Cardiomyocyte Apoptosis Induced in Langendorff Preparation of Isolated Guinea-Pig Heart Perfused with Krebs-Henseleit Solution Deprived of Glucose, with and without Oxygen Supply

Jie Tong, Yongtong Cao[(1)], Yoshihiro Hotta[(2)], Naohisa Ishikawa[(2)], Tomohiro Takeo, Haruaki Nishimaki, Kenji Masuda, Chao Long Yang, Kenji Kasai, Hiroshi Ikeda, and Gen Itoh

First Department of Pathology Second, (1) Department of Surgery and (2) Department of Pharmacology, Aichi Medical University School of Medicine, Japan

Abstract

Whether or not deprivation of glucose and oxygen can induce cardiomyocyte apoptosis was examined. Four groups of Langendorff preparations of guinea-pig hearts were perfused for 6 h with Krebs-Henseleit (K-H) solution deprived of glucose (group A), deprived of oxygen (group B), and deprived of both glucose and oxygen (group C), then examined for the mode of myocardial cell death. The control was perfused with K-H solution.

Results: In group A, myocardial cell death by apoptosis and necrosis was induced. In group C, the apoptotic lesion induced was exaggerated compared to that produced by glucose deprivation alone. In group A and C, decreases in intracellular ATP (ATPi) and intracellular acidosis were observed. In group B and the control, myocardial cells survived with spotted necrotic foci without myocardial-cell apoptosis. A mild decrease in ATPi was observed. However, intracellular pH did not shift to acidity (7.0-7.2).

Conclusion: Myocardial cell apoptosis was not induced by a 6-h stimulus of oxygen-deprivation (hypoxia) alone, but rather by a 6-h stimulus of glucose-deprivation with or without an oxygen supply. Deprivation of both glucose and oxygen intensified the apoptotic lesion. Apoptosis coincided with a decrease in the levels of ATPi and intracellular acidosis.

Key words: acidosis, apoptosis, ATP, deprivation of glucose and oxygen, Langendorff preparation, TUNEL.

Basic Appl Myol 11 (3): 143-150, 2001

It is well known that a significant portion of the myocardial cell-deaths induced by ischemia can be attributed to apoptosis in humans [18, 30, 32], rats and mice [12, 19], as well as cultured cardiomyocytes [13, 27, 41, 42]. It is controversial, however, as to which components of ischemia in vivo induce apoptosis, and what is the mechanism of the apoptosis induced by them. Numerous reports have been released concerning the effects of hypoxia [8, 27, 41] and hypoglycemia on cardiomyocyte-apoptosis [5, 29]. Hypoxia, one of the component-stimuli of ischemia in vivo induced apoptosis in cultured cardiomyocytes [8, 27, 41] via caspase activation and mitochondrial cytochrome c release during hypoxia-mediated apoptosis [8]. In contrast, apoptosis is not induced by ischemia (hypoxia) alone [43], but by a combination of hypoxia and glucose deprivation [29], or of hypoxia and acidosis or reoxygenation [43] in cultured cardiomyocytes, as well as in Langendorff-isolated mouse heart [43]. Thus, the numerous observations of hypoxia-induced apoptosis are inconsistent.

As for glucose, another component-stimuli of ischemia in vivo, many investigators have reported its ability to protect cardiomyocytes from ischemic or hypoxic injury, both in vitro and in vivo [4, 10, 29, 33, 35, 38]. However, only a few studies on apoptosis induced by hypoglycemia or deprivation of glucose have been reported [5, 29]. This suggests that apoptosis was induced by a combination of hypoxia and glucose deprivation (s), and that apoptosis occurred after serum/glucose deprivation in cultured rat cardiac myocytes through the activation of a mitochondrial apoptotic pathway (e), where cytochrome c is released from mitochondria. However, the role of hypoglycemia alone (deprivation of glucose) on the induction of apoptosis has not been documented.

It is well known that intracellular ATP (ATPi) levels are a determinant of the manifestations of cell death [11, 24,

25, 39]. If ATP is rapidly deleted, necrosis will occur [6, 16, 28, 36], whereas apoptosis is induced at reduced ATPi levels [11] under ATP-supplying conditions [7, 11].

Although a few studies using Langendorff-perfused heart preparations have indicated that ischemia accomplished by clamping the aortic cannula caused a decrease in ATPi content and intracellular acidity [22, 37], they did not mention whether or not apoptosis of cardiomyocytes occurred.

Intracellular acidity has been reported to be associated with apoptosis induced by hypoxia/reperfusion [13-15], to be a common characteristic of apoptotic cells [14, 20], and to precede the morphological changes characteristic of apoptosis [14]. Ischemic cardiac myocytes generate excess H^+ through increased anaerobic metabolism, net hydrolysis of ATP, and CO_2 retention [9, 20, 43]. Under conditions of hypoxia or ischemia, by using a culture of cardiomyocytes as well as by clamping the aortic cannula in Langendorff-perfused hearts, extra-cellular protons thus produced are not cleared, resulting in pronounced acidosis [43].

In this experimental system, we used Langendorff-isolated hearts, in which hypoxia and deprivation of glucose were accomplished not by clamping the aortic cannula but by the perfusion of K-H solutions deprived of oxygen and glucose, respectively. Thus, without any influence from the metabolites and ions accumulated in the intercellular space, the role of the induction of apoptosis could be investigated, and the combined effects of hypoxia and deprivation of glucose, in the induction of apoptosis could be examined.

