

Contribution of Myocyte Apoptosis to Myocardial Injury in an *in Vivo* Rabbit Preparation of Ischemia-Reperfusion

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Abstract

Objective: Exposure of the heart to repeated, brief episodes of coronary occlusion/reperfusion prevents lethal myocyte injury. Necrosis and apoptosis, two seemingly distinct mechanisms of cell death caused by ischemia could contribute independently to progressive loss of myocardium. Studies suggest that ischemic conditioning (IC) lessens myocyte injury by decreasing apoptosis. The goal of this study was to examine cell death in rabbit hearts subject to ischemia-reperfusion injury without (nIC) or with pretreatment by IC. **Methods:** In the control study, anesthetized, male rabbits (n = 4/group) underwent 30-min regional coronary occlusion (CO) and either 3, 6 or 24 h reperfusion (REP). In the IC study, rabbits were pretreated by IC (2 cycles of 5-min CO and 10-min REP) before 30-min CO and subsequent REP. Additional groups were evaluated with 60, or 120-min CO followed by up to 96 h REP. Agarose electrophoresis was used to detect DNA laddering and poly (ADP-ribose) polymerase (PARP; chromatin bound nuclear DNA repair enzyme) was assessed in myocardial biopsies. **Results:** Genomic DNA from nIC and IC hearts showed no oligonucleosomal fragmentation. In addition, we did not detect any cleavage of PARP; however, myocardial PARP levels decreased when CO and REP durations were prolonged. **Conclusion:** Absence of genomic DNA fragmentation or PARP cleavage in an *in vivo* preparation of ischemia-reperfusion injury does not support the view that apoptosis contributes markedly to post-ischemic tissue necrosis.

Keywords

Ischemia, Reperfusion, Ischemic Conditioning, Necrosis, Apoptosis, DNA Fragmentation, PARP Cleavage, Rabbits

1. Introduction

Protection of injured cardiomyocytes, the most abundant cardiac cell type in the heart, remains a major goal of therapeutic interventions in response to ischemic injury. Tolerance of cardiac cells to injury varies in relation to metabolic requirements and activation of cellular death pathways for necrosis, apoptosis (*i.e.* programmed cell death) and autophagy. These modes of cell death are regulated by pathways that share initiator and effector molecules and are activated within common sub-cellular sites and organelles [1] [2]. Myocyte necrosis is a major contributor to pathogenesis of myocardial infarction and consequent heart failure [3] [4]. Defining features of necrosis include severe depletion of high-energy phosphates (HEP), disruption of plasma and organelle membranes, cell swelling and inflammation [5]. Apoptosis is a major mechanism for regulated cell death and is crucial for normal cellular development and morphogenesis. Triggers can be either extrinsic via cell surface death receptors, or intrinsic via mitochondrial signaling pathways [6] [7]. Direct involvement of mitochondria is characterized by production of reactive oxygen species, HEPs and activation of poly-[ADP-ribose]-polymerase (PARP) which functions as a molecular switch to regulate cellular HEP levels [8]. Apoptosis is primarily characterized by cellular shrinkage and DNA fragmentation into small apoptotic vesicles that are phagocytosed [9]; a clear distinction from necrosis is the absence of inflammation. Inhibition of apoptosis genes (*i.e.* Bcl-2, p53, etc.) with pharmacologic or non-pharmacologic interventions could protect against ischemic injury [10] [11]. Finally, autophagy is a well-controlled process that necessitates engulfment and destruction of cytoplasmic material and intracellular organelles by autophagosomes [12]. It plays an essential role in development, tissue homeostasis and disease and has been described to have both cytoprotective and cell degradative functions depending on the physiological environment [13] [14]. Strategies targeting specific aspects of the cell death cascade (necrosis, apoptosis, autophagy) are needed to limit negative outcomes in patients.

