Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury

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McCully, James D., Hidetaka Wakiyama, Yng-Ju Hsieh, Mara Jones, and Sidney Levitsky. Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 286: H1923–H1935, 2004. First published January 8, 2003; 10.1152/ajpheart.00935.2003.—Necrosis and apoptosis differentially contribute to myocardial injury. Determination of the contribution of these processes in ischemia-reperfusion injury would allow for the preservation of myocardial tissue. Necrosis and apoptosis were investigated in Langendorff-perfused rabbit hearts (n = 47) subjected to 0 (Control group), 5 (GI-5), 10 (GI-10), 15 (GI-15), 20 (GI-20), 25 (GI-25), and 30 min (GI-30) of global ischemia (GI) and 120 min of reperfusion. Myocardial injury was determined by triphenyltetrazolium chloride (TTC) staining, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), bax, bcl2, poly(ADP)ribose polymerase (PARP) cleavage, caspase-3, -8, and -9 cleavage and activity, Fas ligand (Fasl), and Fas-activated death domain (FADD). The contribution of apoptosis was determined separately (n = 42) using irreversible caspase-3, -8, and -9 inhibitors. Left ventricular peak developed pressure (LVPDP) or SS. Myocardial injury results from a significant increase in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

METHODS

Animals. New Zealand White rabbits (n = 89, 15–20 wk, 3–4 kg) were obtained from Millbrook Farm (Amherst, MA). All animals were housed individually and provided with laboratory chow and water ad libitum. All experiments were approved by the Beth Israel Deaconess Medical Center Animal Care and Use Committee and the Harvard Medical Area Standing Committee on Animals (Institutional Animal Care and Use Committee) and conformed to the National Institutes of Health guidelines regulating the care and use of laboratory animals (NIH Pub. No. 5377-3, 1996). All research was performed in accordance to the American Physiological Society “Guiding Principles in the Care and Use of Animals.”

Langendorff perfusion. Langendorff retrograde perfusion was performed as described by Feinberg et al. (12). All rabbits were anesthetized with ketamine (33 mg/kg) and xylazine (16 mg/kg) and received heparin (200 U/kg) intravenously via the

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 marginal ear vein. The heart was excised and placed in a 4°C bath of Krebs-Ringer solution equilibrated with 95% O2-5% CO2 (pH 7.4 at 37°C), where spontaneous beating ceased within a few seconds. Langendorff retrograde perfusion was performed as previously described (45). In brief, a latex balloon containing a catheter-tipped transducer (Millar Instruments; Houston, TX) was inserted into the left ventricle (LV). The volume of the balloon was maintained using a calibrated microsyringe to provide a constant physiological end-diastolic pressure of 5–10 mmHg during equilibrium, and this balloon volume was maintained for the duration of the experiment (45). The aorta was cannulated with a metal cannula, and the heart was subjected to Langendorff retrograde perfusion at a constant pressure of 75 cmH2O at 37°C. Hearts were paced via the right atrium at 180 ± 3 beats/min throughout the experiment using a Medtronic model 5330 stimulator (Medtronic; Minneapolis, MN). Hemodynamic variables were acquired using the PO-NE-MAH digital data-acquisition system (Gould; Valley View, OH) with an Acquire Plus processor board and LV pressure analysis software (45).

Experimental protocol. The experimental protocol is shown in Fig. 1. Hearts were perfused for 30 min to establish equilibrium hemodynamics. Equilibrium was terminated when heart rate, coronary flow, LV peak developed pressure (LVPDP), and LV end-diastolic pressure (LVEDP) were maintained at the same level for three continuous measurement periods timed 5 min apart. Hearts in the Control group (n = 7) were perfused without global ischemia (GI) at 37°C for 180 min. Rabbit hearts were subjected to 5 min (GI-5; n = 7), 10 min (GI-10; n = 6), 15 min (GI-15; n = 7), 20 min (GI-20; n = 8), 25 min (GI-25; n = 6), or 30 min (GI-30; n = 6) of GI and 120 min of reperfusion. GI was achieved by cross-clamping the perfusion line.

Myocardial function. Myocardial function was assessed by sonomicrometry (Sonomicrometers Digital Ultrasonic Measurement System, Sonometrics; London, Ontario, Canada) using five digital piezoelectric ultrasonic probes (1 mm) with each probe acting as both a transmitter and a receiver. The probes were implanted in the subepicardial layer ~7–10 mm apart in the LV, with one probe placed on the major axis of the heart perpendicular to four probes placed parallel to the minor axis of the heart (including anterior and posterior positions) and secured to the epicardium with a Z-suture using polypropylene stitches (5-0 Prolene, 8580H, Ethicon; Somerville, NJ). The probes were left in place until the end of the experiment. Digital data were inspected for correct identification of end-diastolic and end-systolic points using postprocessing software (SonoView, Sonometrics). Measurements were made over at least three cardiac cycles in normal sinus rhythm and then averaged. LV function was assessed as segment shortening (SS) using the following equation: SS = 100 × (EDL – ESL)/EDL, where EDL is end-diastolic segment length measured at the onset of the positive first derivative of pressure over time (dP/dt) and ESL is end-systolic segment length measured at peak negative dP/dt (45, 46, 48). Wall motion abnormalities were assessed by systolic bulging (SB) and postsystolic shortening (PSS). SB, defined as the bulging of the myocardium after the end of diastole, was determined using the following equation: SB = 100 × (maximum segment length during systole – EDL)/EDL (45, 46, 48). PSS, defined as the shortening after the end of systolic ejection, was determined using the following equation: PSS = 100 × (ESL – minimum segment length during diastole)/EDL. Time course changes in SS were expressed as a percentage of equilibrium values to minimize variability among individual animals (45, 46, 48).