The aim of the present study is to evaluate the respective effect of oxygen-deprivation and glucose-deprivation, and the combined effects of deprivation of oxygen and glucose on the mode of myocardial cell-death, in association with pHi and ATPi content. We provide evidence that hypoxia (deprivation of oxygen) alone does not induce apoptosis, but that deprivation of glucose causes apoptosis in the presence or absence of oxygen. In addition, apoptosis is associated with a low level of ATPi content and intracellular acidosis. Glucose deprivation associated with ATPi depletion and intracellular acidosis may induce apoptosis.

Materials and Methods

Animals

Hartley strain guinea pigs of either sex, weighing between 300-350 g, were used. Throughout the experiments, all animals were handled in accordance with the guidelines for animal experimentation set by the Japanese Association for Laboratory Animal Science.

Preparation of Langendorff-isolated guinea-pig hearts

The procedures for the model of guinea-pig cardiac injury induced by oxygen deprivation, glucose-deprivation, and deprivation of both were those described previously [17] In brief, Hartley strain guinea pigs of either sex were anesthetized with diethyl ether and heparinized (250 IU, i.p.). The heart was rapidly excised, and the aorta was cannulated. The Langendorff hearts were then perfused with Krebs-Henseleit (K-H) solution (pH 7.4, at 37°C) containing (in mM) NaCl 115, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 2.0, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 10. The K-H solution was saturated with a gas mixture containing 95% O_2 and 5% CO_2, and the heart was perfused at a constant pressure of 75 cm H_2O.

Coronary flow during NMR analysis was measured continuously with an in-line flow probe connected to an ultrasonic flow meter (transonic T101, Advance, NY, USA).

A latex balloon was inserted into the left ventricle via the left atrium and connected to a strain-gauge transducer (MIP-510, Baxter, Tokyo, Japan) for measurement of isovolumic left ventricular pressure (LVP). The left ventricular end-diastolic pressure (LVEDP) was adjusted to 10 mm Hg during the equilibration period in each heart, and the volume was unchanged during the experiments.

Experimental protocol

Three experimental groups of Langendorf-isolated guinea pig hearts were respectively perfused with the K-H solution deprived of glucose (group A) (n = 5), oxygen (group B) (n = 5) and both glucose and oxygen (group C) (n = 5). In each group, LVDP, LVEDP and ^{31}-P-NMR were measured. In group A and C, glucose was replaced by 2-deoxy-D-glucose (10 mM). In group B, the solution was saturated with 95% N_2, in place of O_2 at a constant pressure of 75 cm H_2O for NMR measurement.

Isolated guinea-pig hearts of the control group (n = 5), after being perfused with K-H solution for the equilibration, were continuously perfused with K-H solution for 6 h. The solution was pre-saturated with a gas mixture containing 95% O_2 and 5% CO_2, and the hearts were perfused with this solution. After perfusion for 2, 4 and 6 h, each group of hearts was fixed and embedded for light microscopic examination.

Macroscopic detection of myocardial cell death

Immediately after stopping the perfusion at 2, 4 and 6 h, 2% solution of TTC (Sigma Chemical Co.) was perfused retrogradely at 37°C for 5 min and perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered solution (pH 7.4) for 10 min. The heart was sliced transversely (2-3 mm thick), and the slices were immersed in fixatives for an additional 3-4 h at 4°C. Viable myocardium was stained red, and non-viable myocardium remained unstained with TTC [40].

Histological examination and assessment of internucleosomal DNA-cleavage by TUNEL method

The proximal portions of the isolated Langendorff hearts were sliced, fixed for 2 h at room temperature with phosphate-buffered solution containing 2%