To this end, pretreatment by brief, repetitive, periods of acute myocardial ischemia and reperfusion, prior to a prolonged ischemic event (commonly known as ischemic conditioning (IC)), has been documented to markedly delay development of myocyte necrosis in pre-clinical experiments and in humans [15] [16] [17] [18]. IC-mediated protection occurs in two distinct phases, the initial phase is mediated by a host of receptor signaling pathways [19] [20] while the later phase requires transcription of proto-oncogenes, genes related to antioxidant production and stress-related genes [21] [22]. IC also reduces the incidence of ventricular dysrhythmias and potentially affects post-ischemic LV contractile dysfunction. Whether IC reduces ischemia-mediated apoptosis remains to be established [23] [24].

The goal of the present study was to test the hypothesis that IC delays irreversible cellular injury by decreasing apoptosis in our *in vivo* rabbit preparation of ischemia-reperfusion injury. On the basis of our results, we cannot confirm

that IC delays tissue necrosis by limiting apoptosis but suggest that necrosis is the major contributor to cell death during acute myocardial infarction.

2. Materials and Methods

Animals were maintained in accordance with the policies and guidelines of the Canadian Council on Animal Care. The institutional animal care committee at Laval University approved experimental and surgical protocols.

2.1. Animals

Pathogen-free, healthy New-Zealand White male rabbits (2 - 3 Kg body weight; 11 - 12 months of age) from Charles River Laboratories (Québec, Canada) were used. Animals were maintained in single cages under conditions of constant temperature and humidity and kept on a strict 12:12 h dark-light cycle; food (comprising 17.7% crude protein, 3.3% fat and 13.7% crude fiber; (Harlan Laboratories Inc., Montreal, QC) and water were accessible ad libitum. Experiments were carried out, and results reported as described in the ARRIVE guidelines [25].

2.2. Surgical Preparation

Rabbits were pre-medicated with acepromazine (0.5 mg/Kg, IM) and subsequently were anesthetized with pentobarbital sodium (25 mg/Kg, IV). Body temperature was monitored during the experiment with a rectal temperature probe; core temperature was maintained with warming pads placed beneath the animal. After intubation rabbits were ventilated with a positive-pressure small animal ventilator (MDI Inc., Mobile, AL) to maintain arterial blood gases within the physiological range. Standard limb leads of the ECG allowed monitoring of heart rate. Surgical interventions were done under sterile conditions. A left thoracotomy was performed, the heart exposed and a silk suture (3-0; Ethicon, Somerville, NJ) was placed beneath an anterolateral branch of the left main coronary artery midway between the base and apex. The end of the suture was drawn through a polypropylene tube to form a snare for coronary occlusion (CO). Ischemia was confirmed by regional epicardial cyanosis and ST segment elevation of the ECG. Reperfusion (REP) of the infarct-related artery was confirmed by hyperemic blushing of the ischemic region.

2.3. Experimental Protocol

Rabbits were randomly assigned to different groups (22 groups with 4 rabbits per group). Rabbits underwent: 1) no CO or REP (sham group), 2) 30-min CO followed by 3, 6 or 24 h REP (24 rabbits), 2) 30-, 60- or 120-min CO followed by 1, 48 or 96 h REP (64 rabbits). In non-IC (nIC) groups a 20-min wait period was allowed prior to onset of CO; in IC groups, rabbits were exposed to two cycles of 5-min CO and 10-min REP. Heparin or lidocaine were not administered because of their potential cytoprotective actions [17] [18]. Hearts that developed ventri-

cular fibrillation were cardioverted by gentle flicking of the left ventricle (electrical cardioversion was not used) and if unsuccessful, the animal was excluded from the study. At the conclusion of CO-REP (rabbits undergoing 24, 48 or 96 h reperfusion), the chest was closed in layers—pneumothorax was avoided with the use of negative pressure. Rabbits were allowed to recover from anesthesia and subsequently extubated. After REP (for the specified period) rabbits were anesthetized and subsequently killed with an overdose of sodium pentobarbital; the heart was immediately excised, placed on a Langendorff perfusion system (at 65 mm Hg) and perfused with saline to wash out blood from coronary vessels. The infarct-related vessel was re-occluded and Monastryl blue (4%; Sigma Chemical Co., U.S.A.) was injected into the aorta to identify the risk region. Atria were excised and the heart was divided into, 1) basal (non-ischemic region), 2) above (3 mm thick section 1.5 mm above occlusion site parallel to atrioventricular groove) and 3) apical (ischemic zone) sections. Ischemic and non-ischemic myocardial biopsies were separated into endocardial and epicardial regions, snap-frozen in liquid nitrogen and stored at -80°C .

