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Low-flow Ischemia and Hypoxia Stimulate Apoptosis in Perfused Hearts Independently of ReperfusionSilvia Pozzi¹, Giulia Malferrari², Ida Biunno² and Michele Samaja¹¹Department of Medicine, Surgery and Dentistry, University of Milan, San Paolo Hospital, ²ITBA-CNR Milan**Key Words**

Apoptosis • DNA fragmentation • TUNEL • Ischemia • Hypoxia

Abstract

Post-ischemic reperfusion leads to apoptosis-linked loss of myocytes in cultured cells and *in vivo*. We tested the hypothesis that apoptosis develops without reperfusion in Langendorff-perfused hearts exposed to either low-flow ischemia (LFI) or hypoxia (H). Rat hearts were perfused with aminoacid-enriched Krebs-Henseleit buffer and exposed for 6 h to LFI (flow=2 ml/min, PO₂=500±50mmHg, mean±SD), H (10ml/min, 120±15mmHg), or control conditions (C, 10ml/min, 500±50mmHg). At selected times, DNA-fragmentation was measured by agarose-gel electrophoresis and *in situ* TUNEL assay. After 6 h, the ratio (TUNEL-positive)/(total nuclei) was 0.620±0.027, 0.615±0.005, 0.404±0.021 in LFI, H and C, respectively. The ratio was 0.813±0.021 in hearts exposed to 90 min global no-flow ischemia and reperfused (5 h). To assess the role of membrane-diffusible factors, separate experiments were performed recirculating the medium and exposing hearts to LFI or H as above. The degree of apoptosis was the same in both the recirculating

and non-recirculating modes. Thus, apoptosis develops by similar extents and in a time-dependent fashion in crystalloid-perfused rat hearts during LFI or H at the same oxygen shortage (flow•PO₂), even without the reperfusion.

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Introduction

There is a raising interest for apoptosis in cardiovascular pathophysiology. Cardiac myocytes have not definitely lost their capacity to regenerate by mitosis, yet they can not compensate irreversible injury with tissue regeneration as other organs can do. Thus, although it is uncertain if beneficial [1], apoptosis plays an important role in cardiac disease [2]. At present, the oxidative stress associated to the reperfusion after ischemia is perceived to be the main signal triggering apoptosis in isolated or *in vivo* organs [3-10]. Nevertheless, there is some evidence that in isolated cardiomyocytes ischemia or hypoxia alone, without reperfusion, can trigger apoptosis [11-14]. However, there is no uncontroversial analogue in *in vivo* or *ex-vivo* situations. Mouse hearts develop

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www.karger.com/journals/netMichele Samaja
Università di Milano, Dipartimento di Medicina
Chirurgia e Odontoiatria, via di Rudini 8, I-20142 Milano (Italy)
Tel.+39-0289179250/2, Fax - +39-0289179251
E-Mail Michele.Samaja@unimi.it

apoptosis after 4 h coronary artery ligation in the correspondence of hypoxic regions, but independently of p53 [15]. Rat hearts subjected to 2.25 h coronary artery occlusion display apoptosis, but in the correspondence of the inflammatory response caused by neutrophils accumulation [16]. In isolated rat hearts, the translocation of phosphatidylethanolamine and phosphatidylserine, an early sign of apoptosis, develops since ischemia, but the classical signs of apoptosis are seen only after reperfusion [17]. Therefore, it is not yet clear if ischemia or hypoxia alone elicit apoptosis independently of the oxidative stress.

In this study, we test the hypothesis that apoptosis develops in Langendorff-perfused hearts exposed to either sustained low-flow ischemia (LFI) or hypoxia (H) before reperfusion or reoxygenation. Tissue hypoxia (low supply of O₂) is a component of ischemia (low supply of blood), but several of the mechanisms underlying the response to hypoxia and ischemia are different. In hypoxic cardiac myocytes, apoptosis is mediated by the decreased intracellular pH resulting from uncoupled oxidative phosphorylation and intracellular lactate release [14]. In embryonic stem cells, apoptosis is stimulated by mechanisms independent of hypoxia-inducible factor-1 α [18]. The transcription of the gene encoding Nip3, a pro-apoptotic member of the Bcl-2 family of cell death receptors, is increased in hypoxic cells [19]. Therefore, as hypoxia and ischemia share some common features, it may become important to differentiate the effects led by the two stresses, when applied for the same duration and at comparable degree of severity. We will show that apoptosis develops by similar extents and in a time-dependent fashion in crystalloid-perfused rat hearts during LFI or H at the same oxygen shortage (flow•PO₂) without the need of reperfusion.