Infarct size. After reperfusion, the hearts were rapidly removed from the perfusion apparatus and sliced across the long axis of the LV, from apex to base, into 2-mm-thick transverse sections. Tissue samples (~100 mg each) from the apex, LV, and right ventricle were obtained using a standardized grid template from each heart for biochemical and histological analyses (39, 45, 46, 48). The hearts were then incubated in 1% triphenyltetrazolium chloride (TTC; Sigma Chemical; St. Louis, MO) in phosphate buffer (pH 7.4) at 38°C for 20 min.
Infarct areas were enhanced by storage in 10% formaldehyde solution for 24 h before final measurement. A copy of the stained heart slices was traced onto a clear acetate sheet over a glass plate under room light (45, 46, 48). The area of the LV and the area of infarcted tissue were measured by an independent, blinded observer by using planimetry (Scion Image, Scion; Frederick, MD). The volume of the infarcted zone and the area at risk were calculated by multiplying the planimetered areas by the slice thickness (45, 46, 48). Infarct volume was expressed as a percentage of LV volume for each heart (45, 46, 48).

**Caspase inhibitors.** To determine the effects of caspase inhibition on cardioprotection during reperfusion, a separate group of hearts (n = 42; Fig. 1) from GI-20, GI-25, and GI-30 were perfused separately with the cell-permeable irreversible caspase-3 inhibitor z-DEVD.fmk (1.0 μmol/l, Kamiya Biomedical; Seattle, WA; n = 6 each for GI-20, GI-25, and GI-30), the irreversible caspase-8 inhibitor z-IETD.fmk (1.0 μmol/l, Kamiya Biomedical; n = 4 each for GI-20, GI-25, and GI-30), or the irreversible caspase-9 inhibitor z-LEHD.fmk (1.0 μmol/l, Kamiya Biomedical; n = 4 each for GI-20, GI-25, and GI-30) for the first 10 min of reperfusion (0–10 min of reperfusion). Caspase inhibitors were dissolved separately in DMSO (Fisher Scientific; Fair Lawn, NJ). The final concentration of DMSO in Krebs-Ringer solution was <0.1%. DMSO was added to control hearts at the same concentration. The concentrations of z-DEVD.fmk, z-IETD.fmk, and z-LEHD. fmk were determined from preliminary studies using 0.07, 1.0, and 5.0 μmol/l (31). Preliminary studies indicated that 0.07 μmol/l had no effect on infarct size in GI hearts and that 1.0 and 5.0 μmol/l equally decreased infarct size. No difference in functional recovery was observed using either 0.07, 1.0, or 5.0 μmol/l.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.** Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) was performed using the ApopTag detection system (Intergen; Gaithersburg, MD) as previously described (39). In brief, myocardial tissue samples from the apex LV and right ventricle were removed at the end of experiments and flash frozen, and the sections were sectioned (4–6 μm), mounted on glass slides, and used for in situ TUNEL. Five to six slides from each sample were assayed using both TUNEL and propidium iodide staining. Photomicrographs were taken in 10–15 random high-powered (×20) fields using a Zeiss MC80DX camera and exposure meter. All cells were counted on each slide, and TUNEL-positive cells were expressed per 3,000 myocardial cells (39). Myocardial cell specificity was determined on opposite adjacent sequential serial slides (n = 5–6 for each sample) using the cardiac-specific monoclonal antibody for troponin I (Spectral Diagnostics; Toronto, Ontario, Canada) labeled with anti-mouse IgG conjugated to Alexa 350 (Molecular Probes; Eugene, OR). Troponin I was visualized using a DAPI filter (Chroma Technologies; Battleboro, VT). Evaluation of TUNEL and myocyte morphology was performed by a blinded independent examiner. TUNEL-positive cells were expressed per 1,000 myocardial cells (39).

**Western blot analysis.** To detect poly(ADP)ribose polymerase (PARP), nuclear proteins were isolated from pooled tissue samples from apex, LV, and right ventricular tissue samples obtained after 120 min of reperfusion according to the method as described by Andrews and Faller (1). All other antigens were detected in total cytoplasmic protein. Total cytoplasmic protein was isolated from pooled tissue samples from apex, LV, and right ventricular tissue samples obtained after 120 min of reperfusion using SDS-Nonidet P-40 lysis buffer (27). All protein lysis buffers contained the multiprotease inhibitor Complete (2 μM; Boehringer Mannheim) and the cell-permeable caspase inhibitors (z-DEVD.fmk, z-IETD.fmk, and z-LEHD.fmk, 10 μM each, and the nonselective irreversible caspase inhibitor z-VAD.fmk, 50 μM; Kamiya Biomedical). Proteins were separated in parallel (duplicate gels) by 10% SDS-PAGE according to methods previously described (27). In one gel, the immobilized protein bands and molecular mass markers were visualized by Coomassie brilliant blue staining. The other gel was transferred to nitrocellulose membranes as previously described (27). Protein concentration was determined by the method of Lowry et al. (24). Equal amounts of protein (50 μg) were fractionated on 10% SDS-polyacrylamide gels (Invitrogen; Carlsbad, CA) and then electroblotted to nitrocellulose membranes (Invitrogen). Protein equivalency and transfer efficiency were confirmed by Ponceau red staining.

