffer (in mmol/l: NaCl 116, KCl 5.4, $MgSO_4$ 0.8, NaH_2PO_4 1.0, HEPES 20, glucose 5.5; pH 7.35), were layered onto a Percoll density gradient (density, 1.059/1.082), and were centrifuged at $1000\times g$ for 30 min [12]. The myocyte fraction at the Percoll layer interface was collected and washed twice in HEPES buffer. The cells were then resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical Co.) supplemented with 10% fetal bovine serum (FBS) (Bioserum Co.) and 1% antibiotics (5000 μg/ml gentamicin, 5000 μg/ml ampicillin and 100 μg/ml amphotericin B). The myocytes were seeded into 60-mm culture dishes (1×10^5 cells/cm^2) and incubated at 37 °C in humidified atmosphere containing 5% CO_2. 5-Bromo-2′-deoxyuridine (BrdU; 100 μmol/l) was added during the first 48 h to inhibit proliferation of non-myocytes [13]. We routinely obtained contractile, myocytes-enriched, cultures which contained better than 95% myocytes, as assessed by immunofluorescence staining with a monoclonal antibody against β-myosin heavy chain. The myocytes were incubated in DMEM containing 0.5% FBS without BrdU, and all experiments were done 36–48 h after changing to the 0.5% FBS medium.

2.2. Exposure to hypoxia

Prior to hypoxic treatment, the cell medium was replaced with modified Tyrode solution (in mmol/l: NaCl 136.9, KCl 2.68, $Na_2HPO_4\cdot12H_2O$ 8.1, KH_2PO_4 1.47, $CaCl_2$ 0.9, $MgCl_2\cdot6H_2O$ 0.49; pH 7.4) with different glucose concentration (0, 10, 20, 50, 100 mg/dl) to manipulate intracellular ATP levels during hypoxia. No FBS was included in the Tyrode solution. The cardiac myocytes were then transferred to a 37 °C humidified chamber (F-102, Iijima Electronics Co., Aichi, Japan) which was flushed with 5% CO_2 and 95% nitrogen (less than 1% oxygen) for 7 h. To determine the source of ATP production during hypoxia, some myocyte preparations were treated with modified Tyrode solution containing 100

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mg/dl glucose in the presence of 1×10^{-6} mol/l oligomycin, an inhibitor of mitochondrial ATPase. Myocytes incubated in modified Tyrode solution containing 100 mg/dl glucose under normoxic conditions for 7 h were used as a normoxic control.

2.3. ATP content

The ATP content in the myocytes was measured before, and 7 h after, hypoxia. Cardiac myocytes (2.7×10^{6} cells/dish) were treated with 0.25 ml of 0.6 N ice-cold perchloric acid and centrifuged at $1000\times g$ for 5 min at 4 °C. The supernatant was neutralized with KOH to pH 5.0–7.0 and, after 10 min, was centrifuged at $8000\times g$ for 5 min at 4 °C to remove the $KClO_4$. The supernatant was used for the assays. ATP was measured by high-performance liquid chromatography (HPLC: LC-9A liquid chromatograph, Shimadzu, Kyoto, Japan) with a column of STR ODS-M (Shimadzu) [14].

2.4. Determination of cell viability

To monitor cell viability myocytes were grown on Type I collagen-coated glass coverslips. Following 7 h of hypoxia the relative number of living and dead cells was determined using a viability/cytotoxicity kit (Molecular Probes—Catalog number L-3224) [15]. The culture medium was replaced with 2×10^{-6} mol/l calcein acetoxymethyl ester and 4×10^{-6} mol/l ethidium homodimer-1 and the cells were incubated for 45 min at room temperature. Cells with permeabilized membranes (necrosis) take up the ethidium homodimer-1 dye and their nuclei appear red. In contrast, cells with intact membranes exclude the ethidium dye, but take up and hydrolyze the calcein ester and therefore appear green. Cell viability was quantitated by counting the number of necrotic and viable myocytes in 10 random microscopic fields per condition per experiment using a fluorescence microscope.

2.5. Detection of apoptotic myocytes

Desmin content was used to distinguish cardiac myocytes from non-myocytes, as described previously [16]. Cardiac myocytes cultured on Type I collagen-coated coverslips fixed on ice with 4% paraformaldehyde, were permeabilized with ice-cold methanol–acetone (1:1, vol/vol), were blocked with 10% heat-inactivated goat serum and were then incubated with a primary anti-desmin polyclonal antibody (Monosan), followed by secondary FITC-labeled goat anti-rabbit IgG antibody. To visualize the nuclei, 0.5 μg/ml of Hoechst 33258 (Molecular Probes), a general nuclear stain, was added to the secondary antibody mix. The cells were photographed using fluorescence microscopy and dual exposure photography. A total of 800–1000 Hoechst-stained nuclei from 10 random fields (magnification ×200) were counted for each experimental group and the apoptotic cells were identified by the characteristic condensed, fragmented, nuclei.

2.6. Lactate production

Lactate content was measured enzymatically, using the Detaminer LA kit (Kyowa Medics Co. Ltd, Tokyo, Japan). Aliquots of culture media were added to a phosphate-buffered reaction mix containing 0.9 mmol/l *N*-ethyl-*N*-(3-methylphenyl)-*N′*-acetylethylendiamine (EMAE), 0.7 IU/ml lactate oxidase, 3.4 IU/ml peroxidase, and 0.4 mmol/l 4-aminoantipyrine, at pH 6.25. Lactate content was determined spectrophotometrically at 37 °C by measuring quinone absorbance at 555 nm [14].

2.7. Exposure to acidification

To examine the effect of lactate-induced acidification on the development of cell death, L-lactic acid was added into the myocyte culture (Tyrode solution) under normoxic conditions for 7 h, and the percentage of either apoptosis or necrosis and the medium pH were measured. The concentration of L-lactic acid was determined from the original data 7 h after hypoxia (Table 1).

To further demonstrate our hypothesis that intracellular ATP plays an important role in the execution of apoptosis, independently of acidification, we have modified another experimental protocol which we recently reported [8], and examined the effect of energy levels on either apoptosis or necrosis under acidic conditions. Briefly, metabolic inhibition (MI) was achieved by incubating the myocytes with 0.05 μmol/l oligomycin in glucose-free Tyrode solution, pH 7.4, at 37 °C for 1 h. The myocytes were then washed twice with PBS and were incubated for a further 4 h in Tyrode solution containing 0 (MI0 group), 10 (MI10 group), 30 (MI30 group), 50 (MI50 group), or 100 (MI100 group) mg/dl of glucose, without oligomycin, to manipu-

Table 1
Lactate production in myocytes and medium pH

	C	H0	H10	H20	H50	H100	Oligo
Lactate (mmol/l)	0.31±0.02	0.28±0.03†	1.08±0.13*‡	1.73±0.14*§	2.43±0.14$^{*\|}$	3.14±0.18$^{*\|}$	3.89±0.14*¶
pH	7.10±0.032	7.12±0.037	7.08±0.020	7.08±0.020	6.94±0.024$^{*\ddagger\S\|}$	6.92±0.020$^{*\ddagger\S\|}$	6.88±0.020$^{*\ddagger\S\|}$

Myocytes were exposed to 7 h of hypoxia in the presence of 0 mg/dl (H0), 10 mg/dl (H10), 20 mg/dl (H20), 50 mg/dl (H50), 100 mg/dl (H100) of glucose, 100 mg/dl of glucose with 1×10^{-6} mol/l oligomycin (oligo), or normoxic conditions (C), and the lactate content and pH in the medium was determined as described in Methods. (n=8). *P<0.001 vs. C; $^{\dagger}P$<0.05 vs. C; $^{\ddagger}P$<0.001 vs. H0; $^{\S}P$<0.01 vs. H10, $^{\|}P$<0.001 vs. H20, $^{\P}P$<0.001 vs. H100.