Materials and Methods

Heart perfusion

Hearts were isolated and perfused by the Langendorff technique as previously described [20]. Briefly, *ad libitum* fed, male Sprague Dowley rats (250-280 g) were anesthetized with sodium thiopental (10 mg/100 g b.w.) plus heparin (500 IU/100 g b.w.). Excised hearts were mounted on the perfusion system kept at 37°C (ischemia time 15-30 s). The medium (Krebs-Henseleit buffer with 2 mM free Ca⁺⁺, 11 mM glucose, 10 mM HEPES, pH=7.40±0.02 at 37°C) was supplemented with aminoacids (3.5 mL/L Freamine III 8.5%, Clintec Nutrition Clinique, d'Amilly-Montargis, France) and oxygenated by bubbling with either 100% or 21% O₂. A roller pump (Gilson Minipuls 3, France) delivered

the medium to a 8 μ m pore size filter (Nucleopore Corp., Pleasanton, CA), a pre-heater and the aortic cannula. A pressure transducer (Harvard Apparatus model 52-9966, Natick, MA) between the pump and the aortic cannula provided the coronary perfusion pressure. The PO₂ was measured by an O₂ electrode (Yellow Spring Instruments Inc, Yellow Spring, OH).

Study design

Hearts were stabilized for 30 min at coronary flow=10 ml/min with 100% O₂ medium and were subdivided into four groups. Control hearts (C, n=3) were perfused for 6 h under the same conditions of the stabilization. Low-flow ischemia (LFI, n=6) was induced for 6h by decreasing the pump rate to 2 ml/min. Hypoxia (H, n=6) was induced for 6h by switching from the oxygenated (100% O₂) to the aerated (21% O₂) perfusion solution. Ischemia-reperfusion (I/R, n=3) hearts were exposed to global no-flow ischemia (90 min) by switching off the pump, and reperfused for 5 h under the same conditions of the stabilization.

In another set of experiments, the medium was recirculated by redirecting the venous outflow into the reservoir (total volume=800 mL). Hearts were stabilized and assigned to the C, LFI, or H groups (n=4 each) as above.

Biopsies

At selected times, samples were taken for apoptosis detection. Without switching off the pump, the heart apex was resected at about 6 mm from the apex, and dropped into liquid N₂. The specimen, cut into 0.2-0.3 g fragments, was transferred without thawing into sterile tubes and kept at -80°C until use. The remaining part of the heart, still on the perfusion apparatus, was injected through the aorta with 4% formalin (2 ml). Then, a circular 2-mm section perpendicular to the heart axis was done. The section was stored overnight in 4% formalin at ice temperature, and kept under 15% v/v sucrose at that temperature until use. To prevent false positives from long times in formalin [21], we limited formalin fixing to 12-16 h.

DNA extraction and agarose gel electrophoresis of DNA fragments

Myocardial tissue (about 100 mg) was minced, washed several times with cold saline and centrifuged, and the pellet recovered. Each pellet was mixed with 1 mL lysis buffer (7 M urea, 0.3 M NaCl, 10 mM Tris, pH 7.5), added with 1 mL 10% SDS and 100 μ g/mL proteinase K (Ambion-CelBio, Pero, Italy). After incubation (2 h, 55°C), an equal volume of phenol saturated with 1 M Tris at pH 8.0, chloroform and isoamyl alcohol (25:24:1) was added. The suspension was stirred at room temperature and centrifuged at 5000 g for 30 min. The aqueous phase was extracted again with chloroform and isoamyl alcohol (24:1 v/v). DNA was precipitated by adding 0.3 M NaCl and two volumes of 99.8% ethanol, collected by centrifugation at 5000 g for 30 min, and washed in 70% ethanol. The DNA solution was air-dried, re-suspended in 10 mM Tris, 1 mM EDTA and treated with 100 μ g/mL pancreatic RNase (Fluka Chemie, AG, Switzerland) for 1 h at 37°C. DNA concentration was measured by spectrophotometry at λ =260 nm assuming ϵ =0.02 mL/ μ g, and subjected to electrophoresis on 1.2-1.5% agarose gel in 89 mM Tris, 89 mM boric acid, 2 mM EDTA buffer. Ten μ g of DNA was loaded