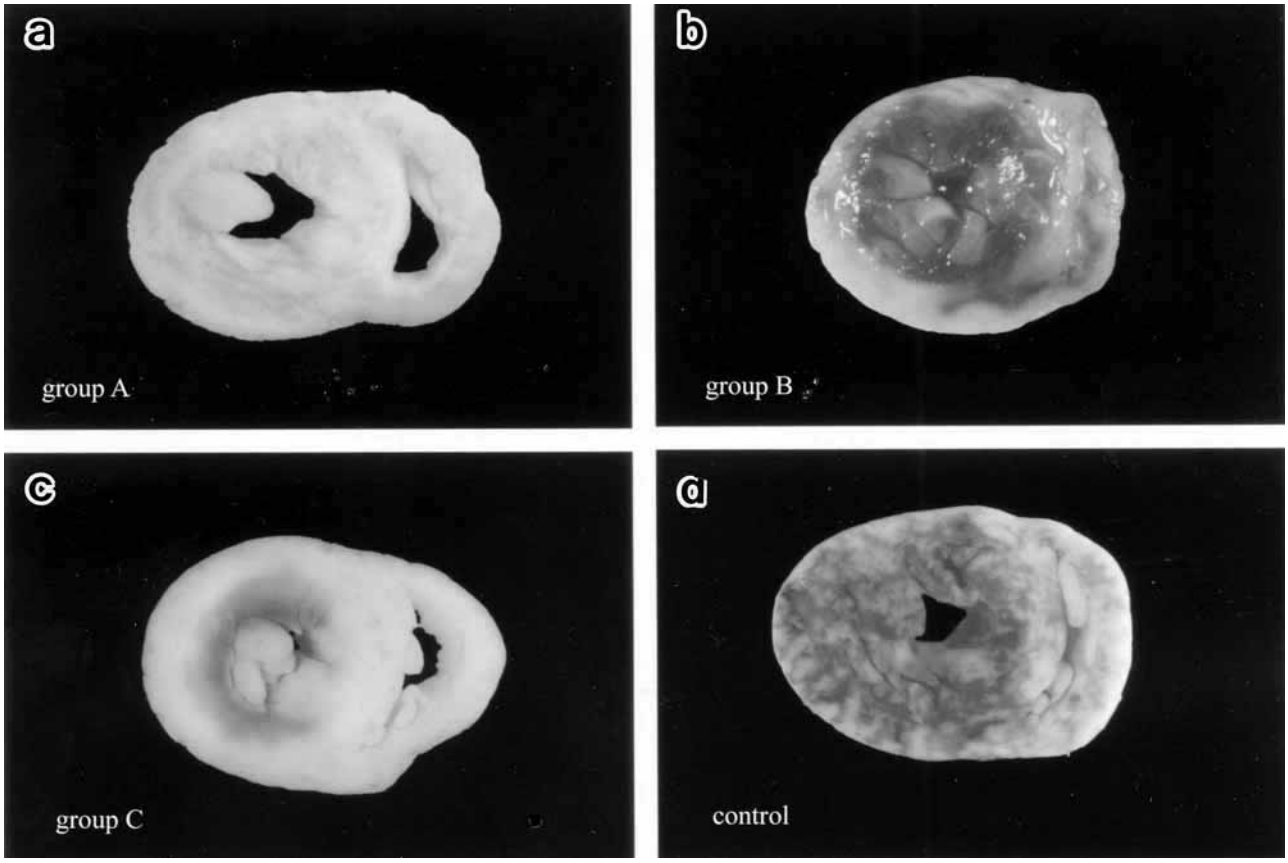

Figure 1. Cross-sections of hearts stained with TTC. a): Heart from group A. Total LVW is not stained with TTC. b): Heart from group B: Almost entire LVW area is stained red with TTC, leaving only the outermost zone of LVW nstained. c): Heart from group C: Total area of LVW is not stained with TTC, but a crescent-shaped area at the mid-portion of the free left ventricular wall is stained red with TTC. d): Heart from control group. Total area of LVW is stained red with TTC, except for multiple, unstained spotted foci

paraformaldehyde and embedded in paraffin for subsequent routine histological examination and the TUNEL method. A set of 2 sections of each sliced-heart was deparaffinized and stained with hematoxylin and eosin, and subjected to the TUNEL method.

Measurement of left ventricular developed pressure (LVDP)

LVDP indicating left ventricular contractility was calculated by subtracting the LVEDP from left ventricular systolic pressure. Myocardial temperature was maintained at 37 ± 0.5°C by a water-jacketed perfusion line and a continuous stream of air around the sample tube of NMR.

Measurement of ^{31}-P-NMR and data analysis

In all the perfusion experiments, ^{31}-P-NMR spectra were monitored along with simultaneous recording of ventricular pressure as described previously [17]. In brief, the heart connected to the Langendorff perfusion line was placed in a standard 20 mm NMR tube with the apex approximately 2.5 cm from the bottom of the tube, which was then inserted into the NMR coil. The effluent was removed from a level above the heart by a peristaltic pump, leaving the heart submerged in a fixed volume of the perfusate.

^{31}-P-NMR spectra were obtained at 161.8 MHz on a GSX 400 spectrometer (JEOL Datum Co. Ltd., Tokyo, Japan) equipped with a 9.4-tesla vertical-bore magnet. For each spectrum, 90 free-induction decays (4 min) were accumulated after 45-degree flip-angle pulses (18 μsec) by use of 4096 data points and 15.015 KHz spectral width at a repetition time of 2 sec. Accumulated free-induction decays were exponentially filtered, resulting in 30-Hz line broadening.

Measurement of ATP, PCr and pHi

PCr, Pi and β-ATP were quantified by comparison with a capillary tube of standard methylenediphosphonic acid (MDP, 0.25 M) fixed inside the NMR tube. Phosphate peaks expressed as percentages of control values were determined by measuring the area under each resonance peak, and the relative intensities of each peak were later used for quantitative analysis. Datum Station ALICE software (JEOL DATUM, Tokyo, Japan) was used to determine the area under each peak using a personal computer. β-ATP/Pi ratio was calculated.