Membranes were blocked for 2 h at room temperature with 5% nonfat milk in Tris-buffered saline (25 mM Tris, 137 mM NaCl, and 2.7 mM KCl) containing 0.05% Tween 20 and then incubated overnight with shaking at 4°C with specific antibodies according to methods previously described (28). Immunoblotting was performed using actin (monoclonal antibody, anti-actin clone AC-40, Sigma Chemical; 1:1,000 dilution), bax (polycyonal antibody, bax-N-20, Santa Cruz Biotechnology; Santa Cruz, CA; 1:200 dilution); bcl2 (polycyonal antibody, bcl2-N-19, Santa Cruz Biotechnology; 1:200 dilution); caspase-3 (monoclonal antibody, CCP-32, Ab-3, anti-caspase-3, Oncogene; Boston, MA; 1:200 dilution); caspase-8 (monoclonal antibody, caspase-8 p-20, D8, Santa Cruz Biotechnology; 1:200 dilution); caspase-9 (monoclonal antibody, Calbiochem; San Diego, CA; 1:1,000 dilution); Fas (polycyonal antibody F-335, Santa Cruz Biotechnology; 1:200 dilution); Fas ligand (FasL; polycyonal antibody, AB-1, Oncogene; 1:1,000 dilution; Fas-activated death domain (FADD) antibody, Upstate Biotechnology; Lake Placid, NY; 1:1,000 dilution); and PARP (monocyonal antibody P ARP, Ab-2, Oncogene; 1:1,000 dilution). After being washed, the blots were incubated for 1 h with appropriate horse radish peroxidase-conjugated antibodies, and the blots were detected using ECL Plus (Amersham Pharmacia Biotech; Piscataway, NJ). Semiquantitative analysis of one-dimensional gels and Western blots was performed using an LKB Ultrascan XL laser densitometer (Pharmacia) using the LKB GelScan XL software program for one-dimensional analysis previously described (27).

**Caspase-like activity.** Caspase-like activity was determined in total cytoplasmic proteins isolated in SDS-Nonidet P-40 lysis buffer containing protease (2 μM; Complete, Boehringer Mannheim) without the addition of caspase inhibitors. Caspase-3, -8, and -9 activity was determined separately using the appropriate colormetric analysis kit, pNA standards, and inhibitors according to the manufacturer’s instructions (Chemicon; Temecula, CA).

**Wet weight-to-dry weight ratios.** LV tissue samples (~0.1 g) obtained from the apex from all experimental groups were weighed (wet weight), dried at 80°C for 24 h for reweighing.
(dry weight), and then used for the determination of wet weight-to-dry weight ratios, as previously described (45).

Statistical analysis. Statistical analysis was performed using the SAS (version 6.12) software package (SAS Institute; Cary, NC). Means ± SE for all data were calculated for all variables. Statistical significance was assessed using repeated-measures ANOVA with group as a between-subjects factor and time as a within-subjects factor. If this overall test was significant, then one-way ANOVA was performed at individual time points, and, when significant, post hoc comparisons were made between groups at a time point. Dunnett’s test was used for comparisons between control and other groups to adjust for the multiplicity of tests. The Bonferroni correction was used for comparisons between groups other than the Control group. A one-way ANOVA was used for infarct size. Linear regression analysis was performed to determine the relation between SS and infarct size. Differences in regression lines between groups were compared by analysis of covariance. *P < 0.05 was used for statistical significance.

RESULTS

Equilibrium hemodynamics. No significant difference in LVPDP, LVEDP, +dP/dt, or coronary flow was observed within or between groups at the end of equilibrium (Fig. 2A and Table 1).

Hemodynamics during reperfusion. Ventricular fibrillation occurred in GI-20, GI-25, and GI-30 hearts at the onset of reperfusion. The incidence of ventricular fibrillation was 37.5% in GI-20 hearts, 50.0% in GI-25 hearts, and 66.7% in GI-30 hearts. The duration of ventricular fibrillation was 3.8 ± 1.2 min in GI-20 hearts, 4.1 ± 0.7 min in GI-25 hearts, and 5.4 ± 1.0 min in GI-30 hearts. There was no significant difference in the duration of ventricular fibrillation among GI-20, GI-25, and GI-30 hearts. No ventricular fibrillation was observed at the onset reperfusion in GI-5, GI-10, and GI-15 hearts. LVPDP in GI-25 and GI-30 hearts was significantly decreased throughout 120 min of reperfusion (P < 0.05 vs. control; Fig. 1). LVPDP in GI-20 was significantly decreased.