Table 1. Arterial PO₂, coronary flow and O₂ supply (PO₂ • coronary flow • O₂ solubility coefficient) in the groups under study (mean±SD). The assumed value for the O₂ solubility coefficient was 1.4•10⁻⁶ M/mmHg. *, significant (P<0.05) difference from control.

Group	Arterial PO ₂ mmHg	Coronary flow ml/min	O ₂ supply μmoles/min/heart
Control	500±50	10.0±0.1	7.0±0.7
Low-flow ischemia	500±50	2.0±0.1*	1.4±0.1*
Hypoxia	120±15*	10.0±0.1	1.7±0.2*

together with 1μL of dye (0.25% w/v xilenol, 30% w/v glycerol) and stained with 0.5 μg/mL ethidium bromide. The gel was photographed under UV light. A standard constituted by a 100 bp DNA ladder (Amersham Pharmacia Biotech, Little Chalfont, UK) was used as size marker.

In-situ detection of DNA fragmentation

We used the *in situ* cell death detection kit Terminal-deoxynucleotidyl-Transferase (TdT) mediated dUTP Nick End-Labeling (TUNEL-POD kit, Roche Diagnostics, Switzerland). Formalin-fixed specimens were embedded in Optimum Cutting Temperature Compound (Bio Optica, Milan, Italy) and kept under freezing plate for solidification. Two 5 μm-thick cryosections were obtained from each specimen (Cryostate CM 1510, Leica, Germany). Sections were washed with PBS (2.7 mM KCl, 137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄), and incubated sequentially with 20 μg/ml proteinase K (Clontec Laboratories, Palo Alto, CA, USA) in 10 mM Tris at pH 8.0 (30 min at room temperature), 3% H₂O₂ in methanol to inhibit endogenous peroxidase (5 min at room temperature), 0.1% Triton X-100 in 3.4 mM Na citrate for permeabilization (2 min at 4°C), TUNEL reaction mixture (Roche Label solution and TdT) (60 min at 37°C, in a humidified chamber). Positive controls were obtained by incubating sections with 0.9 mg/mL DNaseI (Roche Diagnostics, Switzerland) diluted in 1mM MgSO₄ in PBS (20 min at 37°C) before addition of TUNEL reaction mixture. Peroxidase was revealed by 3,3'-diaminobenzidine (Histo Mark Black Peroxidase System, TPL, MD, USA). After staining, sections were counterstained with Meyer hematoxylin (Fluka Chemie, Switzerland), dehydrated through crescent alcoholic scale (80% and 96% ethanol; Fluka Chemie, Switzerland) and immersed 4 times in xylene (Fluka Chemie, Switzerland) for 5 min each. Negative controls were obtained by incubating sections with label solution without TdT.

Images

Both TUNEL- and hematoxylin-eosin-stained sections were analyzed using a light microscope (Zeiss Axiolab, Germany) at 40x magnification. The images obtained using two consecutive sections treated with the two staining procedures were first superimposed to assure that stained nuclei belong to cardiac myocytes. Sixteen images were randomly acquired per each section using a CCD Color Camera C4200 (Hamamatsu, Japan). Images were transferred to a PC using Image Grabber 24 (Neotech, Eastleigh, England). Stored images were analyzed with Adobe PhotoShop (Adobe Systems, San Jose, CA, USA) and adjusted using the autolevel function. The degree of apoptosis, or apoptotic index, was assumed proportional to the ratio (number of TUNEL-

positive) / (number of TUNEL-positive + number of hematoxylin-stained nuclei).

Statistics

Data are expressed as mean±SD. The significance level was P=0.05 (two-tailed). To detect significant differences among the three groups considered, we first performed one-way ANOVA test. If significant, the differences between selected pairs of data were tested using the Fischer comparison procedure (StatView, Abacus Concepts, Berkeley, CA).