2.4. DNA Fragmentation Analysis

Frozen myocardial biopsies (200 to 500 mg) were homogenized on ice for 15 seconds at 10,000 rpm (PT 1200, Kinematica AG, Switzerland) in 10 volumes of Tris-HCl (10 mM, pH 7.4), EDTA (1.0 mM) buffer; 1 volume of lysis buffer was added before incubation for 16 h at 37°C . RNAase A was added (final concentration 20 $\mu\text{g}/\text{mL}$) and then incubated for 2 h at 37°C . Saturated NaCl (0.1 mL) was then added and the solution was vigorously mixed. Samples were centrifuged at 13,000 rpm at 4°C for 5-min. DNA was extracted in phenol:chloroform: isoamyl alcohol (25:24:1); precipitated in 2 volumes of 95% ethanol and then centrifuged at 2000 rpm for 3-min. DNA was resuspended in extraction buffer and the concentration determined by spectrophotometry (260 nm). To detect DNA internucleosomal cleavage, 10 μg of each sample was loaded onto 1.5% agarose gel with 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide. A Hind III digest was run in parallel as a molecular size standard. DNA in the gel was visualized and photographed under UV light; qualitative analysis of DNA fragmentation was performed by analysis of the pattern of low-molecular weight DNA (*i.e.*, multiples of 180 base pair fragments).

Thymus from neonatal rat and rabbits given a bolus of 1 mg/Kg dexamethasone (*i.p.*) and allowed to recover for up to 8 h was used as a positive control for DNA fragmentation analysis. Tissue biopsies were prepared as described.

2.5. Poly (ADP-Ribose) Polymerase Cleavage

Frozen myocardial biopsies were homogenized on ice using a Polytron homogenizer in 20 volumes of buffer (Tris-HCl 62.5 mM pH 6.8; urea, 6M; glycerol, 10%; SDS, 2%; freshly prepared β -mercaptoethanol, 5%; protease inhibitors including: leupeptin, 10 $\mu\text{g}/\text{ml}$; antipain, 10 $\mu\text{g}/\text{ml}$; pepstatin, 10 $\mu\text{g}/\text{ml}$; PMSF, 0.35

mg/ml) as previously described [26]. Homogenate was sonicated (Ultrasonic homogenizer; Cole Parmer Instruments Co., Illinois, U.S.A.) on ice for 30 sec; 30 µg was separated by Western analysis using PAGE-SDS electrophoresis and transferred to PVDF membranes. Bound antibody was visualized with a sheep anti-mouse Ig conjugated to horseradish peroxidase secondary antibody (Roche Diagnostics, Montreal, CAN) and enhanced chemiluminescence (ECL, Boehringer Mannheim, Laval, CAN). Thymus from dexamethasone-treated neonatal rabbits was used as a positive control.

2.6. Data Analysis

Statistical comparisons were done using one-way ANOVA with a post-hoc analysis by Tukey's test; significance was defined as $p \leq 0.05$.