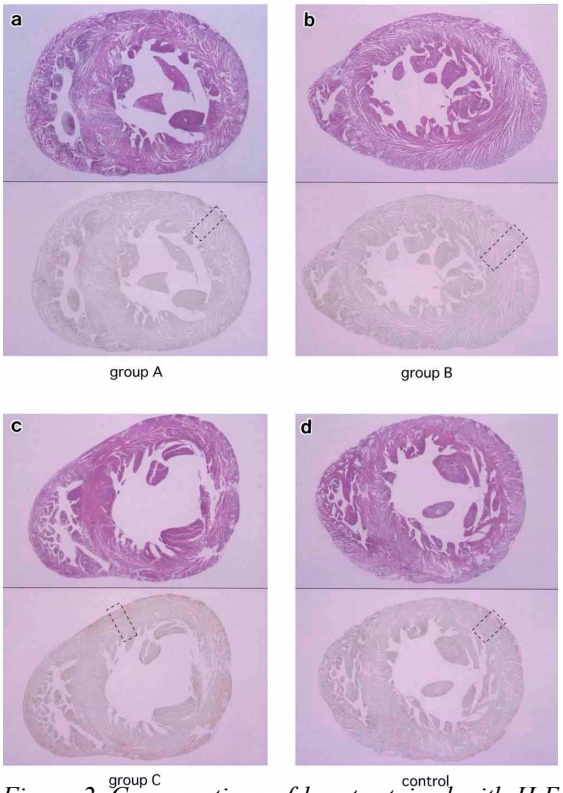

Figure 2. Cross-sections of hearts stained with H-E and TUNEL method. H-E (upper) and TUNEL (lower). Areas enclosed by square are observed at higher magnification illustrated in Fig. 3. a):Hearts from group A. b): Hearts from group B. c): Hearts from group C. d): Hearts from control group

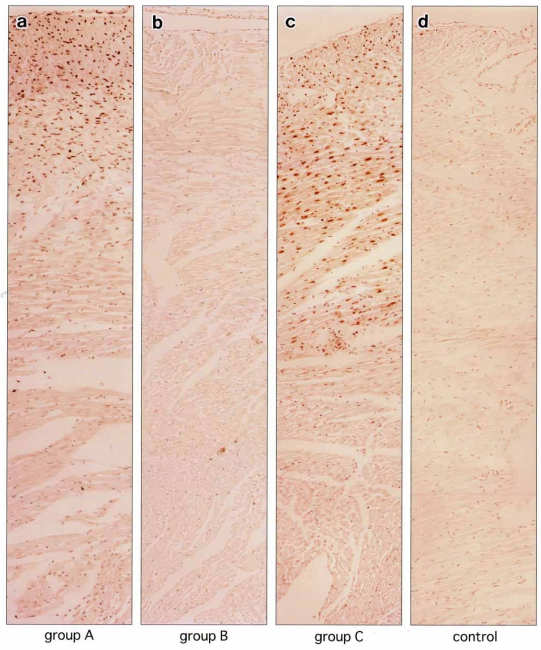

Figure 3. Higher magnification of areas enclosed with a square illustrated in Fig. 2. a): Group A: Around 1/3 of the outer portion of LVW is stained positive with TUNEL. b): Group B: Cardiomyocytes are negatively stained with TUNEL. c): Group C: Cardiomyocytes in outer two-thirds of LVW are stained positive with TUNEL. d): Control group: Cardiomyocytes are negatively stained with TUNEL

The intracellular pH (pHi) was calculated from the chemical shift between the phosphatecreatine (PCr) and inorganic phosphate (Pi) resonances using the following equation: $pH = 6.90 - \log((\delta 0\text{-}5.85)/(3.29\text{-}\delta 0))$ where $\delta 0$ is the chemical shift of Pi from PCr expressed as parts per million (ppm).

Statistical analysis

All data are presented as means ± SEM unless otherwise specified. Hemodynamic data of the perfused hearts of groups A, B, and C are expressed as a percentage of those of the control group. We analyzed NMR data at 4, 8, 12, and 30 min as well as 1, 2, 3, 4, 5, and 6 h after the onset of the respective perfusions. The unpaired *t* test or Dunnett's method was used for comparison of means between the groups. Statistical significance was defined as $p < 0.05$.

Results

Morphological detection of myocardial cell death and assessment of internucleosomal DNA-cleavage by TUNEL method

In group A, the total area of the cross section was negatively stained with TTC 6 h after initiation of perfusion (Fig. 1a), indicating myocardial cell death. By light microscopic examination, TUNEL-positive cardiomyocytes (apoptotic myocardial cells) were detected at the outer two thirds of the left ventricular wall, while myocardial cells unstained with TUNEL method (necrotic cardiomyocytes) were localized at the remaining inner zone of the left ventricular wall (Fig. 2a, 3a).

In group B, the total area was stained red with TTC at the cross section of the hearts with unstained narrow areas in the outermost zone of LVW (zone of cell death) (Fig. 1b). Using TUNEL, the myocardial cells corresponding to the area stained red with TTC were not stained with TUNEL, indicating that the unstained cells remain alive, while myocardial cells corresponding to those unstained with TTC were not stained with TUNEL, indicating they were necrotic (Fig. 2b, 3b).

In group C, the total area of the LVW was not stained with TTC, but only a small, island-like area at the midportion of the free left ventricular wall was stained red with TTC (Fig. 1c). Using TUNEL, the myocardial cells corresponding to those unstained with TTC at the outer portion (1/3-1/2) of the LVW were stained positive, indicating apoptotic myocardial cells, while those corrsponding to the subendocardial area of the LVW unstained with TTC were negatively stained by TUNEL, indicating ne-

crotic myocardial cells. The myocardial cells corresponding to the mid-portion of the free left ventricular wall stained red with TTC were not stained with TUNEL, indicating surviving myocardial cells (Fig. 2c, 3c).

In the control group, the total area of LVW was stained red with TTC with multiple, spotted unstained foci (Fig. 1d). The TUNEL method revealed that myocardial cells corresponding to those positively stained with TTC were negatively stained with TUNEL, indicating surviving myocardial cells. Myocardial cells corresponding to those of spotted foci unstained with TTC were negatively stained with TUNEL, indicating necrotic myocardial cells (Fig.-2d, 3d).

No guinea pig hearts of groups A, B, C or control were stained positive with TUNEL within 4 h after initiation of the perfusion-experiments.