Results

Low-flow ischemia and hypoxia

Table 1 shows arterial PO₂ and coronary flow under the various experimental conditions. The O₂ supply was calculated as flow•arterial PO₂•O₂ solubility coefficient in water (1.4•10⁻⁶ moles/l [22]). During LFI and H, the O₂ supply was 20% of control. There was no significant difference in O₂ supply between LFI and H.

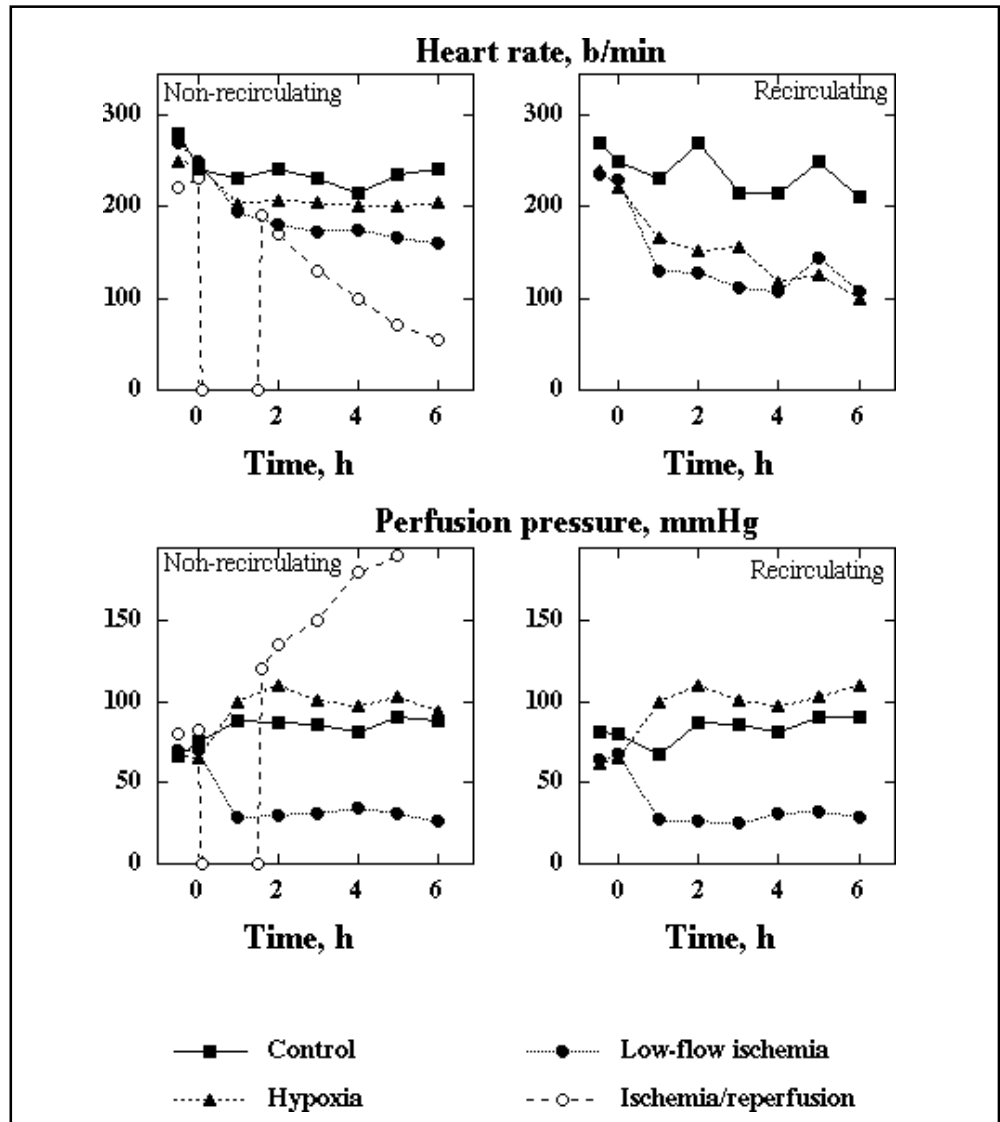
In control hearts, the heart rate did not change appreciably during 6 h perfusion (Figure 1). Either LFI or H acutely decreased heart rate, yet hearts kept contracting for the whole duration of LFI or H. In H hearts, heart rate was intermediate between LFI and C. Hearts exposed to I/R displayed progressive deterioration during the reperfusion.