![Figure 2](http://ajpheart.physiology.org/)

Fig. 2. A: effects of GI on left ventricular (LV) peak developed pressure (LVPDP; mmHg); B: segmental shortening (SS) during equilibrium and reperfusion; C: linear regression analysis of infarct size (% of area at risk) and SS (% of equilibrium) at the end of 120 min of reperfusion; D: infarct size (% of LV mass) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cell number (per 1,000 myocytes) after GI and reperfusion. Control hearts were perfused without GI at 37°C for 180 min. Rabbit hearts were subjected to GI-5, GI-10, GI-15, GI-20, GI-25, or GI-30 and 120 min of reperfusion. All results are shown as means ± SE; n = 6–8 hearts/group. *Significant difference (P < 0.05) vs. Control hearts. SS was significantly (P < 0.05) and inversely related to infarct size. NS, not significant.
during the early phase of reperfusion (10–30 min of reperfusion) and at 90–120 min of reperfusion (P < 0.05 vs. Control hearts; Fig. 1). LVEDP in GI-30 hearts was significantly increased during the first 30 min of reperfusion (P < 0.05 vs. Control hearts; Table 1). After 60 min of reperfusion, no significant difference in +dP/dt was observed between Control and GI hearts (Table 1). No significant difference in coronary flow was observed between Control and all GI hearts throughout reperfusion (Table 1).

SS during reperfusion. Myocardial function assessed by SS was significantly decreased in GI-30 hearts throughout reperfusion (P < 0.05 vs. Control hearts). At the end of reperfusion, SS in GI-20, GI-25, and GI-30 hearts was significantly decreased compared to Control hearts (P < 0.05; Fig. 2B). No significant differences in SS were observed between Control and GI-5, GI-10, or GI-15 hearts during reperfusion. No abnormal myocardial wall motion defined as SB or PSS was observed in any group during equilibrium and reperfusion.

Infarct size. Infarct size expressed as a percentage of LV volume was 2.1 ± 0.4% in Control hearts. No significant difference in infarct size was observed among Control, GI-5 (1.8 ± 0.3%), GI-10 (1.9 ± 0.3%), and GI-15 hearts (2.6 ± 0.3%) (Fig. 2C). Infarct size was significantly increased to 6.8 ± 1.5% in GI-20 hearts, 12.5 ± 1.0% in GI-25 hearts, and 23.8 ± 1.0% in GI-30 hearts (P < 0.05 vs. Control).

Relationship between infarct size and SS. To determine the relationship between morphological injury and functional injury, infarct size (percentage of area at risk) and SS (percentage of equilibrium) at the end of 120 min of reperfusion were compared using linear regression (Fig. 2C). The linear regression equation was y = −2.4323x + 100, and the correlation coefficient was 0.878. Our results indicate that SS was significantly (P < 0.05) and inversely related to infarct size.

TUNEL. At the end of reperfusion, only minimal (<5 cells/1,000 myocytes) TUNEL-positive cells were observed in Control, GI-5, GI-10, and GI-15 hearts. The number of TUNEL-positive cells was significantly increased (P < 0.05 vs. Control hearts) to 12.3 ± 2.3 cells/1,000 myocytes in GI-20 hearts, 19.3 ± 3.1 cells/1,000 myocytes in GI-25 hearts, and 28.1 ± 5.2 cells/1,000 myocytes in GI-30 hearts (Fig. 2D).

Apoptotic protein cleavage and activity. The antiapoptotic protein bcl-2 was significantly decreased (P < 0.05 vs. Control hearts) in GI-20, GI-25, and GI-30 hearts (Fig. 3, A and B). There was no significant difference in bcl-2 levels among GI-20, GI-25, and GI-30 hearts (Fig. 3, A and B). The proapoptotic protein bax was significantly increased (P < 0.05 vs. Control hearts) at GI-5 and remained elevated to GI-30 (Fig. 3, A and B). The bcl-2/bax ratio was significantly increased (P < 0.05 vs. Control hearts) at GI-5 and remained elevated to GI-30 (Fig. 3B). No significant difference in the bcl-2/bax ratio was observed among GI-20, GI-25, or GI-30 (Fig. 3B).

Caspase-9 cleavage was first evident in GI-5 hearts. Caspase-3 cleavage was first evident in GI-10 hearts, and PARP cleavage was first evident in GI-15 hearts (Fig. 3A). Caspase-3 and -9 and PARP cleavage were evident in GI-30 (Fig. 3A).

Caspase-9-like activity (pmol Ac-LEHD-pNA/μg protein−1·min−1) was significantly increased (P < 0.05 vs. Control hearts) in GI-15, GI-20, GI-25, and GI-30 hearts (Fig. 3B). Caspase-3 activity was significantly increased (P < 0.05 vs. Control hearts) in GI-20, GI-25, and GI-30 hearts (Fig. 3B). No alteration in Fas, FasL, FADD, or caspase-8 cleavage or activity was observed in any group (Fig. 4).
Effect of caspase inhibition on functional recovery and infarct size. To determine the effects of apoptosis on infarct size, a separate study (n = 42) was performed using the caspase inhibitors z-DEVD.fmk (caspase-3), z-IETD.fmk (caspase-8), and z-LEHD.fmk (caspase-9), separately. No arrhythmia was observed in GI-20 + z-DEVD.fmk, GI-20 + z-IETD.fmk, or GI-20 + z-LEHD.fmk hearts. One rabbit in the GI-25 + z-DEVD.fmk group and two rabbits each in the
GI-25 + z-IETD.fmk and GI-25 + z-LEHD.fmk groups suffered ventricular fibrillation at the onset of reperfusion. The duration of these events ranged from 2.8 to 3.6 min. Ventricular fibrillation was observed in two hearts each in the GI-30 + z-DEVD.fmk, GI-30 + z-IETD.fmk, and GI-30 + z-LEHD.fmk group and ranged from 2.6 to 3.3 min. No significant difference was observed in the incidence of ventricular fibrillation between GI-20, GI-25, and GI-30 hearts and GI-20, GI-25, and GI-30 hearts treated with caspase inhibitors. The caspase-8 inhibitor z-IETD.fmk had no effect on caspase-3-like activity or TUNEL-positive cell number compared with GI-20, GI-25, and GI-30 hearts.