Changes in left ventricular developed pressure (LVDP) and left ventricular end diastolic pressure (LVEDP)

LVDP and LVEDP were measured 4, 8, 12, 30 (min), 1, 2, 3, 4, 5 and 6 (h) after initiation of perfusion (Fig. 4a).

In group A, LVDP decreased to 73.5 ± 3.9% at 4 min, then decreased further to 1.0 ± 1.0% at 4 h and was undetectable at 5 h. In group B, LVDP decreased to 54.1 ± 2.1% at 4 min, and then decreased gradually to 19.1 ± 1.2% at 6 h. In group C, LVDP decreased dramatically to 8.3±2.8% at 4 min and then decreased to 3.3±0.3% at 12 min. LVDP was not detected thereafter. In the control group, LVDP decreased gradually to 60.4 ± 0.7% 6 h after initiation of perfusion with the K-H solutions.

Changes in ATPi content

Changes in ATPi content were monitored by measuring ^{31}-P-NMR spectra during the whole period of each experimental group. The β-ATP/Pi ratio was calculated. Time-sequential changes in β-ATP/Pi ratio of groups A, B, C and control shown in Fig. 4b. Four min after the initiation of perfusion, ATP content decreased to 89.4 ± 3.6% (group A), 87.8 ± 5.3% (group B) and 81.8 ± 4.9% (group C). In group C, however, ATP content decreased dramatically thereafter to 64.4 ± 5.6% (at 8 min), 53.8 ± 5.1% (at 12 min), 16.8 ± 1.3% (at 30 min) and 13.7 ± 4.2% (at 1 h). By contrast, in groups A and B, ATP content decreased gradually to 63.6 ± 6.4% (at 30 min), 32.8 ± 6.8% (at 1 h) and 16.1 ± 3.6% (at 6 h), (group A), and 85.2 ± 1.8% (at 30 min), 64.5 ± 3.0% (at 1 h), and 41.9 ± 2.6% (6 h), (group B). In the control group, ATP content was 92.3 ± 1.9% (1 h), 89.1 ± 1.9% (2 h), 85.4 ± 1.1% (3 h), 77.2 ± 2.2% (4 h), 73.5 ± 1.9% (5 h), and 72.6 ± 1.3% (6 h).

Changes in pHi

In group A, though the pHi shifted sharply to pH 6.3 at 1 h, it remained 6.2-6.4, thereafter. In group C, the pHi revealed a sharp shift from 7.0 to 6.2 during the first 12 min of the perfusion, and thereafter pHi remained be-

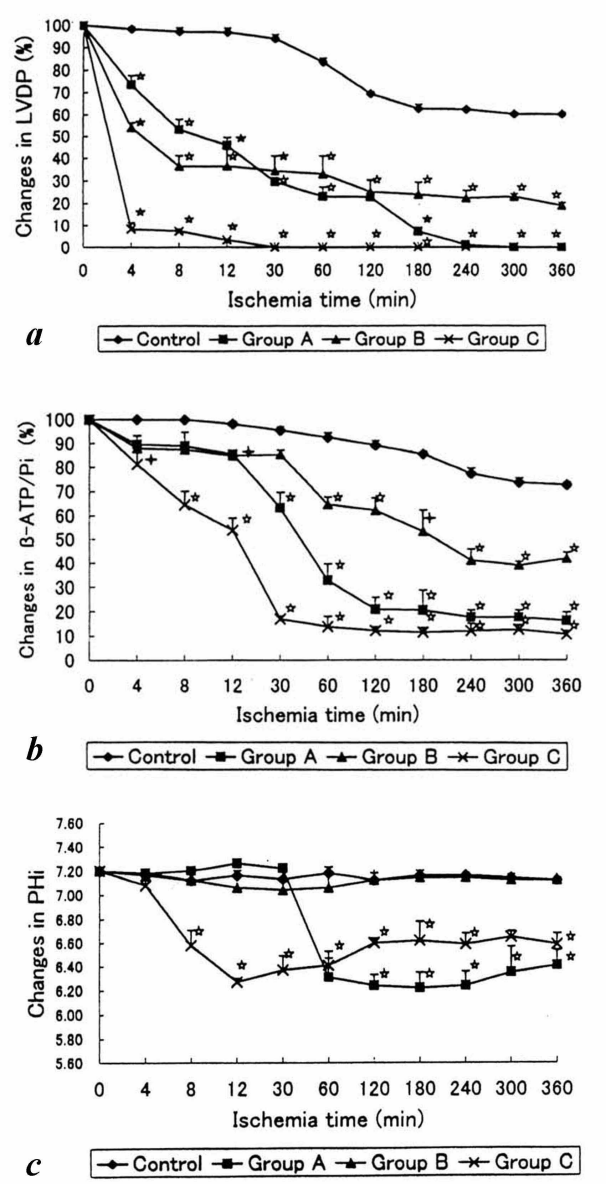

Figure 4. a: Changes in left ventricular developed pressure (LVDP). Undetectable LVDT began at 30 min in group C, and 240 min in group A after initiation of perfusion, while LVDP was detectable in group B even 360 min after initiation of perfusion. LVDP in control group remained above 60% 360 min after. b: Changes in β-ATP/Pi: β-ATP/Pi of groups A and C fell to less than 20% from 120 through 360 min after initiation of perfusions, while that of group B and control remained above 39% during the whole experimental period. c: Changes in intracellular pH (pHi). The pHi of groups A and C remained within 6.2 and 6.6 from 60 through 360 min after the initiation of perfusion, while pHi of group B and control remained between 7.0 and 7.2 throughout the experimental period.

tween 6.3 and 6.6, while in the groups supplemented with glucose (B and control), the pHi ranged within 7.0 to 7.2 in the presence or absence of oxygen (Fig. 4c).