In pump-perfused hearts, the coronary perfusion pressure is an index of vascular vasoconstriction (Figure 1). In control hearts, the coronary perfusion pressure increased slightly, but stabilized after 1 h. In H hearts, the pressure was higher than in C. In LFI heart, the pressure decreased proportionally to the lower flow rate. In both LFI and H, however, the perfusion pressure was maintained for the whole duration of LFI or H. In I/R hearts, however, steadily increasing perfusion pressure indicated performance deterioration.

Apoptosis in ischemic and hypoxic hearts

Apoptosis is characterized by cleavage of genomic DNA into fragments of 180 bp or multiple, which can be detected as DNA ladder by gel electrophoresis. To detect

Fig. 1. Myocardial dynamics in the various groups of hearts examined in this study (n=2-4/group). The left and right panels describe hearts perfused in the open and closed circuit, without and with medium recirculation, respectively. The S.D. (not shown for clarity) averaged $\pm 10\%$.



DNA laddering, high molecular weight DNA was extracted from frozen cardiac tissue at selected times during the experiments. However, in hearts perfused for 6 h under control, LFI or H conditions, DNA fragmentation was barely visible (Figure 2).

In situ detection of DNA fragments by the TUNEL assay yielded the results shown in Figure 3. This method is based on the new 3'-OH DNA ends generated by DNA fragmentation. In contrast, normal nuclei, with relatively low number of DNA 3'-OH ends, were not stained. The images obtained from two consecutive sections treated with the two staining procedures (TUNEL- and hematoxylin-eosin) were superimposed and analyzed to obtain the apoptotic index. This value increased almost linearly with time in hearts exposed to either low-flow ischemia or hypoxia. In contrast, it remained essentially

constant in control hearts (Figure 4). Hearts exposed to ischemia-reperfusion displayed a higher apoptotic index than LFI and H hearts ($P < 0.05$).

Medium recirculation

In separate experiments, we aimed at assessing whether substances released from ischemic or hypoxic hearts affect the degree of apoptosis. Recirculating the medium could address this question at the expense of greater function deterioration, as evident from faster decrease in heart rate and increase in coronary perfusion pressure (Figure 1). Nevertheless, the apoptotic index was unchanged with respect to data obtained without recirculation. The recirculating setup, although finally leading to heart arrest, typically for $t > 9$ h (not shown), allowed to monitor H^+ release. The medium pH was

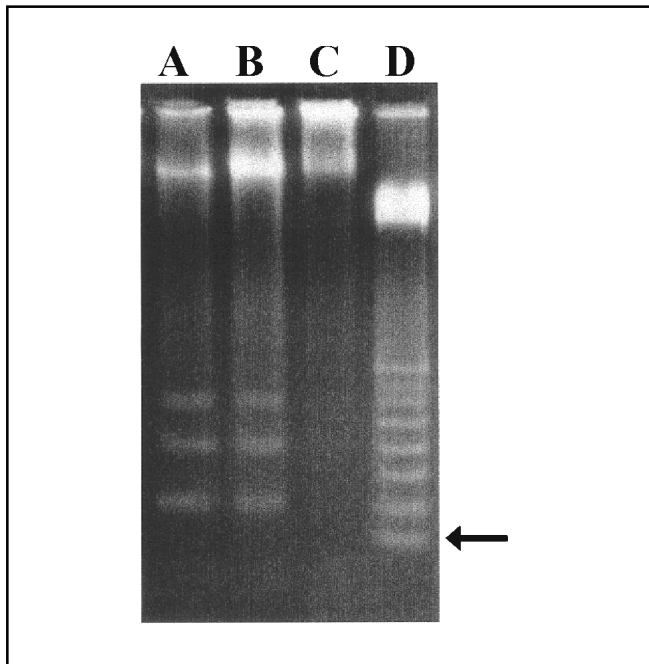


Fig. 2. Agarose gel electrophoresis of myocardial DNA. All lanes were loaded with 10 μ g DNA together with 1 μ L of dye and stained with 0.5 μ g/mL ethidium bromide. The gel was photographed under UV light. A standard constituted by a 100 bp DNA ladder (Amersham Pharmacia Biotech, Little Chalfont, UK) was used as size marker. Lanes are as follows (from left to right): hearts exposed for 6 h to low-flow ischemia (A), hypoxia (B), control conditions (C), standard fragments (C) constituted by a 100 bp DNA ladder (Amersham Pharmacia Biotech, Little Chalfont, UK), with arrowhead indicating 100 bp.

checked every 30 min and titrated to 7.4 with 1M NaOH. The total amount of base, equivalent to H^+ release by the heart, was assumed as an index of myocardial energy expenditure (Figure 5), which was more marked for H than LFI.