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late the intracellular ATP content. The medium (Tyrode solution) pH was adjusted to 6.8 at the start of this 4 h incubation to induce cell death. Myocytes that were incubated in 100 mg/dl of glucose Tyrode solution (pH 6.8) for 4 h but without the prior MI were designated as the MI(−) group. We then determined the type of cell death by histochemical methods.

2.8. DNA agarose gel electrophoresis

Apoptotic internucleosomal DNA strand breaks were assessed by agarose gel electrophoresis of low-molecular-weight genomic DNA [17]. Briefly, detached cells in the culture medium were collected by centrifugation. The adherent cells were lysed with a lysis buffer (10 mmol/l EDTA, 10 mmol/l Tris–HCl [pH 7.4], 0.5% Triton X-100), scraped, pooled with the detached cells, and centrifuged at 18 000×*g* for 20 min. The supernatant was treated with RNase A (400 μg/ml) for 1 h at 37 °C and then with proteinase K (400 μg/ml) for 1 h at 37 °C. The DNA was precipitated with NaCl (0.5 mol/l) and 50% (vol/vol) isopropanol at −20 °C. The precipitated DNA was centrifuged at 18 000×*g* for 15 min and the isopropanol was removed. The nucleic acid pellet was resuspended in 1×10^{-2} mmol/l Tris–HCl (pH 7.4), 1×10^{-3} mmol/l EDTA (pH 8.0). The samples were normalized for cell number and subjected to electrophoresis on 2% agarose gel. Gels were stained with SYBR green (Molecular Probes). The DNA bands were detected under ultraviolet (UV) light.

2.9. Measurement of mitochondrial membrane potential

Loss of mitochondrial membrane potential was assessed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes). Coverslip-grown myocytes were incubated in PBS containing 10 μmol/l JC-1 at 37 °C for 5 min. Fluorescence was viewed at 527 and 590 nm using excitation at 480 nm.

2.10. Immunoblotting

For detection of cytochrome c and Bcl-2-associated X protein (Bax), myocytes were scraped and pelleted by centrifugation at 800×*g* for 5 min. The cells were resuspended in 150 μl of cold lysis buffer (in mmol/l: sucrose 250, HEPES 20, KCl 10, $MgCl_2$ 1, EDTA 1, EGTA 1, dithiothreitol 1, phenylmethanesulfonyl fluoride [PMSF] 1; pH 7.5) and were incubated for 5 min on ice. The cells were homogenized with 10 strokes of a pestle and the suspension was centrifuged at 750×*g* for 10 min at 4 °C to sediment the nuclei. The supernatant was centrifuged at 12 000×*g* for 10 min at 4 °C to collect the mitochondrial fraction. The supernatant was further centrifuged at 100 000×*g* for 60 min at 4 °C, and the resultant supernatant was used as the cytosolic fraction [18].

To detect the cleavage of procaspase-3, myocytes were scraped off the plates and were pelleted by centrifugation at 800×*g* for 5 min. The cells were resuspended in 50 μl of cold lysis buffer (2×PBS, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS; pH 7.4) and were incubated for 10 min on ice. The suspension was centrifuged at 14 000×*g* for 10 min at 4 °C and the supernatant was collected for analysis.

Immunoblotting was performed using standard protocols. Samples containing equal amounts of protein were subjected to electrophoresis on 12% SDS–polyacrylamide gel and blotted onto polyvinylidine difluoride membrane (ATTO. AE-6665, Tokyo, Japan). After blocking with 5% skim milk in PBS containing 0.1% Tween-20 at room temperature for 1 h, the membranes were probed with antibodies specific to cytochrome c (Pharmingen. 7H8.2C12, San Diego, CA, USA), Bax (Santa Cruz Biotechnology. sc-526, CA, USA) or caspase-3 (Santa Cruz Biotechnology. sc-7148) at 4 °C overnight, followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG (all from Amersham, Little Chalfont, UK) at room temperature for 1 h. Detection of chemoluminescence was performed with ECL Western blot detection kits (Amersham), according to the supplier's recommendation.

2.11. Caspase-3 activity

The activity of caspase-3 in the hypoxic myocytes was determined spectrophotometrically with the cystein protease p32 (CPP32) assay kit (MBL) by the release of the chromophore *p*-nitroanilide following hydrolysis of substrate Asp–Glu–Val–Asp (DEVD)-*p*-nitroanilide as previously described [19]. Briefly, 2.7×10^{6} cells were solubilized, and equal amounts of protein lysates were reacted with 200 μmol/l DEVD-*p*-nitroanilide at 37 °C for 2 h. The activity was read in a microtiter plate reader at 400 nm.

2.12. Statistics

Data are expressed as mean±S.E.M. from at least eight separate experiments. Differences were analyzed by one-way ANOVA combined with Scheffé's test, and a *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. ATP content

The intracellular ATP concentration in the myocytes at 7 h after hypoxia or normoxia is shown in Fig. 1. The ATP content in hypoxic myocytes cultured in Tyrode solution containing 0, 10, 20, or 50 mg/dl glucose was 14.9%, 30.5%, 58.6%, and 81.0% of normoxic levels, respectively.

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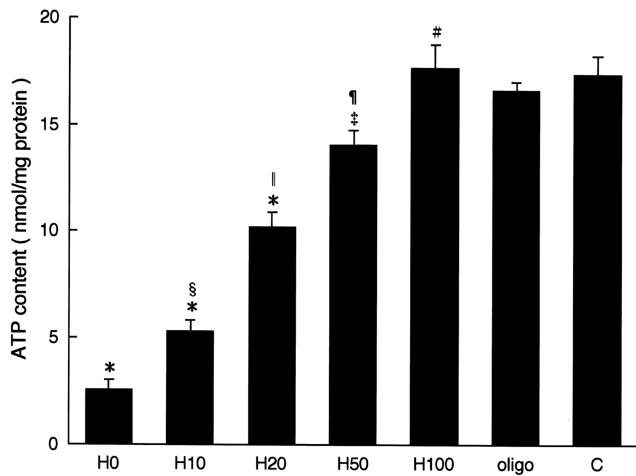
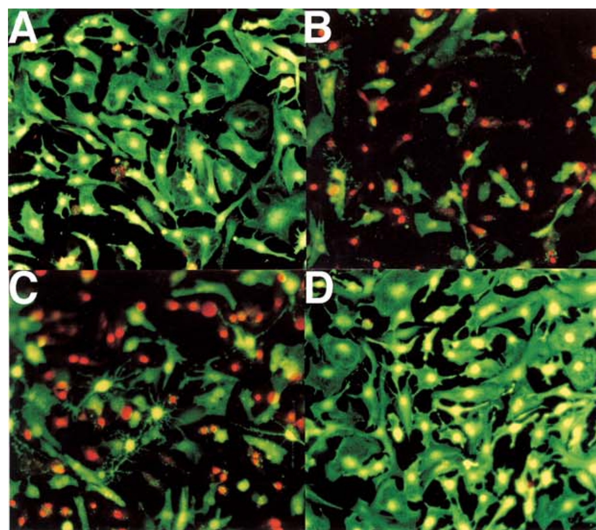


Fig. 1. ATP content in myocytes. Myocytes were exposed to 7 h of hypoxia in the presence of 0 mg/dl (H0), 10 mg/dl (H10), 20 mg/dl (H20), 50 mg/dl (H50), 100 mg/dl (H100) of glucose, 100 mg/dl of glucose with 1×10^{-6} mol/l oligomycin (oligo), or normoxic conditions (C), and the ATP content in the myocytes was determined as described in Methods. ($n=8$). *$P<0.0001$ vs. C; ‡$P<0.05$ vs. C; §$P<0.01$ vs. H0; ‖$P<0.0001$ vs. H10; ¶$P<0.001$ vs. H20; #$P<0.001$ vs. H50.