3. Results

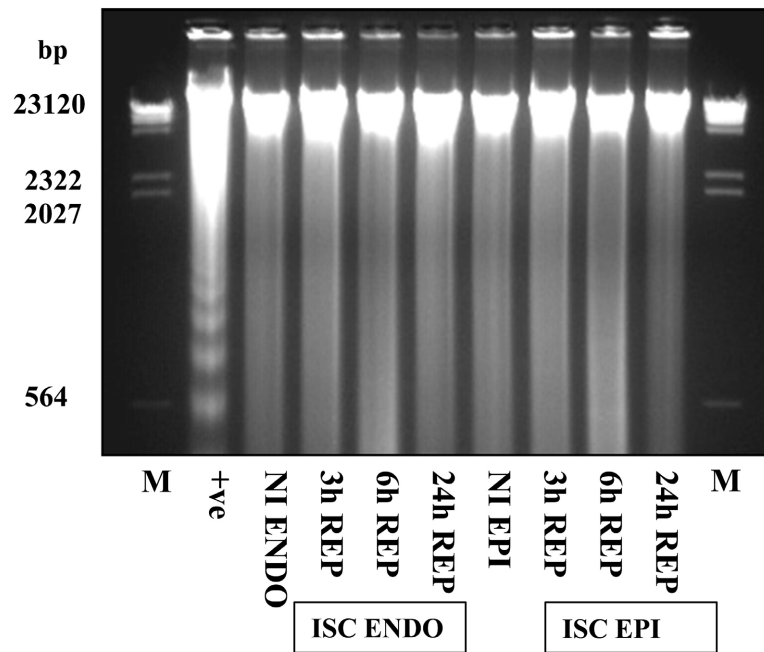
Risk region averaged 55% (as percent of LV area) in this experimental model; infarct size (as percent of area at risk) was markedly smaller (~50%; $p \leq 0.001$) in IC treated hearts compared to nIC in rabbits subject to 30-min CO and prolonged REP. With longer durations of CO protection afforded by IC was lost as has been reported elsewhere [27] [28].

3.1. DNA Fragmentation Electrophoresis

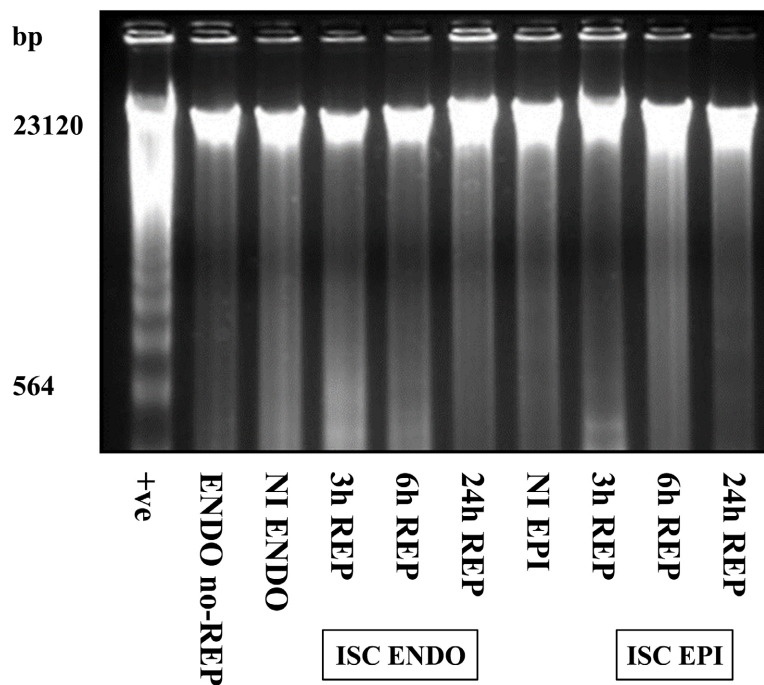
Representative agarose gels of DNA from ischemic myocardium pretreated, or not, by IC are shown in **Figure 1** and **Figure 2**. A typical DNA fragmentation ladder was obtained in positive control tissues from dexamethasone-treated neonatal animals. However, apoptosis-related internucleosomal DNA fragmentation was not clearly observed in any of the myocardial biopsies from hearts subjected to the different durations of CO-REP. In contrast, we observed significant background smearing that is associated with random DNA fragmentation and necrotic cell death. The time periods selected for CO and REP were based on earlier studies documenting significant protection against apoptosis-mediated cell death; longer REP times were evaluated since infarct size is well defined by 96 hours.