Discussion

No single model represents perfectly the cell death signalling properties in adult ventricular myocytes [23]. Transformed cell lines are defective at one or more control points. Actively proliferating myocytes from embryonic sources are more vulnerable than non-cycling cells. Primary cultures of neonatal and adult rat ventricular myocytes lack contractile activity and cell-matrix communication. Thus, establishing a model of apoptosis in isolated organs is important because it represents a com-

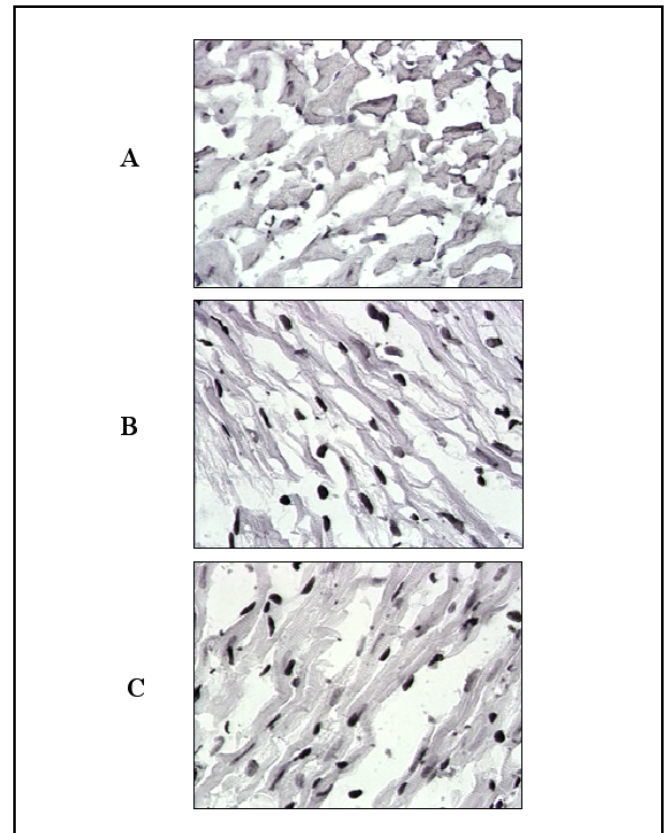


Fig. 3. Staining of myocardial sections by *in situ* TUNEL assay in control hearts (A), hearts exposed to 6 h low-flow ischemia (B), and hearts exposed for 6 h to hypoxia (C, from top to bottom). TUNEL-positive nuclei are visualized as brown, with hematoxylin staining used for counting total nuclei. Original magnification x40.

promise between the simplicity of cultured cells and the complexity of *in vivo* organs. Also, the metabolic interrelationships are intact, and pathophysiological stimuli are applied independently of the neuro-hormonal system. Finally, the control of dynamic and metabolic variables and the access to biochemical, physiological and morphological techniques are optimal.

Lack of lymphocytes in the perfusion media prevents phagocytosis of apoptotic cells and suppresses inflammatory responses. The prolonged permanence of apoptotic cells might explain in part their large number. Lack of red cells decreases total O_2 supply, thereby potentially inducing hypoxia. However, high PO_2 and coronary flow led O_2 supply in controls to compare well with 9-10 μ moles/min/g reported for normally perfused hearts [24]. Although not fully reflecting performance, heart rate, a good index of myocardial viability, fell markedly in association with O_2 supply reduction.