There was no significant difference in LVDPD or dP/dt within or among GI-20, GI-25, and GI-30 hearts treated with caspase inhibitors (z-DEVD.fmk, z-IETD.fmk, or z-LEHD.fmk) and GI-20, GI-25, and GI-30 hearts after 10 min of equilibrium or during 120 min of reperfusion (Table 2). There was no significant difference in LVEDP, SS, or coronary flow within or among GI-20, GI-25, and GI-30 hearts treated with caspase inhibitors (z-DEVD.fmk, z-IETD.fmk, or z-LEHD.fmk) and GI-20, GI-25, and GI-30 hearts after 10 min of equilibrium or during 120 min of reperfusion (results not shown).

There was no significant decrease in infarct size between GI-20 and GI-20 + z-DEVD.fmk (6.0 ± 0.7%) and GI-20 + z-LEHD.fmk hearts (7.2 ± 0.7%; Fig. 5A). However, the caspase-3 (z-DEVD.fmk) and caspase-9 (z-LEHD.fmk) inhibitors significantly decreased infarct size in GI-25 + z-DEVD.fmk hearts to 9.0 ± 0.8%, in GI-25 + z-LEHD.fmk hearts to 9.8 ± 1.2% (P < 0.05 vs. GI-25), in GI-30 + z-DEVD.fmk hearts to 15.5 ± 1.7%, and in GI-30 + z-LEHD.fmk hearts to 17.7 ± 1.5% (P < 0.05 vs. GI-30). The caspase-8 inhibitor z-IETD.fmk had no effect on infarct size with either GI-20 + z-IETD.fmk (6.7 ± 1.7%), GI-25 + z-IETD.fmk (12.76 ± 1.2%), or GI-30 + z-IETD.fmk (26.67 ± 1.7%) compared with GI-20, GI-25, or GI-30. The combined use of the caspase inhibitors z-DEVD.fmk and z-LEHD.fmk to inhibit caspase-3 and -9 failed to provide any further reduction in infarct size at either GI-20 (6.6 ± 1.64), GI-25 (9.4 ± 1.5), or GI-30 (16.4 ± 1.2) (results not shown).

Linear regression analysis of infarct size (percentage of area at risk) and SS (percentage of equilibrium) at the end of 120 min of reperfusion for GI-20, GI-25, and GI-30 were compared using linear regression (Fig. 2C). The linear regression equation was y = 2.432x + 100, and the correlation coefficient was 0.087. Our results indicate that SS was significantly (P < 0.05) and inversely related to infarct size.

Both the caspase-3 inhibitor (z-DEVD.fmk) and the caspase-9 inhibitor (z-LEHD.fmk) significantly decreased caspase-3-like activity at GI-25 and GI-30 (Fig. 5C) and significantly decreased the TUNEL-positive cell number (Fig. 5B).

Wet weight-to-dry weight ratios. The wet weight-to-dry weight ratio after 180 min of perfusion in control hearts was 5.95 ± 0.1. No significant difference in the wet weight-to-dry weight ratio was observed within or between groups (results not shown).

DISCUSSION

Myocardial ischemia-reperfusion injury significantly compromises myocardial function and increases morbidity and mortality (2, 4, 7). Therefore, the investigation of strategies for...
the amelioration or preservation of myocardial cell viability has been a matter of great interest in both clinical and experimental settings (2, 3, 7, 10, 16, 31, 36, 37, 39).

Two morphologically distinct pathways contribute to myocardial ischemia-reperfusion injury: necrosis and apoptosis. In this study, we investigated the contribution of necrosis and apoptosis to myocardial injury using a linear model of ischemia-reperfusion. The induction of apoptosis has been shown to involve activation of death receptors such as Fas by specific ligands (FasL) resulting in the proteolytic cleavage and activation of caspase-8, which in turn results in the proteolytic cleavage and activation of caspase-9 and the cellular apoptosis caspase (30).