In contrast, hypoxic cells cultured in Tyrode solution containing 100 mg/dl glucose contained ATP levels similar to those in the normoxic controls. The presence of oligomycin in the hypoxic Tyrode solution containing 100 mg/dl glucose did not alter the ATP content from control levels.

3.2. Cell viability

The effect of hypoxia on cell viability is illustrated in Fig. 2. The fraction of viable cells (green fluorescence) was consistently greater than 95% under normoxic conditions. However, the percentage of necrotic cells (red nuclei) increased dramatically after 7 h of hypoxia in the glucose-depleted Tyrode solution. The nuclei of the necrotic cells appeared condensed, suggesting that an apoptotic mechanism had been initiated prior to the onset of necrosis. Increasing the concentration of glucose in the hypoxic Tyrode solution resulted in a corresponding decrease in necrosis and, at 100 mg/dl glucose, cell viability was not different from the normoxic control. Quantitation of the percent necrotic cells showed that after 7 h of hypoxia in glucose-depleted Tyrode solution, roughly 63% of the cells were necrotic (Fig. 2). However, increasing the glucose concentration in the hypoxic Tyrode solution to 10 and 20 mg/dl resulted in a corresponding decrease in necrosis to 43% and 20%, respectively. At 50 mg/dl of glucose, there was no detectable necrosis in the hypoxic cultures. The above results with calcein acetoxymethyl ester and ethidium homodimer-1 were well correlated with the data of creatine kinase activity released into



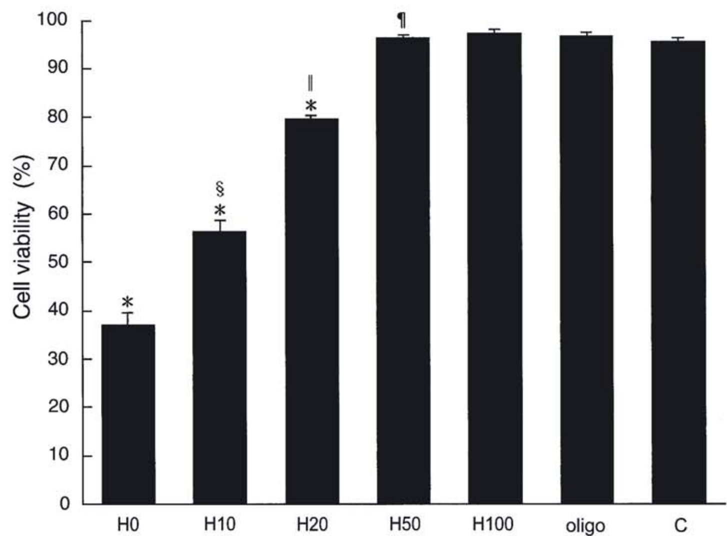


Fig. 2. Histochemical determination of cell viability. Cardiac myocytes were exposed to 7 h hypoxia in the presence of 0 mg/dl (H0), 10 mg/dl (H10), 20 mg/dl (H20), 50 mg/dl (H50), 100 mg/dl (H100) of glucose, 100 mg/dl of glucose with 1×10^{-6} mol/l oligomycin (oligo), or normoxic conditions (C). The myocytes were then labeled with calcein acetoxymethyl ester and ethidium homodimer-1 and visualized by fluorescence microscopy as described in Methods, and then scored as either viable (green cytosolic fluorescence of calcein) or non-viable (red nuclear fluorescence of ethidium). Left panel shows representative micrographs (magnification ×200). (A) Myocytes maintained under normoxic conditions. (B) Myocytes following 7 h of hypoxia in the absence of glucose. (C) Myocytes following 7 h of hypoxia in the presence of 10 mg/dl glucose. (D) Myocytes following 7 h of hypoxia in the presence of 100 mg/dl glucose. Right panel shows the percent viable cells present in 10 random microscopic fields per condition per experiment, which were determined using fluorescence microscopy, as described in Methods. ($n=8$). *$P<0.0001$ vs. C; §$P<0.0001$ vs. H0; ‖$P<0.0001$ vs. H10; ¶$P<0.001$ vs. H20.

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the medium (ΔCK), suggesting that live/dead cell assay can properly evaluate cell viability (data not shown).

3.3. Myocyte apoptosis

Histochemical nuclear staining with Hoechst 33258, and immunohistochemical staining of cellular desmin revealed apoptotic myocytes with typical fragmented nuclei and condensed chromatin 7 h after hypoxia, as illustrated in Fig. 3. When compared to normoxic myocytes, myocytes exposed to hypoxia in the presence increasing concentrations of glucose showed a corresponding increase in the numbers of apoptotic cells. Quantitation of this effect demonstrated that the percentage of apoptotic myocytes in glucose-depleted hypoxic Tyrode solution (1.13±0.13%) was not significantly greater than normoxic control (0.98±0.17%). In contrast, as the glucose content in the hypoxic solution was increased, there was a corresponding and significant increase in the percent apoptotic cells. Thus, at 20, 50, and 100 mg/dl of glucose, the percent apoptosis increased to 4.3±0.42, 9.44±0.49, and 12.0±0.66%, respectively (Fig. 3). Oligomycin in the presence of glucose did not significantly affect this hypoxia-induced apoptosis. Furthermore, there was a statistically significant positive correlation between ATP content and myocyte apoptosis ($r=0.873$, $P<0.0001$), as shown in Fig. 4.