Fig. 4. Time-course of the ratio (number of TUNEL-positive) / (number of TUNEL-positive + number of hematoxylin-stained nuclei) in hearts exposed to control conditions, low-flow ischemia, hypoxia, and global no-flow ischemia followed by reperfusion (no medium recirculation). This ratio increased almost linearly with time in heart exposed to either low-flow ischemia or hypoxia. In contrast, it remained essentially constant in control hearts. Hearts exposed to ischemia-reperfusion displayed higher ratio than hearts exposed to low-flow ischemia or hypoxia ($n=2-4$ /point, the bar represents S.D.).

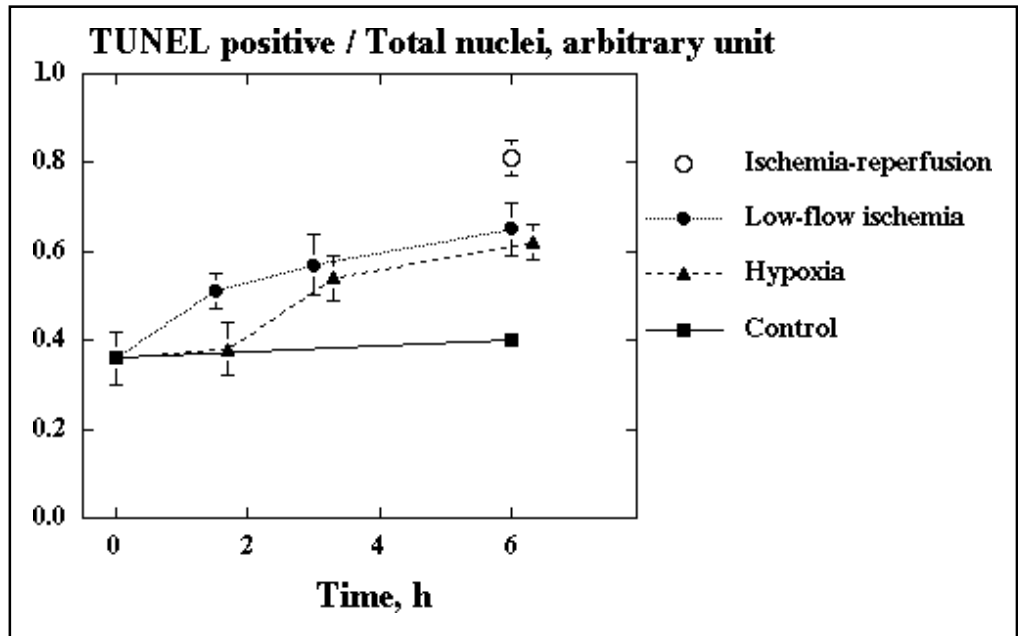
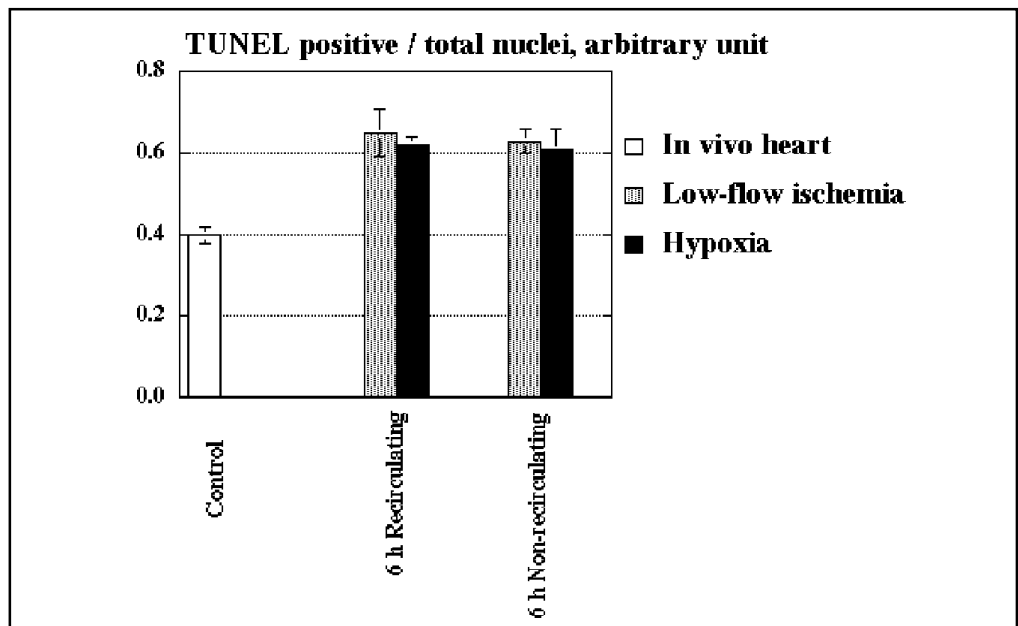