3.2. Poly-[ADP-Ribose]-Polymerase Cleavage

PARP was evaluated in myocardial biopsies from rabbits undergoing 30-min CO followed by 3, 6 or 24 h REP (**Figure 3**). Detection of the 89 kDa fragment of PARP in ischemic myocardium is a marker of caspase-3 enzyme activity; as such, cleavage of the PARP fragment is considered a sensitive marker of apoptosis. PARP levels decreased progressively in myocardial tissues from nIC and IC groups when REP was extended to 24 h. PARP cleavage was not observed in any of the myocardial biopsies examined (the response to dexamethasone in thymus tissue was used as a positive control). We were also concerned that negative findings were caused by the freezing and tissue storage protocol (*i.e.*, -80°C).

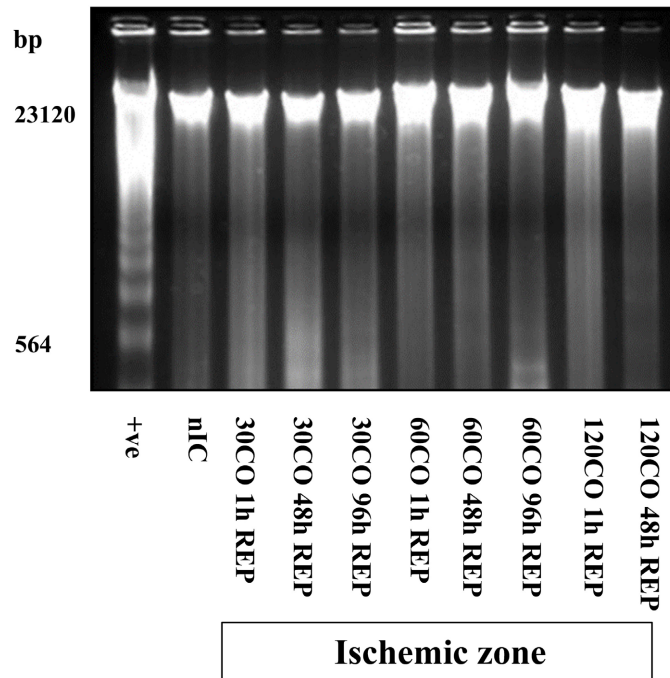


(A)

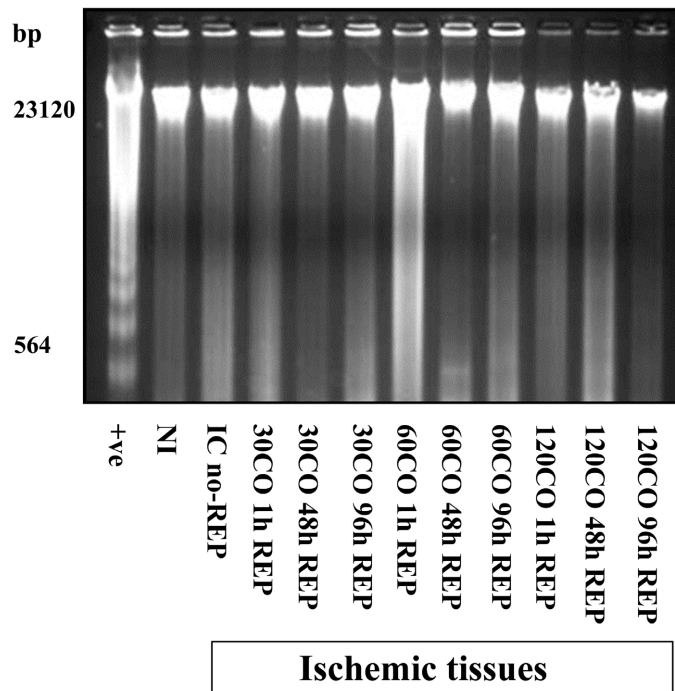


(B)

Figure 1. DNA electrophoresis (1.5% agarose) of myocardium from nIC and IC groups subject to 30-min CO. λ Hind III digest was used as a molecular size standard (M). (A) nIC group; lane 1, thymus (+ve control); lane 2, non-ischemic endocardium (NI ENDO); lanes 3 - 5, ENDO from ischemic zone after 30-min CO with 3, 6 or 24 h REP, respectively; lane 6, non-ischemic epicardium (NI EPI); lanes 7 - 9, EPI from ischemic zone after 3, 6 or 24 h REP, respectively. (B) IC group; lane 1, thymus (+ve control); lane 2, NI ENDO; lane 3, ENDO from rabbits after IC without REP; lanes 4 - 6, ENDO after 3, 6 or 24 h REP, respectively; lane 7, nIC EPI; lane 8, EPI from rabbits subjected to IC without REP; lanes 9 - 11, EPI after 3, 6 or 24 h REP.