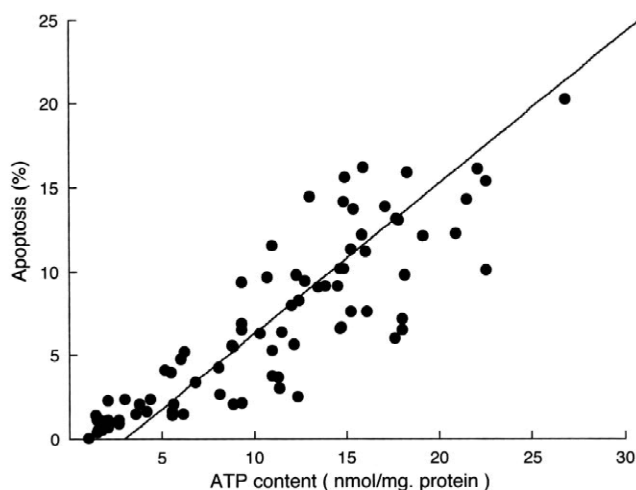
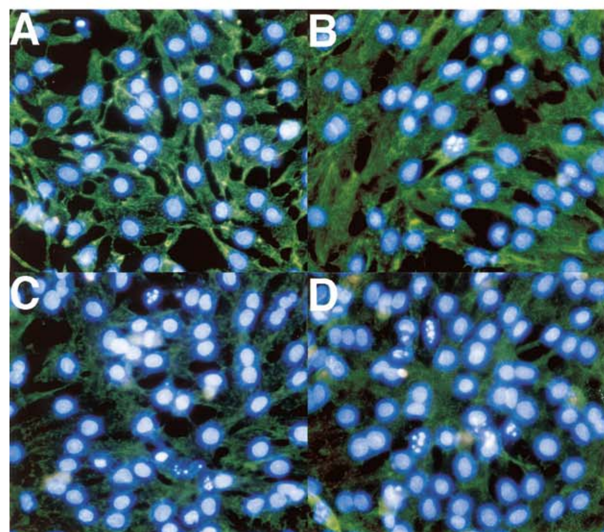


Fig. 4. Scatterplot showing the relation between ATP content and the percentage of apoptotic myocytes after 7 h of hypoxia in the presence of increasing concentrations of glucose. The relation was $y=0.685x-0.497$, $r=0.873$, $P<0.0001$.

Fig. 5 illustrates the DNA fragmentation pattern in cardiac myocytes following 7 h of normoxia or hypoxia. The normoxic myocytes did not exhibit any detectable DNA fragmentation. However, DNA isolated from myocytes exposed to 7 h of hypoxia under glucose-free conditions showed a strong DNA smear, characteristic of necrosis, with very little of the internucleosomal DNA fragmentation normally associated with apoptosis. In con-



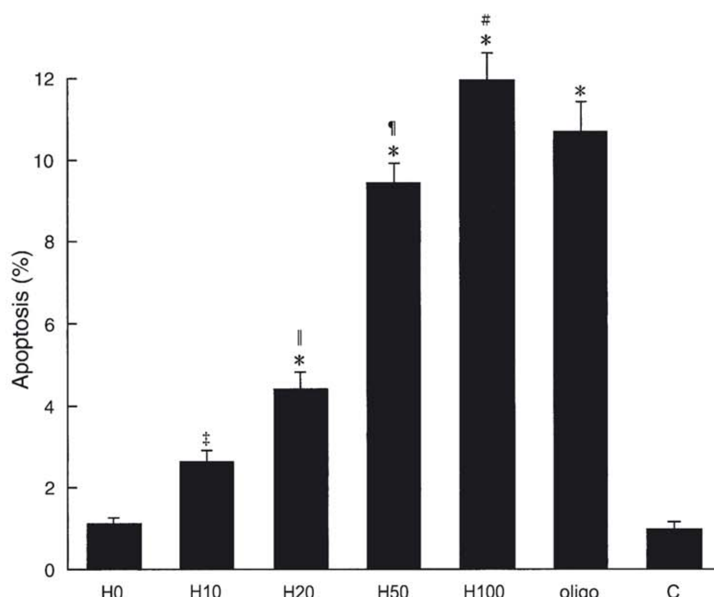


Fig. 3. Immunohistochemical determination of myocyte apoptosis. Cardiac myocytes were exposed to 7 h of hypoxia in the presence of 0 mg/dl (H0), 10 mg/dl (H10), 20 mg/dl (H20), 50 mg/dl (H50), 100 mg/dl (H100) of glucose, 100 mg/dl of glucose with 1×10^{-6} mol/l oligomycin (oligo), or normoxic conditions (C). The cells were fixed and incubated with anti-desmin primary antibody and FITC-tagged secondary antibody as described in Methods. The nuclei were simultaneously stained with Hoechst 33258. Left panel shows representative micrographs (magnification ×400). (A) Myocytes maintained under normoxic conditions. (B) Myocytes following 7 h of hypoxia in the presence of 10 mg/dl glucose. (C) Myocytes following 7 h of hypoxia in the presence of 50 mg/dl glucose. (D) Myocytes following 7 h of hypoxia in the presence of 100 mg/dl glucose. Right panel shows percent apoptosis in cardiac myocytes. Apoptotic myocytes were identified by their fragmented nuclei and condensed chromatin, and the percent apoptotic cells was calculated as described in Methods. ($n=8$). *$P<0.0001$ vs. C; ‡$P<0.05$ vs. C; ‖$P<0.05$ vs. H10; ¶$P<0.0001$ vs. H20, #$P<0.0001$ vs. H50.

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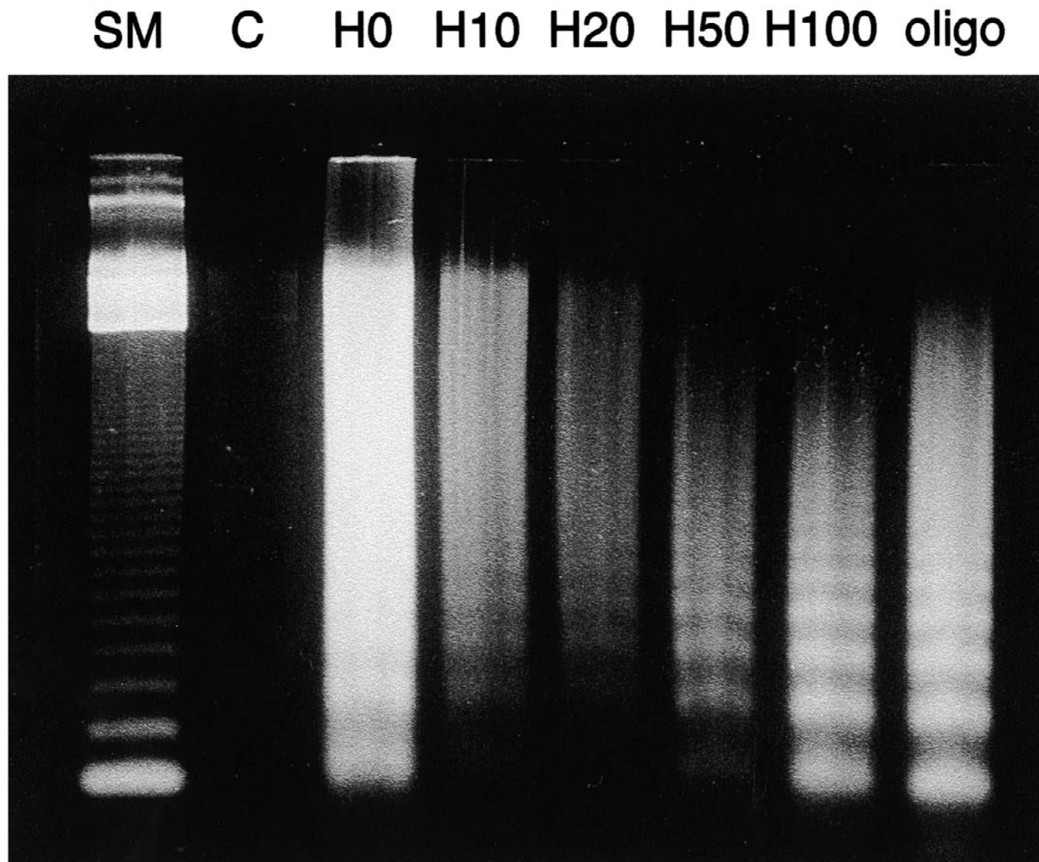