Fig. 5. Ratio (number of TUNEL-positive) / (number of TUNEL-positive + number of hematoxylin-stained nuclei) in, from left to right, control hearts, and hearts exposed to either low-flow ischemia or hypoxia in the recirculating and non-recirculating mode. The bar represents the S.D. ($n=2-4$ /group).



The heart rate fall was more pronounced in the recirculating than non-recirculating experiments. This might be due either to glucose depletion or to lactate accumulation in the medium. The maximal glucose utilization in anoxic hearts ($15-20 \mu\text{moles/g}_{\text{dw}} \cdot \text{min}$, or $1.95-2.60 \mu\text{moles/g}_{\text{ww}} \cdot \text{min}$ [25]) corresponds to 0.7 millimoles uptake during a 6h perfusion (average heart weight 0.95g). The glucose concentration in the recirculating medium (total volume= 800mL) would therefore decrease from 11 to 10.1mM , which is irrelevant when compared with the K_M of hexokinase for glucose ($<0.1\text{mM}$). In

contrast, lactate concentration would increase to about 2mM , and extracellular lactate is known to depress cardiac function [26].

DNA fragments visualization, although a late event not always occurring in the presence of apoptosis [27], is nevertheless a hallmark of apoptosis. The TUNEL technique has been criticized for lack of standardization and uncritical use and interpretation of data [28]. In addition, TUNEL-positivity often reflects a range of cellular conditions wider than apoptosis only [29]. Nevertheless, this technique is one of the most used to monitor apoptosis

in the myocardium. As the only variable is heart exposure to LFI or H of varying duration, the apoptotic index is intended relative to the effects exerted by the stresses rather than the true expression of apoptotic cells number [30]. Webster et al showed that H triggers apoptosis only when combined with acidosis [14], but in the present study with perfusion medium pH maintained constant by titration, the degree of apoptosis did not change between LFI and H. Thus, acidosis was not needed for apoptosis in this model.

After excluding studies with cultured cells, most investigations into the effects of ischemia on apoptosis concerned hearts exposed to global no-flow ischemia. Yet, as ischemia reflects a situation where the energy supply does not meet the demand [31], the most common condition related to ischemia involves restricted, rather than abolished, coronary flow.

Comparing the derangement caused by LFI and H helps addressing the mechanisms underlying apoptosis in myocardial tissue. Despite matched O₂ supply, heart rate and H⁺ release were higher in H than LFI hearts. This is consistent with previous observations indicating myocardial down-regulation during LFI but not during H, perhaps for the low coronary flow that decreases the washout of membrane-diffusible factors, including lactate, which depresses cardiac function [32]. Myocardial down-regulation during LFI implies preservation of high-energy phosphates [33], improved recovery [34] and greater tolerance to sustained O₂ supply reduction [35]. Despite such critical differences, the degree of apoptosis

was similar in LFI and H. The degree of apoptosis is therefore apparently independent of the metabolic alteration extent, and is triggered by non-diffusible factors belonging to common paths in LFI and H. One of these is tissue hypoxia, which may represent an important first messenger of apoptosis that acts independently of the oxidative stress originated from the reperfusion of ischemic tissue.

Conclusion

The Langendorff-perfused heart is a suitable model to study apoptosis and to test various hypotheses on apoptosis insurgence during or after ischemia or hypoxia. Hearts exposed to 1-h ischemia plus reperfusion display a relevant degree of apoptosis, in agreement with several reports. But low-flow ischemia and hypoxia without reperfusion also stimulate apoptosis, although to a lesser extent. As apoptosis was independent of medium recirculation, and LFI and H were applied at the same O₂ supply, yet with different coronary flows, the shortened O₂ supply, rather than the bioenergetic derangement or the oxidative stress, appears a key trigger of apoptosis.

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