Discussion

In the present study using Langendorff guinea-pig hearts, we demonstrated that apoptosis was not induced by oxygen-deprivation alone, but that the deprivation of glucose induced myocardial cell death mainly by apoptosis in the presence or absence of oxygen. The deprivation of both oxygen and glucose exaggerated the apoptotic lesion morphologically.

Glucose deprivation coincided with the decrease in ATPi content and intracellular acidosis in the presence or absence of oxygen.

Sakamoto et al. [37] and Koretsune et al. [22], using Langendorff heart preparations, have demonstrated a severe decrease in ATPi content and severe acidosis [22, 37]. However, these reports, in which ischemia was induced by clamping the aortic cannula have not dealt with the mode of cardiac myocyte-death, apoptosis or necrosis. Clamping the aortic cannula in these experiments did not clear the H^+ or the metabolites that had accumulated in the extracellular spaces, resulting in not only an accumulation of extracellular H^+ but a decrease in extracellular pH (pHo). Decreased pHo will be paralleled by a corresponding, or even greater, drop in pHi, causing intracellular acidosis [23, 43], that results in decreased production of intracellular ATP.

In our experiments, however, hypoxia and the deprivation of glucose accomplished under continuous perfusion prevented the accumulation of extracellular H^+ and metabolites, resulting in attenuating the ATPi depletion and the shift of pHi to acidity

The numerous observations of hypoxia-induced apoptosis are inconsistent. Some investigators have suggested that hypoxia induced apoptosis in cultured cardiomyocytes [5, 27, 29, 41]. However, others have indicated, in primary culture of rat cardiomyocytes, that apoptosis was not induced by hypoxia alone [29], but that hypoxia induced apoptosis in the absence of glucose [29] or that apoptosis was induced by a combination of hypoxia and glucose deprivation [29], or of hypoxia and acidosis or reoxygenation [43].

Hypoxia has been known to cause the intracellular inhibition of oxidative phosphorylation, a switch to glycolytic metabolism, and increased production of lactic acid, resulting in a lower intracellular pH [1, 2, 31] and a significant reduction in ATP levels [1,2,21,31]. Though little is known about the mechanism of hypoxia-induced apoptosis, apoptosis has been acknowledged to be induced at reduced levels of ATP under ATP-supplying conditions, while necrosis is induced under ATP-depleting conditions [11, 24, 25]. Accordingly, intracellular ATP levels determined whether cell death occurred by apoptosis or necrosis [11, 24, 25]. Intracellular acidosis associated with ATPi depletion has been shown to correlate with apoptosis in myocardial hypoxia/reperfusion [15, 20, 43], and to be a common characteristic of apoptotic cells [3, 14, 26, 34]. The morphological changes characteristic of apoptosis have been reported to be preceded by intracellular acidification [14].

ATPi content and pHi obtained from our experiments, however, did not reflect the respective levels of apoptosis and necrosis, nor the respective levels of necrosis and surviving, but rather the total levels of the mixed condition of apoptosis and necrosis, or necrosis and survival, or necrosis, apoptosis and survival. In group B of our experiments, where oxygen was omitted from a glucose supplement, myocardial cells survived. In group A, where glucose was omitted under oxygen supply, myocardial cell-death was by both apoptosis and necrosis. The findings obtained from groups A and B were, as far as ATPi content is concerned, compatible with those reported previously [3, 11, 14, 15, 20, 24-26, 34, 43]. In group C, however, deprivation of both glucose and oxygen, compared to glucose deprivation alone, induced severe ATPi depletion, which had been speculated to cause myocardial cell-necrosis, but in fact induced intensified lesions of myocardial cell-apoptosis with necrosis. These findings obtained from group C suggested that myocardial cell-apoptosis is induced by glucose-deprivation not via ATPi depletion alone, but by a combination of ATPi depletion and other factors, including pHi. They also suggested that oxygen deprivation, when it coexists with glucose-deprivation might intensify the apoptotic lesion produced by glucose deprivation alone.

A few reports have indicated that in cultured neonatal rats, glucose deprivation induced apoptosis in cardiac myocytes [5, 29]. Serum and glucose deprivation induced cardiac myocyte apoptosis via the mitochondrial death pathway, namely, the translocation of cytochrome c from mitochondria to the cytosol, coinciding with activation of both caspase-9 and caspase-3 [5]. Though these reports did not demonstrate data on ATPi levels or pHi, they undoubtedly indicated a mechanism of apoptosis-induction by glucose-deprivation.

Recent studies have suggested that glycolysis and oxidative phosphorylation play fundamentally different roles in energy-requiring processes; oxidative phosphorylation provides energy for force generation, whereas glycolysis preferentially maintains transmembrane ion gradients [44]. Decreased glycolysis and decreased ATPi are likely to induce a loss of transmembrane gradients of H^+, intracytoplasmic acidosis, and the destruction of the mitochondrial membrane system, all of which may serve as a death trigger during glucose deprivation by promoting the release of cytochrome c coinciding with the activation of caspase 9 and caspase 3 [5].