Fig. 5. Agarose gel electrophoresis of DNA. Myocytes were exposed to 7 h of hypoxia in the presence of 0 mg/dl (H0), 10 mg/dl (H10), 20 mg/dl (H20), 50 mg/dl (H50), 100 mg/dl (H100) of glucose, 100 mg/dl of glucose with 1×10^{-6} mol/l oligomycin (oligo), or normoxic conditions (C), and low-molecular-weight genomic DNA was isolated from the myocytes and loaded onto 2% agarose gel as described in Methods. The normoxic myocytes did not exhibit any detectable DNA fragmentation (C). However, DNA isolated from myocytes exposed to 7 h of hypoxia under glucose-free conditions showed a strong DNA smear, with very little of the internucleosomal DNA fragmentation (H0). In contrast, DNA isolated from myocytes exposed to hypoxia in glucose-containing Tyrode solution showed extensive apoptotic DNA fragmentation. The intensity of the DNA ladder increased proportionately with increasing glucose concentrations (H10–H100, oligo). Results are representative of 3 independent experiments. SM; size marker.

trast, DNA isolated from myocytes exposed to hypoxia in glucose-containing Tyrode solution showed extensive apoptotic DNA fragmentation, and the characteristic DNA 'ladder' formation. The intensity of the DNA ladder increased proportionately with increasing glucose concentrations (Fig. 5). Oligomycin in the presence of glucose did not appear to alter the DNA fragmentation pattern.

3.4. Lactate production and acidification

Lactate concentration in Tyrode solution was measured at 7 h after hypoxia or normoxia. As shown in Table 1, lactate production during hypoxia was significantly lower than the normoxic control in the glucose-free cultures. In contrast, lactate production significantly increased with increasing glucose concentrations in hypoxic Tyrode solution. The presence of oligomycin further potentiated lactate production during hypoxia. This lactate production resulted in a mild but significant decrease in the medium pH (Table 1).

Acidification by L-lactic acid significantly increased the myocyte apoptosis in a pH-dependent fashion (Fig. 6). The maximal percentage of apoptosis was 7.9±0.3%, when the concentration of L-lactic acid was 3.14 mmol/l in the medium. In contrast, we confirmed that myocyte necrosis estimated by acetoxymethyl ester and ethidium homodimer-1 did not significantly increase by acidification (data not shown).

Fig. 7 illustrates the time course change of myocyte ATP content and the percentage of apoptotic myocytes 4 h after acidification treatment. Subsequent incubation of the MI-treated cells for 4 h with varying concentrations of glucose resulted in a concentration-dependent restoration of ATP, such that 0, 10, 30, 50, and 100 mg/dl of glucose increased the ATP to 2.9±1.7, 25.6±4.1, 39.7±4.5, 53.7±4.8, and 65.3±2.1% of baseline, respectively. Moreover, the frequency of apoptotic cells increased with increasing glucose concentration. The percentage of apoptotic nuclei 4 h after acidification increased significantly to 6.4±0.3, 7.3±0.4, 8.3±0.2, and 8.9±0.3% in the MI10, MI30, MI50, and MI100 groups, respectively. In contrast, myocyte necrosis did not increase by acidification in each

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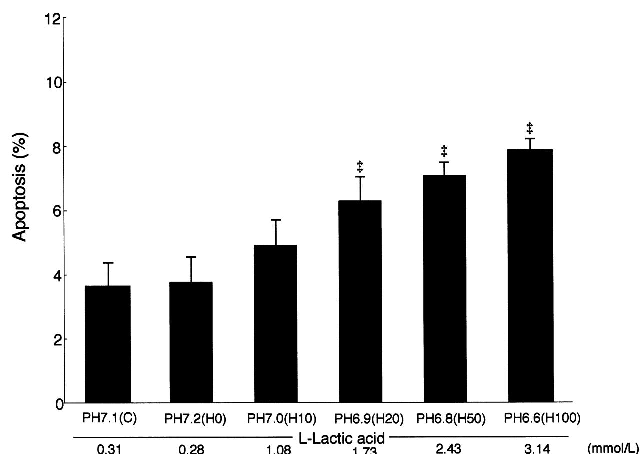

Fig. 6. Lactate and myocyte apoptosis. Myocytes were incubated in Tyrode solution containing 0.31 mmol/l, 0.28 mmol/l, 1.08 mmol/l, 1.73 mmol/l, 2.43 mmol/l, and 3.14 mmol/l of L-lactic acid under normoxic conditions for 7 h. The myocytes were then stained with an anti-desmin polyclonal antibody and Hoechst 33258, and the percent apoptotic cells was calculated as described in Methods. The concentration of L-lactic acid was determined from the original data (C, H0, H10, H20, H50, and H100) 7 h after hypoxia (Table 1). After the addition of L-lactic acid, the medium pH was changed to 7.1, 7.2, 7.0, 6.9, 6.8, and 6.6, respectively, as indicated in the figure. ($n=8$). ‡$P<0.05$ vs. L-lactic acid 0.31.

group (data not shown). In addition, we confirmed that there was no significant change in the medium pH after 4 h of incubation.

3.5. Mitochondrial membrane potential

The green-fluorescent dye JC-1 exists as a monomer at low membrane potential. However, at higher potentials, JC-1 forms red-fluorescent 'J-aggregates'. The emission of this dye can therefore be used to monitor mitochondrial membrane potential in apoptotic cardiac myocytes [20]. Under aerobic control conditions, the myocytes showed red–orange mitochondrial staining, indicative of normal high membrane potentials (Fig. 8). Hypoxia caused loss of mitochondrial membrane potential in a time-dependent fashion and resulted in a significant loss of membrane potential after 5 h of hypoxia (Fig. 8A). Moreover, myocytes exposed to 7 h hypoxia in the presence or absence of 100 mg/dl glucose showed green fluorescence indicating the loss of mitochondrial membrane potential (Fig. 8B).

3.6. Translocation of bax and cytochrome c

Fig. 8A illustrates the time-dependent effects of hypoxia on the intracellular localization of Bax and cytochrome c in the myocytes in the presence of 100 mg/dl glucose. Bax was modestly expressed in the cytosolic fraction of the myocytes. Hypoxia induced superexpression of Bax in a time-dependent fashion. Moreover, hypoxia caused an increase in Bax immunoreactivity in the mitochondrial fraction in the first 3 h. This mitochondrial Bax immunoreactivity was depressed after 5 h of hypoxia. In contrast, prior to hypoxia, cytochrome c was detected exclusively in the mitochondrial fraction. Hypoxia caused a time-dependent decrease in cytochrome c immunoreactivity in the mitochondrial fraction, with a concomitant increase in the cytosolic fraction, reaching a maximum level after 7 h of hypoxia.