The second apoptotic pathway, the intrinsic pathway, involves signals originating from numerous other stimuli, which are potently inhibited by bc2l (15). The mitochondrion has been implicated as a major regulator of the intrinsic pathway (15). The mechanisms modulating the intrinsic pathway remain to be fully elucidated, but recent investigations have shown that cellular ATP depletion initiates the translocation of bax, a proapoptotic bc2l family member protein, from the cytosol to the outer mitochondrial membrane by mechanisms that remain unclear (22, 42). The translocation of bax causes mitochondrial dysfunction and swelling and induces the efflux of cytochrome c to the cytosol. The efflux of cytochrome c to the cytosol occurs by a mechanism that is thought to involve changes in mitochondrial permeability caused by the opening of the permeability transition pore in the mitochondrial inner membrane or mitochondrial hyperpolarization followed by swelling and membrane rupture (22, 42). Cytochrome c on release to the cytosol activates caspase, which is normally expressed as latent zymogens that are cleaved and reassociated to generate catalytically active heterodimers that are potently inhibited by bc2l (22, 42). It is the activation of the effector caspase, caspase-3, that is the common event initiated by the multiple different stimuli that induce apoptosis. Caspase-3 is primarily responsible for the cleavage of PARP, a nuclear enzyme that is catalytically activated by DNA strand interruptions (4, 15, 22).

Our results indicate that activation of the intrinsic apoptosis pathway occurs early. The proapoptotic protein bc2l is significantly increased and the proteolytic cleavage of caspase-9 is evident in GI-5 hearts, caspase-3 cleavage is evident in GI-10 hearts, and PARP cleavage is evident in GI-15 hearts (Fig. 3, A–C). Caspase-9-like activity is significantly increased at GI-15, and caspase-3-like activity is significantly increased at GI-20. Neither caspase-3- nor -9-like activities are significantly increased at this time point. These results suggest that caspase-3 and -9 activity reach a plateau once induced. Support for this observation comes from Suzuki et al. (44), who reported in a rat cardiomyocyte culture that, once activated, caspase-3 activity remains stable.

These results are in agreement with TUNEL-positive cell data that demonstrate that only minimal (<20 cells/1,000 myocytes) TUNEL-positive nuclei are observed in Control, GI-5, GI-10, and GI-15 hearts. In GI-20, GI-25, and GI-30 hearts, where caspase-3-like activity is significantly increased, TUNEL-positive nuclei are significantly increased. Our data indicate that the induction of proapoptotic protein cleavage occurs in a sequential manner and is evident before biochemical detection by TUNEL.

### Table 2. Effects of caspase inhibitors on hemodynamics

<table>
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<tr>
<td>GI-20+LEHD</td>
<td>108.4 (3.6)</td>
<td>61.2 (2.8)</td>
<td>79.1 (3.2)</td>
<td>92.0 (3.2)</td>
<td>91.1 (4.9)</td>
<td>93.3 (3.7)</td>
</tr>
<tr>
<td>GI-25+LEHD</td>
<td>108.6 (3.2)</td>
<td>61.6 (4.5)</td>
<td>76.3 (3.5)</td>
<td>93.3 (4.8)</td>
<td>93.6 (5.7)</td>
<td>90.0 (4.7)</td>
</tr>
<tr>
<td>GI-30+LEHD</td>
<td>109.5 (3.6)</td>
<td>60.6 (4.6)</td>
<td>77.2 (5.6)</td>
<td>90.2 (6.9)</td>
<td>93.6 (6.3)</td>
<td>89.0 (4.3)</td>
</tr>
</tbody>
</table>

Results are means (with SEs in parentheses). Hemodynamic data are shown during equilibrium and during reperfusion (10–120 min of reperfusion) for GI-20, GI-25, and GI-30 hearts perfused separately with Krebs-Ringer solution containing either 1.0 mmol/l z-DEVD.fmk, (GI-DEVD) to inhibit caspase-3 activity, 1.0 mmol/l z-IETD.fmk (GI-IETD) to inhibit caspase-8 activity, or z-LEHD.fmk (GI-LEHD) to inhibit caspase-9 activity. All caspase inhibitors were perfused for 10 min at the immediate start of reperfusion.
Fig. 5. Effects of caspase inhibition on cardioprotection during reperfusion in GI-20, GI-25, and GI-30 hearts perfused separately with the cell-permeable irreversible caspase-3 inhibitor DEVD, the irreversible caspase-8 inhibitor IETD, or the irreversible caspase-9 inhibitor LEHD for the first 10 min of reperfusion (0–10 min of reperfusion) on infarct size (%LV mass; A), TUNEL-positive cell number (per 1,000 myocytes; B), and caspase-3-like activity (C). All results are shown as means ± SE for n = 4–6 for each sample. *Significant difference (P < 0.05) vs. the matched GI group. The irreversible caspase-3 inhibitor DEVD and the irreversible caspase-9 inhibitor LEHD each significantly decreased TUNEL-positive cell number and caspase-3-like activity in GI-20, GI-25, and GI-30 hearts and infarct size in only GI-25 and GI-30 hearts. Caspase-8 inhibition had no effect on TUNEL-positive cell number, caspase-3-like activity, or infarct size.
Our results indicate that no significant differences in infarct size, LVDP, or SS was observed among Control, GI-5, GI-10, and GI-15 hearts. These results are in agreement with earlier investigations indicating that 15–20 min of GI is required to induce stunning in the isolated perfused rabbit heart (49). When GI time was increased to 20, 25, or 30 min, infarct size was significantly increased in a quadratic manner (~2-fold) from GI-15 (2.6 ± 0.3%) to GI-20 (6.8 ± 1.5%), from GI-20 to GI-25 (12.5 ± 0.98%), and from GI 25 to GI-30 (23.8 ± 0.67%). This increase in myocardial injury is the result of a significant increase in both necrosis and apoptosis (P < 0.05 vs. Control hearts for each), as determined by TTC staining and TUNEL-positive nuclei (Fig. 2C), and is associated with a significant decreases in LVDP.