Though data in this study have failed to shed light on the signaling pathway and mechanisms of apoptosis, they

are compatible with the views that decreased ATPi levels and decreased pHi may contribute to apoptotic cell-death [15, 20, 43], and that glucose-deprivation causes myocardial cell apoptosis [5, 29] in the absence of oxygen [29]. In addition, our data support the hypothesis that disturbed glycolysis induces the destruction of the mitochondrial membrane system, thus serving as a death trigger by promoting the release of cytochrome c [5]. This is because glycolysis disturbed primarily by glucose deprivation might cause further destruction of the mitochondrial membrane system, thereby triggering a death signal.

However, the combined deprivation of glucose and oxygen induced an exaggerated apoptotic lesion. Why this combined deprivation of both glucose and oxygen exaggerated the apoptotic foci remains unclear. Further studies are needed to clarify this point.

Acknowledgment

The authors would like to thank Mr. M. Takeuchi, Mr. N. Igari and Mrs. Y. Matsubara for their skillful technical assistance, and Mr. T Miyake and H. Nakagome for their assistance with the photographs.

Address correspondence to:

Gen Itoh, M.D., Ph.D., First Department of Pathology, Aichi Medical University School of Medicine, 21 Karimata Nagakute-cho Aichi-gun Aichi-ken 480-1195, Japan, Email: genitoh@aichi-med-u.ac.jp.

References

[1] Allen DG, Orchard CH: Myocardial contractile function during ischemia and hypoxia. *Circ Res* 1987; 60: 153-168.

[2] Allen DG, Lee JA, Smith GL: The consequences of simulated ischemia on intracellular Ca^{2+} and tension in isolated ferret ventricular muscle. *J Physiol* 1989; 410: 297-323.

[3] Barry MA, Eastman A: Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch Biochem Biophys* 1993; 300: 440-448.

[4] Bekheit S, Isber N, Jani H, Butrous G, Boutjdir M, El-Sherif N: Reduction of ischemia-induced electrophysiologic abnormalities by glucose-insulin infusion. *J Am Coll Cardiol* 1993; 22: 1214-1222.

[5] Bialik S, Cryns VL, Miyata S, Wollowick AL, Srinivasan A, Kitsis RN: The mitochondrial apoptotic pathway is activated by serum and glucose deprivation in cardiac myocytes. *Circ Res* 1999; 85 (5): 403-414.

[6] Buja LM, Eigenbrodt ML, Eigenbrodt EH: Apoptosis and necrosis: basic types and mechanisms of cell death. *Arch Pathol Lab Med* 1993; 117: 1208-1214.

[7] Buja LM. Entman ML: Modes of myocardial cell injury and cell death in ischemic heart disease. *Circulation* 1998; 98: 1355-1357.

[8] de-Moissac D: Gurevich RM, Zheng H, Singal PK, Kirshenbaum LA: Caspase activation and mitochondrial cytochrome c release during hypoxia-mediated apoptosis of adult ventricular myocytes. *J Mol Cell Cardiol* 2000; 32 (1): 53-63.

[9] Dennis SC, Gevers W, Opie LH: Protons in ischemia: where do they come from, where do they go? *J Moll Cell Cardiol* 1991; 23: 1077-1086.

[10] Eberli FR, Weinberg EO, Grice WN, Horowitz GL, Apstein CS: Protective effect of increased glycolytic substrate against systolic and diastolic dysfunction and increased coronary resistance from prolonged global underperfusion and reperfusion in isolated rabbit hearts perfused with erythrocytes suspension. *Cir Res* 1991; 68: 466-81.

[11] Eguchi Y, Srinivasan A, Tomaselli KJ, Shimizu S, Tsujimoto Y: ATP-dependent steps in apoptosis signal transduction. *Cancer Research* 1999; 59: 2174-2181.

[12] Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL: Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 1994; 94: 1621-1628.

[13] Gottlieb RA, Gruol DL, Zhu JY, Engler RL: Preconditioning rabbit cardiomyocytes; role of pH, vascular proton ATPase, and apoptosis. *J Clin Invest* 1996; 97: 2391-2398.

[14] Gottlieb RA, Giesing HA, Zhu JY, Engler RL, Babior BM: Cell acidification in apoptosis: granulocyte colony stimulating factor delays programmed cell death in neutrophiles by up-regulating the vacuolar H(+)-ATPase. *Proc Natl Acad Sci USA* 1995; 92: 5965-5968.

[15] Gottlieb RA, Engler RL: Apoptosis in myocardial ischemia-reperfusion. *Ann N Y Acad Sci* 1999; 30: 874412-26.

[16] Hochachka PW, Buck LT, Doll CJ, Land SC: Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc Natl Acad Sci USA* 1996; 93 (18): 9493-9498.

[17] Hotta Y, Ando H, Takeya K, Sakakibara J: Direct measurement of increased myocardial cellular ^{23}NaNMR signals in perfused guinea-pig heart induced by dihydroouabain and grayanotoxin-I. *Moll Cell Biochem* 1994; 139: 59-70.

[18] Itoh G, Tamura J, Suzuki M, Suzuki Y, Ikeda H, Koike M, Nomura M, Jie Tong, Ito K: DNA fragmentation of human infarcted myocardial cells demonstrated by the nick end labeling method and DNA agarose gel electrophoresis. *Am J Pathol* 1995; 146: 1325-1331.