(A)



(B)

Figure 2. DNA electrophoresis (1.5% agarose) of myocardium from nIC and IC groups after CO-REP. (A) nIC group; lane 1, thymus (+ve control)); lane 2, NI ENDO; lanes 3 - 5, ENDO after 30-min CO and 1, 48 or 96 h REP, respectively; lanes 6 - 8, ENDO after 60-min CO and 1, 48 or 96 h REP, respectively, lanes 9 - 10, ENDO after 120-min CO and 1 or 48 h REP. (B) IC group; lane 1, thymus (+ve control); lane 2, control NI ENDO; lane 3, ENDO from rabbits after IC without REP; lanes 4 - 6, ENDO after 30-min CO and 1, 48 or 96 h REP, respectively; lanes 7 - 9, ENDO after 60-min CO and 1, 48 or 96 h REP; lanes 10 - 12, ENDO after 120-min CO and 1 or 48 h REP.

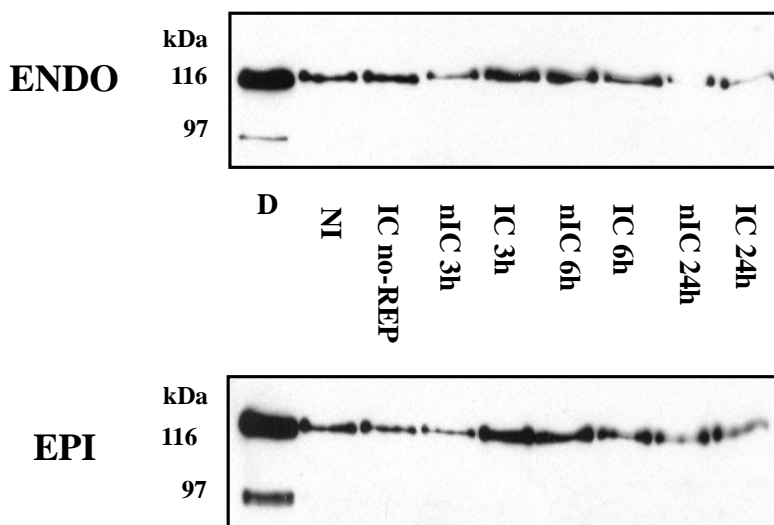


Figure 3. Immunodetection of PARP (Western blot analysis) in endocardial (ENDO; panel A) and epicardial (EPI; panel B) biopsies from ischemic zone after 30-min CO. PARP cleavage (+ve control) in thymus is shown (lane D). NI ENDO is shown in lane 1; lane 2, ENDO (ischemic zone) after IC without CO or REP, lanes 3, 5 and 7, ENDO after nIC and REP for 3, 6 or 24 h; lanes 4, 6 and 8, ENDO after IC and REP for 3, 6 or 24 h. Note that myocardial PARP levels decrease with prolonged REP.

Consequently, PARP activity was assessed in fresh/frozen myocardial biopsies from rabbits subjected to either no ischemia (*i.e.*, absolute controls) or 30-min CO followed by REP. PARP cleavage was detected in positive control thymus as well as in brain biopsies (data not shown) but not in myocardial biopsies; this suggests that freezing the tissues did not account for the negative results.

4. Discussion

Results of this study show that ischemic necrosis is primarily responsible for cell death produced by ischemia-reperfusion injury. We were unable to show significant DNA fragmentation or PARP cleavage in myocardium (biopsies from endocardium or epicardium layers) from rabbits subject to nIC or IC prior to acute regional CO and REP.