We were unable to detect any change in Fas, FasL, FADD, or caspase-8 cleavage (Fig. 4). We were also unable to detect any change in caspase-8 cleavage despite the use of a series of commercially available caspase-8 antibodies. To augment our results, we also determined caspase-8-like activity. Our results (Fig. 4) indicate that no caspase-8-like activity was detected in our model. In support of this conclusion are the results shown in Fig. 5A, which show that inhibition of caspase-8 activity using the caspase-8-specific inhibitor z-IETD.fmk had no effect on infarct size compared with GI-20, GI-25, or GI-30.

Apoptosis and necrosis are morphologically distinct pathways contributing to myocardial infarct size following ischemia and reperfusion (2, 3, 7, 16, 36). The induction and final commitment of the apoptosis pathways to irreversible myocardial injury can be modulated through the use of inhibitors of the apoptosis pathways (29, 33). Previous reports have indicated that the induction and apoptotic signals irreversibly lead to cell death (30). This concept has been recently challenged in studies that have shown that caspase inhibitors successfully attenuate apoptotic injury in vitro and in vivo (14, 17, 29, 30). To determine the contribution of apoptosis and necrosis to myocardial cell death, we used three irreversible tetrapeptide caspase inhibitors (z-DEVD.fmk, caspase-3; z-IETD.fmk, caspase-8; and z-LEHD.fmk, caspase-9) either separately or in combination to inhibit apoptosis. All the caspase inhibitors are cell permeable and exert an irreversible inhibition on their selective caspases. The exact mechanism of this inhibition has been reported to be modulated through the fluoromethylketone (fmk) functional group, which binds to the active site cysteine residue of caspase enzymes as well as cathepsin cysteine protease enzymes (38). There is controversy over the optimal protocol for the administration of caspase inhibitor to attenuate apoptosis (11, 19, 29, 33). Recent reports have shown that the apoptosis process would be accelerated during the reperfusion (14, 29, 51). This speculation is supported by Freude et al. (13) and Zhao et al. (51), who have independently shown that apoptotic cell death is initiated by ischemia and that reperfusion is essential for the completion of the apoptotic cascade. Thus we investigated the effect of caspase inhibition at the onset of reperfusion.

In this study, we used 1.0 μmol/l of z-DEVD.fmk, Z-IETD.fmk, or z-LEHD.fmk or 1.0 μmol/l each of z-DEVD.fmk and z-LEHD.fmk for the first 10 min of reperfusion to modulate apoptosis in the heart subjected to normothermic ischemia-reperfusion injury. In a previous study, 0.07 μmol/l Ac-DEVD.cmk was used to inhibit caspase-3 activity in isolated rat hearts (3, 29). The authors showed that this concentration significantly decreased infarct size. The concentration of each tetrapeptide caspase inhibitor in our study was determined based on preliminary investigations that showed that 1.0 μmol/l would decrease infarct size and TUNEL-positive cells without any harmful effect on hemodynamics throughout reperfusion (results not shown). Our preliminary studies indicated that 0.07 μmol/l was ineffective in decreasing the TUNEL-positive cell number. The difference between studies is most likely due to differences in the experimental protocol and/or animal species.

Our results indicate that inhibition of caspase activity during early reperfusion significantly decreases myocardial ischemic-reperfusion injury. Our results shown in Fig. 5, B and C, show that the irreversible tetrapeptide caspase inhibitors z-DEVD.fmk (caspase-3) and z-LEHD.fmk (caspase-9) significantly decreased TUNEL-positive cell number and significantly decreased caspase-3-like activity such that no difference compared with control was observed. Similar results were observed for both z-DEVD.fmk and z-LEHD.fmk. The combined use of z-DEVD.fmk and z-LEHD.fmk to inhibit both caspase-3 and -9 activity did not show any additive effect, with both TUNEL-positive cell number and caspase-3-like activity being similar to that achieved by individual usage (results not shown). The caspase-8-specific inhibitor z-IETD.fmk failed to decrease either caspase-3-like activity or TUNEL-positive cell number.

Neither z-DEVD.fmk nor z-LEHD.fmk significantly decreased infarct size in GI-20 hearts (Fig. 5A) despite significantly decreasing caspase-3-like activity and TUNEL-positive cell number in GI-20 hearts. This inability to decrease infarct size would suggest that the apoptotic contribution to infarct size is minimal at this time point. However, in GI-25 and GI-30 hearts, both z-DEVD.fmk and z-LEHD.fmk significantly decreased infarct size (P < 0.05) as determined by TTC staining. In GI-25 hearts, z-DEVD.fmk and z-LEHD.fmk decreased infarct size by ~3.5% and 2.7%, respectively (absolute relationship), whereas in GI-30 hearts z-DEVD.fmk and z-LEHD.fmk decreased infarct size by ~8.3% and 6.1%, respectively (absolute relationship). These results indicate that caspase-mediated apoptosis significantly contributes to total myocardial cell death after GI-20. These results further suggest that caspase-mediated apoptosis increases exponentially as GI time is increased. This is in agreement with earlier investigations by us and others indicating that a direct relationship between infarct size and ischemic time exists (9, 46).