[19] Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Reed JC, Olivetti G, Anversa P: Apoptotic and necrotic myocyte cell deaths are independ-

ent contributing variables of infarct size in rats. *Lab Invest* 1996; 74: 86-107.

[20] Karwatowska-Prokopczuk E, Nordberg J A, Li HL, Engler RL, Gottlieb RA: Effect of vacuolar proton ATPase on pHi, Ca^{2+}, and apoptosis in neonatal cardiomyocytes during metabolic inhibition/recovery. *Circ Res* 1998; 82: 1139-1144.

[21] Kirshenbaum LA, Singal PK: Changes in antioxidant enzymes in isolated cardiac myocytes subjected to hypoxia-reoxygenation. *Lab Invest* 1992; 67: 796-803.

[22] Koretsune Y, Marban E: Mechanism of ischemic contracture in ferret hearts: relative roles of $[Ca^{2+}]i$ elevation and ATP depletion. *Am J Physiol* 1990; 258 (Heart Circ Physiol 27): H9-H16.

[23] Lazdunski M, Frelin C, Vigne P: The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *J Mol Cell Cardiol* 1985; 17: 1029-1042.

[24] Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P: Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* 1997; 21: 185 (8): 1481-1486.

[25] Leist M, Single B, Naumann H, Fava E, Simon B, Kuehnle S, Nicotera P: Inhibition of mitochondrial ATP generation by nitric oxide swtches apoptosis to necrosis. *Exp Cell Res* 1999; 249: 396-403.

[26] Li J, Eastman A: Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. *J Biol Chem* 1995; 270: 3203-3211.

[27] Long X, Boluyt MO, Hipolito ML, Lundberg MS, Zhen JS, O'Neill L, Cirielli C, Lakatta EG, Crow MT: p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiomyocytes. *J Clin Invest* 1997; 99: 2635-2643.

[28] Majno G, Joris S: Apoptosis, oncosis and necrosis; an overview of cell death. *Am J Pathol* 1995; 146: 3-15.

[29] Malhotra R, Brosius III FC: Glucose uptake and glycolysis reduce hypoxia-induced apoptosis in cultured neonatal rat cardiac myocytes. J Biol Chem 1999; 274: Issue 18; 12567-12575.

[30] Misao J, Hayakawa Y, Ohno M, Kato S, Fujiwara T, Fujiwara H: Expression of Bcl-2 protein, an inhibitor of apoptosis, and Bax, an accelerator of apoptosis, in ventricular myocytes of human hearts with myocardial infarction. *Circulation* 1996; 94: 1506-1512.

[31] Neely JR, Grotyohann LW: Role of glycolytic products in damage to ischemic myocardium. *Circ Res* 1984; 55: 816-824.

[32] Olivetti J, Quaini F, Sala R Lagrasta C, Corradi D, Bonacina E, Gambert SR, Cigola E: Anversa P. Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. *J Moll Cell Cardiol* 1996; 28: 2005-2016.

[33] Owen P, Dennis S, Opie LH: Glucose flux rate regulates onset of ischemic contracture in globally underperfused rat hearts. *Cir Res* 1990; 66: 344-54.

[34] Peres-Sala D, Collado-Escobar, Mollinedo F: Intracellular acidification suppresses lovastatin-induced apoptosis in HL-60 cells through the inactivation of a pH-dependent endonuclease. *J Biol Chem* 1995; 270: 6235-6242.

[35] Ramasamy R, Hwang YC, Whang-J, Bergmann SR: Protection of ischemic hearts by high glucose is mediated, in part, by GLUT-4. *Am J Physiol Heart Circ Physiol* 2001; 281 (1): H290-297.

[36] Reimer KA, Ideker RE: Myocardial ischemia and infarction: anatomic and biochemical substrates for ischemic cell death and ventricular arrythmias. *Human Pathol* 1987; 18: 462-475.

[37] Sakamoto K, Ishikawa M, Koga K, Urushidani T, Nagao T: Energy preserving effect of *l-cis* Diltiazem in isolated ischemic and reperfused guinea pig hearts: a ^{31}P-NMR study. *Jpn J Pharmacol* 2000; 83: 224-232.

[38] Schaffer, SW, Croft CB, Solodushko V: Cardioprotective effect of chronic hyperglycemia: effect on hypoxia-induced apoptosis and necrosis. *Am J Physiol Heart Circ Physiol* 2000; 278: H1948-1954.

[39] Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsujimoto Y: Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* 1996; 13 (1): 21-29.

[40] Takashi E, Aschraf M: Pathologic assessment of myocardial cell necrosis and apoptosis after ischemia and reperfusion with molecular and morphological markers. *J Mol Cell Cardiol* 2000; 32: 209-224.

[41] Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, Marumo F, Hiroe M: Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ Res* 1994; 75: 426-433.

[42] Umansky SR, Cuenco GM, Khutzian SS, Barr PJ, Tomei LD: Postischemic apoptotic death of rat neonatal cardiomyocytes. *Cell Death Differ* 1995; 2: 235-241.

[43] Webster KA, Discher DJ, Kaiser S, Hemandez O, Sato B, Bishopric NH: Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. *J Clin Invest* 1999; 104 (3): 239-252.

[44] Weiss JN, Lamp ST: Cardiac ATP-sensitive K+ channels–evidence for preferential regulation by glycolysis. *J Gen Physiol* 1989; 94: 911-935.