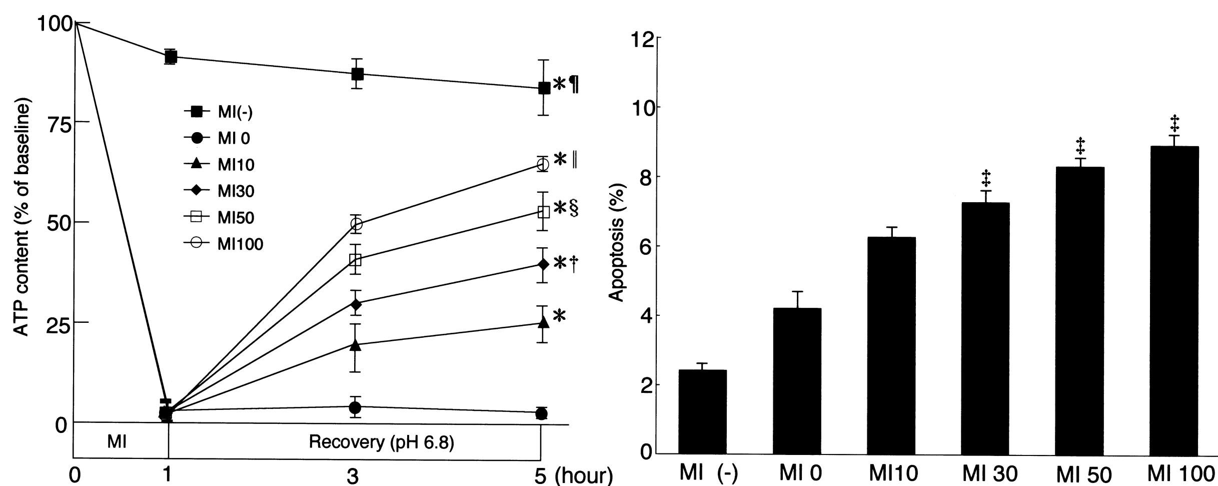

Fig. 7. Acidification and ATP-dependent-apoptosis. Cardiac myocytes were subjected to metabolic inhibition (MI) for 1 h, followed by a 4 h-incubation (Recovery) in medium (Tyrode solution) containing 0 (MI0 group), 10 (MI10 group), 30 (MI30 group), 50 (MI50 group), or 100 (MI100 group) mg/dl of glucose. The medium pH was adjusted to 6.8 at the start of this 4 h incubation to induce cell death. One group of myocytes was not subjected to MI but was incubated in medium containing 100 mg/dl glucose (MI[−] group). Left panel shows time course change of ATP content in the myocytes. The ATP content in the myocytes was determined as described in Methods. Right panel shows percent apoptosis in cardiac myocytes. After metabolic inhibition (MI) followed by acidification treatment, the myocytes were stained with an anti-desmin polyclonal antibody and Hoechst 33258, and the percent apoptotic cells was calculated as described in Methods. ($n=8$). *$P<0.01$ vs. MI0; †$P<0.05$ vs. MI10; §$P<0.05$ vs. MI30; ‖$P<0.05$ vs. MI50; ¶$P<0.01$ vs. MI100; ‡$P<0.05$ vs. MI(−).

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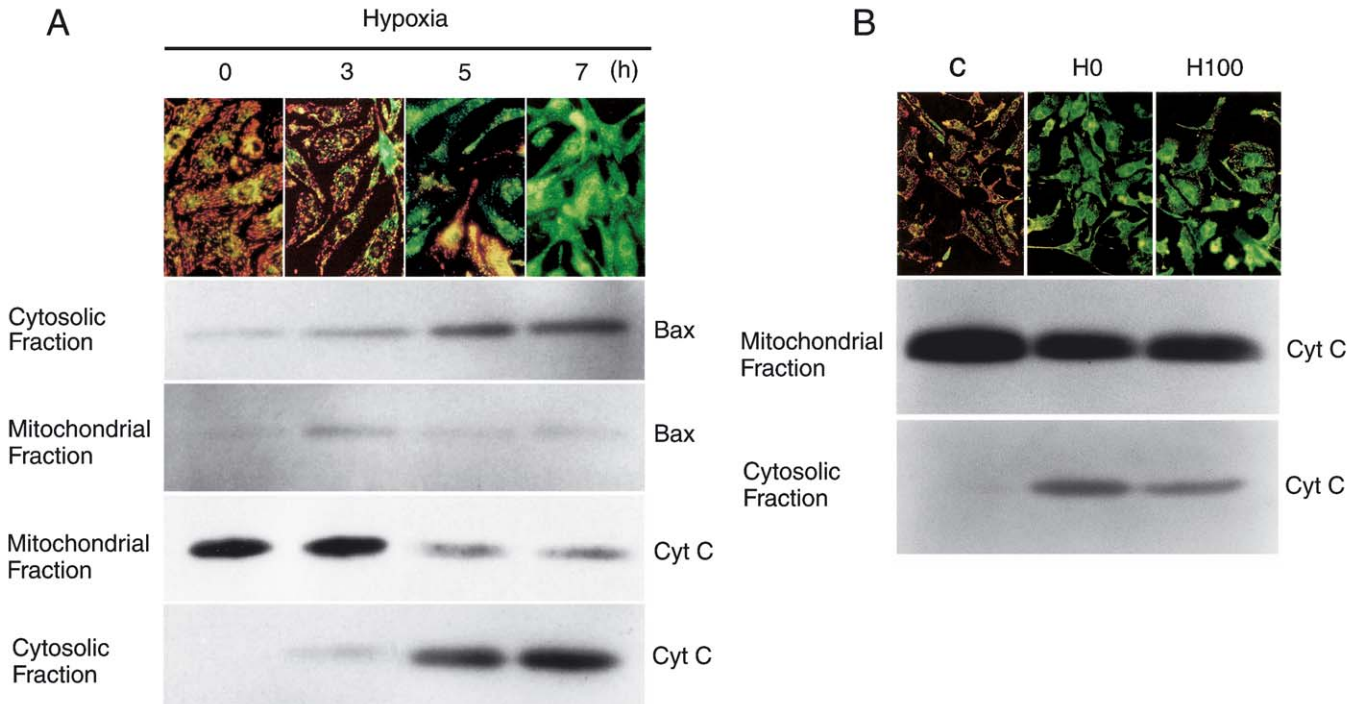

Fig. 8. (A) Time course of mitochondrial transmembrane potential, translocation of Bax and cytochrome c release. Myocytes were exposed to 7 h of hypoxia in the presence of 100 mg/dl of glucose, and were harvested at the given time intervals. Myocytes were stained with JC-1 as described in Methods, and fluorescence was monitored at 527 and 590 nm. Mitochondrial and cytosolic fractions were prepared, and aliquots containing 15 μg protein were subjected to Western blot analysis and were probed with antibodies for Bax and cytochrome c (Cyt C) as described in Methods. Results are representative of three independent experiments. (B) Loss of mitochondrial membrane potential and cytochrome c release from mitochondria. (A) Myocytes were exposed to 7 h of hypoxia in the presence of 0 mg/dl (H0) and 100 mg/dl (H100) of glucose, or normoxic conditions (C). The cells were then stained with JC-1 as described in Methods, and fluorescence was monitored at 527 and 590 nm. The mitochondrial and cytosolic fractions were isolated, and 15 μg of protein from each fraction was run on an SDS–polyacrylamide gel. Cytochrome c (Cyt C) was detected by means of Western blotting as described in Methods. Results are representative of three independent experiments.

To examine the effect of intracellular ATP content on the translocation of cytochrome c, a similar analysis was done in both glucose-depleted and glucose-supplemented cells (Fig. 8B). In normoxic control, cytochrome c was detected only in the mitochondrial fraction. However, after 7 h of hypoxia, cytochrome c was released from the mitochondrial fraction and simultaneously appeared in the cytosolic fraction. This effect was observed under conditions of both high (H100) or low (H0) ATP content.