Of importance is our data indicating that, whereas caspase-3 and/or -9 inhibition significantly decreased apoptosis and total infarct size at GI-25 and GI-30, there was no effect on postischemic functional recovery (Table 2 and Fig. 6). These results are in agreement with the studies of Mocanu et al. (29), who reported that in the isolated rat heart infarct size was significantly reduced by 28% using the caspase-3 inhibitor Ac-DEVD.cmk during early reperfusion. These results are in contrast with those of Okamura et al. (33), who have shown that neither a caspase-3 inhibitor (DEVD-aldehyde) nor a caspase-1 inhibitor (YVAD-aldehyde) administered intravenously 5 min before ischemia reduced infarct size, as evaluated by TTC staining. It is most likely that the differences observed in our study are the result of the efficacy of the caspase inhibitors (fmk vs. aldehyde) and the time of administration of inhibitors (reperfusion vs. ischemia). An alternative argument may be made for lack of sufficient recovery time. In this investigation, we used 2 h of...
reperfusion. It is possible that our results indicate that stunning is prolonged and the antistunning effects associated with decreased apoptosis would be evident at some later time point during reperfusion. It is reasonable to assume that decreased infarct size and the positive inotropic effects of salvaged tissue would be beneficial.

In this study, we attempted to determine of the contribution of necrosis and apoptosis in ischemia-reperfusion injury. To do this, we estimated apoptosis using various methods including TUNEL and necrosis by TTC staining. The measurement of apoptosis by TUNEL has been generally accepted by investigators and is the only method for the identification of apoptotic cell evaluation in large tissue samples. TUNEL is a sensitive but nonspecific methodology for the identification of apoptotic nuclei that utilizes the 3’ hydroxyl termini present at DNA strand breaks for the identification of DNA degradation by enzymatic labeling of termini with modified nucleotides. The significance of TUNEL as an indicator of myocyte apoptosis has recently been challenged as terminal deoxynucleotide transferase allows for the labeling of both single-strand (necrosis) and double-stranded (apoptosis) DNA breaks with free 3’ hydroxyl termini, suggesting that TUNEL may not be a specific pathologic indicator and that additional evidence is required to confirm apoptosis (36).

An alternative method for the detection of apoptosis is through the use of DNA gel electrophoresis, but this method is not quantitative (16, 37). Previous reports have indicated that oligonucleotide laddering, a marker of apoptosis, reported to occur from the initial cleavage of nuclear DNA into larger nucleosomal fragments (50–300 kb) before degradation into polynucleosomal fragments (multimers of ~200 bp), is apparent 2–4 h after reperfusion (25, 32). We have previously used DNA fragmentation for the determination of apoptosis in the blood-perfused regional ischemic sheep model (39). However, in the present experiments, we were unable to clearly detect oligonucleotide laddering due to the presence of DNA smear at GI-20, GI-25, and GI-30 (results not shown).

TTC staining has been a standard for the measurement of infarct size and has been used previously for the assessment of infarct size resulting from apoptosis and necrosis (6, 29, 33, 50, 52). The assessment of infarct size by TTC staining is accurate as long as sufficient time has been allowed for the washout of dehydrogenases and other cofactors from apoptotic and necrotic tissue (21). We have previously demonstrated that 2 h of reperfusion is sufficient to determine infarct size in the Langendorff-perfused rabbit heart model (45).

The time of reperfusion may affect TUNEL-positive cell number as previous studies have indicated that apoptosis may occur for several hours after reperfusion (2, 3, 7, 16). In our protocol, we used 3 h of total perfusion including 2 h of reperfusion based on previous studies indicating that perfusion longer than 3 h resulted in decreased myocardial contractile function in Control groups (45). This protocol may have underestimated the apoptotic component at GI-20, GI-25, and GI-30. It is important to note the isolated perfused heart model relies on locally released mediators such as tumor necrosis factor-α to trigger apoptosis and participate in the pathogenesis of necrosis. The absence of blood and blood constituents may have modulated the extrinsic apoptosis response such that no extrinsic apoptosis effects are observed; however, we found no detectable caspase-8 activity or alteration in Fas, FasL, FADD, or caspase-8 cleavage in the in situ pig heart model of acute myocardial infarction (18). The effects of inflammatory mediators were not determined in this model.

The use of crystalloid compared with blood perfusion should also be considered with regard to the overall effect on infarct size. Previous investigation has shown that infarct size in buffer-perfused hearts is significantly greater than in crystalloid-perfused hearts, and as such our results may overestimate the effects of ischemia time on absolute myocardial injury (34, 35).

In summary, our results indicate that myocardial injury results from a significant increase in both necrosis and apoptosis (P < 0.05 vs. Control heart for each), which is evident by TUNEL, TTC staining, and caspase-3-like activity at GI-20. Proapoptotic factors are evident early during ischemia but do not significantly contribute to infarct size before GI-25. The contribution of necrosis to infarct size at GI-20, GI-25, and GI-30 is significantly greater than that of apoptosis. The effects of apoptosis can be significantly decreased by caspase inhibition during early reperfusion, but this protection does not improve immediate postischemic functional recovery.
REFERENCES