Metabolic changes that occur within ischemic myocardium after acute CO produces a rapid depletion of high-energy phosphate (HEP) reserves; restoration of blood flow to the ischemic tissues by percutaneous coronary interventions allows recovery of HEP production by intact mitochondria depending on the overall duration of ischemia. As such, cellular energy levels play a determinant role in the interchange between necrosis (low HEP levels) and apoptosis (high HEP levels). Apoptosis could also be pre-activated during ischemia but executed during reperfusion due to its dependence on HEP production [29]. Furthermore, total depletion of HEP in ischemic myocardium produces structural changes which differ morphologically and mechanistically from both necrosis and apoptosis [30]. IC reportedly limits depletion of HEP reserves in ischemic myocardium [31] [32].

Agarose electrophoresis results did not show clear-cut DNA laddering but rather significant DNA smearing that is consistent with previously reported results [33] [34]. However, absence of DNA laddering does not necessarily rule out presence of apoptosis [35] [36]. We showed a progressive decrease in PARP levels in ischemic myocardium with extended periods of ischemia; however, we did not find extensive cleavage of PARP which puts into question the presence of apoptosis. Activation of caspase-3 (and other caspases [35]) during ischemia-reperfusion normally results in significant cleavage of PARP [36] and its activation causes excessive depletion of HEP, slowed glycolysis and greater activation of pro-inflammatory mediators [37] [38].

Piot *et al.* reported a close correlation between infarct size and DNA fragmentation in rats [24] [39]; they also suggested that apoptosis and necrosis pathways were interrelated. Ohno *et al.* showed that myocyte damage during coronary occlusion proceeds from reversible to irreversible oncosis with or without DNA fragmentation [40] caused by activation of endonucleases, or the dying cascade; however, it is unclear whether apoptosis is an ischemia or reperfusion phenomenon [41] [42]. Reactive oxygen intermediates which are considered important mediators of ischemia-reperfusion injury also play a role in onset of apoptosis [43] [44] [45]. Moreover, peroxynitrite, formed by reactions between superoxide anion and nitric oxide, is a potent activator of PARP [46] which contributes to cardiomyocyte death. Pharmacologic blockade of PARP activation limits infarct size in rodents [47]. The present study shows lower levels of PARP in ischemic myocardium when reperfusion is extended to 24 h possibly due to a greater number of irreversibly injured myocytes in the ischemic risk zone. Absence of PARP cleavage suggests that myocyte cell death was not due to apoptosis.

This study has some limitations; despite the growing consensus that apoptosis contributes markedly to cell death after ischemia-reperfusion our findings do not support the hypothesis. Species differences are probably not responsible as we used an anesthetized rabbit experimental model as did Gottlieb *et al.* [41]; in our experiments reperfusion was extended to 96 h and the severity of acute ischemia was greater (CO up to 120 min). Shorter durations, or greater severity of acute ischemia (due to higher metabolic demand) might have produced different results but this remains speculative. Advantages of using PARP cleavage as a marker of apoptosis-mediated cell death include: 1) ability to detect caspase-3 activity (*i.e.* presence of 89-kDa fragment) which increases during ischemia and 2) detection during early stages even within small cell populations [26]. We fully expected to observe apoptosis-mediated myocardial cell death but were unable to confirm with two commonly used detection methods. Histochemical evaluation by TUNEL staining was also done but results were inconclusive (positive reactions were primarily confined to non-myocytes as previously documented [48] [49]).

Significant attention is being directed to mechanisms that regulate cell death as well as therapeutic interventions that may limit loss of different cardiac cell

types (myocytes and non-myocytes) after acute ischemia. The present findings show significant necrosis, not apoptosis, in ischemic myocardium. Furthermore, we were unable to confirm the anti-apoptotic capacity of IC; however, protection afforded by IC when ischemia is of relatively short duration is unquestioned. An improved understanding of the relationships between different modes of cell death (necrosis, apoptosis, autophagy, etc.) and their role in pathogenesis of cardiac disease is essential to allow development of novel pharmacologic and non-pharmacologic interventions.

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Conflicts of Interest

The author has no conflict of interest to declare for these studies.

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