3.7. Caspase-3 activation

Caspase-3 activity in myocytes exposed to 7 h of hypoxia and glucose-deprivation did not significantly increase, compared with that of the normoxic controls, as shown in the left panel of Fig. 9. However, the activity of caspase-3 was found to increase further with increasing concentrations of glucose concentration in the medium. For example, caspase-3 activity in myocytes subjected to 7 h hypoxia in the presence of 100 mg/dl glucose was increased by 3.5-fold over control. Oligomycin increased caspase-3 activity slightly but significantly when compared to 100 mg/dl glucose alone. The addition of an inhibitor of caspase-3, DEVD-CHO, during hypoxia suppressed the activity of caspase-3 to normoxic levels, confirming the presence of activated caspase-3 in our samples (data not shown). The activation of caspase-3 was also analyzed by Western blot. As shown in the right panel of Fig. 9, procaspase-3 (32 kD) was constitutively expressed in the myocytes. No cleavage of procaspase-3 was apparent in normoxic myocytes or in myocytes exposed to 7 h of hypoxia in the absence of glucose. However, significant cleavage of the procaspase and the concomitant production of the 17 kD active caspase-3 was seen in myocytes exposed to 7 h of hypoxia in the presence of 100 mg/dl glucose.

4. Discussion

The present study demonstrates that intracellular ATP levels play an important role in determining whether hypoxic cardiac myocytes die by necrosis or apoptosis. Using an in vitro model of cultured neonatal rat heart myocytes exposed to 7 h of hypoxia, we show that when the extracellular medium is depleted of glucose, the intracellular ATP levels drop precipitously and cell death occurs predominantly through necrosis, as confirmed by

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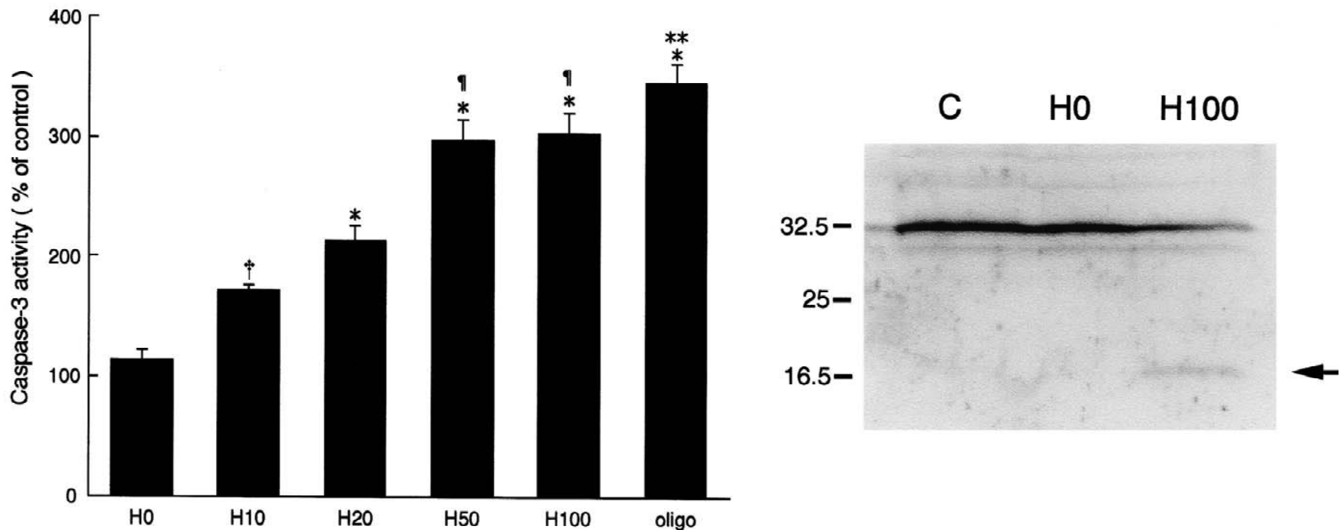

Fig. 9. Caspase-3 activation in cardiac myocytes. Left panel shows caspase-3 activity in myocytes exposed to 7 h of hypoxia. Myocytes were exposed to 7 h of hypoxia in the presence of 0 mg/dl (H0), 10 mg/dl (H10), 20 mg/dl (H20), 50 mg/dl (H50), 100 mg/dl (H100) of glucose, 100 mg/dl of glucose with 1×10^{-6} mol/l oligomycin (oligo), or incubated under normoxic conditions (C), and the activities of caspase-3 were determined as described in Methods. ($n=8$) Right panel shows Western blot analysis of caspase-3 activation. Myocytes were exposed to 7 h of hypoxia in the presence of 0 mg/dl (H0) and 100 mg/dl (H100) of glucose, or normoxic conditions (C). Cell extracts containing 100 mg protein were subjected to blot analysis as described in Methods. Results are representative of three independent experiments. *$P<0.0001$ vs. C; †$P<0.001$ vs. C; ¶$P<0.001$ vs. H20; **$P<0.05$ vs. H100.

the intense nuclear staining by ethidium that are indicative of loss of cell membrane integrity, as well as by the smearing observed in the DNA agarose gels that is characteristic of the random degradation of genomic DNA during necrosis. However, when glucose was added in increasing concentrations to the hypoxic medium, the level of intracellular ATP showed a corresponding increase, and cell death reverted gradually from necrosis to apoptosis. Eventually, with glucose concentrations at or above 50 mg/dl, the ATP levels in the hypoxic cells reached those observed in the normoxic controls. At that ATP concentration, cell death assumed predominantly apoptotic features, as illustrated by the activation of caspase-3, the appearance of distinctive fragmented nuclei, and the presence of characteristic DNA ladders in agarose gels which are indicative of apoptotic internucleosomal DNA fragmentation [21].

Our data therefore appear to confirm previous studies with other cell types that have shown a similar dependence of apoptosis on ATP [9,10]. However, such a correlation has not yet been shown in cardiac myocytes, although the available data strongly support the notion that ATP regulates apoptosis in cardiac myocytes [22]. In fact, although they are in general agreement with our current findings, recent studies that have addressed this question in cardiac myocytes have produced ambiguous data. For example, a recent study by Taimor et al., in which isolated adult rat cardiac myocytes were subjected to prolonged hypoxia in glucose-free medium, showed an 80% depletion of creatine phosphate and ATP accompanied by primarily necrotic cell death, in agreement with our data [23]. However, reoxygenated cells continued to die primarily by necrosis despite the restoration of normal creatine phosphate levels. The absence of an increase in apoptosis in this study may have been attributable to the apparent inability of the reoxygenated cells to re-synthesize ATP, which remained at 40% of control [23].

Another recent study by Malhotra and Brosius examined the effects of prolonged hypoxia on cultured neonatal rat heart myocytes and found that, even under conditions of glucose deprivation and presumed ATP depletion, cell death occurred primarily by apoptosis [24], an apparent conflict with the concept that ATP depletion promotes necrosis. However, this apparent discrepancy may be attributable to the fact that, in the Malhotra and Brosius study, 5% serum was included in the hypoxic glucose-deprived medium. It seems possible that the serum may have provided the cells with substrates for glycolytic ATP production which may have supported the apoptotic machinery. Since no determination of ATP content was done in the Malhotra and Brosius study, this possibility cannot be excluded at this time. Still another recent manuscript by Bialik et al. has shown that neonatal myocytes cultured in medium (RPMI 1640) deprived of glucose and serum show a depletion of ATP to 30% of control but nevertheless demonstrate extensive apoptotic cell death [25]. These data are surprising because the apoptosis occurred 24 h after the initiation of the metabolic inhibition, a much longer duration than our 7 h. However, is it important to note that this study maintained normoxic conditions throughout, and that neither this study, nor that of Malhotra and Brosius examined in a quantitative fashion the incidence of necrotic cell death. It is therefore entirely possible that necrosis may have contributed significantly to

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these observations. Finally, a study by Webster et al. has demonstrated that prolonged (72 h) hypoxia in the presence of glucose caused the depletion of ATP to 60% of control and resulted in approximately 11% apoptosis [26]. These data are in general agreement with ours. However, the level of apoptosis observed in our studies following comparable depletion of ATP (e.g. 4.3% apoptosis observed with 60% residual ATP) was predictably lower because of the shorter (7 h) period of hypoxia.

Although the ability of hypoxia to cause apoptosis in isolated cardiac myocytes has been demonstrated in a number of studies, the mechanism of this injury remains largely unresolved [27,28]. Apart from ATP depletion, hypoxia involves time-dependent changes in a variety of intracellular parameters such as energy levels and ionic balances. A number of possible hypoxia-mediated injurious pathways have been proposed recently, such as an increase in the expression of Fas mRNA and Fas protein [27,29], hypoxia-inducible factor-1α [30], Bax [31], interleukin-1β [32] or reactive oxygen intermediates [33]. Of particular relevance is the likelihood that an hypoxia-induced decrease in intracellular pH contributed to the incidence of apoptosis. The recent study by Webster et al. demonstrated that hypoxia-induced apoptosis in cultured cardiac myocytes was strongly promoted by a decline in cellular pH [26]. In fact, we have observed in the present study that the increased lactate production observed with increasing glucose content resulted in a significant decline in medium pH. Our data support the contention of Webster et al. [26] that this decline in pH promotes apoptosis, although the percentage of apoptosis induced by acidification was lower than that by hypoxia alone. In contrast, we have demonstrated that as the intracellular ATP levels were increased after MI, there was a corresponding and significant increase in the percentage of apoptotic myocytes induced by acidification treatment. In addition, we have confirmed that combination of acidification (pH 7.4–6.5) with 7 h of hypoxia did not increase any apoptosis under ATP-depleting conditions (data not shown). These evidences strongly suggest that intracellular ATP levels play a crucial role in the active execution of the apoptotic program, independently of cellular acidification, although there still remains the difficulty in separating ATP effect from pH effect on apoptosis under hypoxic conditions.

Our study shows that, in the presence of adequate glucose, ATP production can be maintained at normoxic levels even during hypoxia, or in the presence of the mitochondrial inhibitor oligomycin, as has also been shown by others [34]. Combined with our observation of a significant increase in lactate production during hypoxia, our data therefore indicate that the hypoxic myocytes fuel the apoptotic machinery solely by means of glycolytic processes, as has previously been demonstrated in other cell types [35]. Besides the absence of oxygen, another reason that the injured myocytes may be forced to rely on glycolysis for ATP production may be the mitochondrial permeability transition. Permeability transition, the opening of calcium-dependent pores in the inner mitochondrial membrane, is known to accompany hypoxic cell injury, and has been observed in both apoptosis and necrosis [36,37]. Recent reports have suggested that one of the triggers of mitochondrial permeability transition is translocation of Bax, a pro-apoptotic protein, from cytosol into mitochondria, and Bax may form selective channels for cytochrome c release [38]. In the present study, we have shown that hypoxia induced the time-dependent expression of Bax in the cytosol and the translocation of Bax into the mitochondria prior to the release of cytochrome c. The consequence of the permeability transition is the loss of matrix components, such as cytochrome c, which causes an uncoupling of the mitochondria and prevents the generation of ATP. Thus, our data demonstrate the translocation of Bax into the mitochondria, the loss of mitochondrial membrane potential and the concomitant release of cytochrome c into the cytosol of hypoxic myocytes, thereby confirming the occurrence of a mitochondrial permeability transition. Similar release of cytochrome c was observed previously in hypoxic myocytes in vitro [25] and in vivo [39]. Interestingly, it is the release of cytochrome c into the cytosol of hypoxic myocytes which may provide the link between ATP and myocyte apoptosis [22]. A number of studies have demonstrated that cytochrome c utilizes ATP or dATP to initiate the cascade of events leading to the activation of the caspases, including caspase-3, which are the enzymatic executioners of apoptosis [40,41]. The significant increase in caspase-3 activity observed by us in the apoptotic myocytes appears to confirm the involvement of this cytochrome c-dependent cascade in our model.

The implications of our data with regards to the origin and etiology of necrotic and apoptotic cell death in the ischemic heart are particularly intriguing. The notion that apoptosis and necrosis are distinct forms of cell death with different mechanisms is becoming less tenable. Instead, it is becoming increasingly likely that necrosis and apoptosis share the same initial mechanisms [6]. Data from our laboratory and others suggest that apoptosis is the preferred and early mode of cell death in injured myocardium, presumably because of its non-inflammatory nature [1–4]. It is therefore only when the cells become depleted of ATP, or when the apoptotic machinery is disabled through persistent injury, that apoptosis ceases and the cell dies by necrosis. The timing of this interruption of apoptosis presumably dictates the degree to which the dying cells display the characteristics of both necrosis and apoptosis. Our present data provide support for a common initial pathway for both apoptosis and necrosis. We show that the loss of mitochondrial membrane potential and cytochrome c release occurred in both the necrotic and apoptotic cells, indicating that mitochondrial permeability transition occurred prior to the bifurcation of the injured cells to apoptosis or necrosis.

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The clinical ramifications of a common mechanism for apoptosis and necrosis are likely to be profound. Our present data suggest that the apoptotic machinery in these cells was arrested during the ischemia-associated depletion of ATP, and that the subsequent replenishing of the cellular energy during reperfusion re-initiated the interrupted apoptotic process. Moreover, our data suggest that 'Reperfusion Injury', the postulated ability of reperfusion-associated phenomena to lethally injure myocytes at the onset of reperfusion, may in fact be an ATP-induced acceleration of apoptosis. On the other hand, several previous reports have indeed shown that ischemia results in a significant increase in TUNEL-positive myocytes in vivo. However, it is very difficult to compare such studies with our own tests, simply because no data have been presented to show the actual level of ATP in the cells or regions of the ischemic heart that showed either apoptosis or necrosis in vivo. Such determinations would be exceedingly difficult to perform in view of the inherent difficulty of correlating levels of microcirculation and ATP production in selected regions of the ischemic myocardium in vivo. Thus, there still remain some study limitations in our in vitro culture model systems, as compared with organ level investigations, and future investigation will be necessary to demonstrate our hypothesis in whole organ models.

In conclusion, our study has demonstrated for the first time a significant correlation between ATP content in hypoxic myocytes and the incidence of apoptosis in these cells. Although hypoxia involves many proapoptotic factors including acidification, our data suggest that intracellular ATP levels are an important determinant in the regulation of myocyte apoptosis, independently of cellular acidification. We further demonstrate that both apoptosis and necrosis appear to have a common mechanism, a fact which may have significant implications in the development of future therapies to combat the effects of myocardial ischemia.

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