Dopamine Treatment of Postischemic Contractile Dysfunction Rapidly Induces Calcium-Dependent Pro-Apoptotic Signaling

Christof Stamm, MD; Ingeborg Friehs, MD; Douglas B. Cowan, PhD; Hung Cao-Danh, PhD; Yeong-Hoon Choi, MD; Lennart F. Duebener, MD; Francis X. McGowan, MD; Pedro J. del Nido, MD

Background—Ischemia and adrenergic stimulation of cardiomyocyte cultures have been shown to induce apoptotic cell death. We hypothesized that in a model of contractile dysfunction following ischemia, a commonly used catecholamine such as dopamine augments cardiomyocyte apoptosis via activation of calcium-dependent signaling cascades.

Methods and Results—Isolated perfused rabbit hearts were subjected to 45 minutes of normothermic ischemia with cardioplegic arrest. Hearts were reperfused for 120 minutes with unmodified perfusate (control), perfusate containing 20 nM dopamine, dopamine/H11002,3-butanedione monoxime (BDM), a MgATPase-inhibitor, or the calcium-sensitizing inotrope ORG 30029. Ischemia-reperfusion alone caused contractile dysfunction without significant myocardial necrosis (left ventricular pressure-volume curves; 1% triphenyltetrazolium chloride staining; creatine kinase release) or apoptosis (terminal deoxynucleotidyl transferase-mediated nick end labeling [TUNEL] analysis; immunoblotting for poly(ADP-ribose) polymerase [PARP] cleavage; activation of caspases-3, -8, and -9; expression of Bax/Bcl-2). Intracellular calcium [Ca2+], measured by rhod-2 spectrofluorometry was increased in dopamine-reperfused hearts. Although postischemic dopamine treatment improved contractility, the number of apoptotic cardiomyocytes was significantly higher than in untreated postischemic hearts (32.5±9 versus 5.5±1.6/1000 nuclei, P<0.01). Further evidence of dopamine-stimulated apoptosis included PARP cleavage, activation of mitochondrial-derived caspase-9, and the terminal effector caspase-3. Dopamine also increased cellular content of pro-apoptotic Bax while decreasing anti-apoptotic Bcl-2. Simultaneous treatment with BDM suppressed contractility without affecting [Ca2+]i and did not reduce dopamine-stimulated apoptotic markers. When contractility was increased without elevating [Ca2+]i using ORG 30029, no activation of pro-apoptotic signaling cascades was found. Dopamine infusion in nonischemic hearts did not result in cardiomyocyte apoptosis.

Conclusions—Postischemic dopamine treatment of contractile dysfunction activates pro-apoptotic signal cascades, most likely via a calcium-dependent process and mitochondrial damage.

Key Words: ischemia ■ apoptosis ■ inotropic agents ■ calcium

Postoperative myocardial contractile dysfunction is commonly treated with inotropic drugs to maintain adequate cardiac output. Catecholamine-type inotropic drugs improve myocardial contractility mainly by increasing calcium entry into the cell via cAMP-mediated protein kinase A phosphorylation of sarcolemmal L-type calcium channels. It has been well established that calcium cycling is impaired and the free cytosolic calcium concentration is significantly elevated in the postischemic heart.1 When catecholamines are given postischemia, there is the potential for further and sustained elevation of cytosolic calcium resulting in activation of proteolytic enzymes and mitochondrial damage, eventually leading to cell membrane disruption and necrosis. However, mitochondrial calcium accumulation may also lead to opening of the mitochondrial permeability transition pore with release of pro-apoptotic factors from the intermembrane space followed by “programmed” cell death (apoptosis).2,3 In contrast to reversible postischemic contractile dysfunction (myocardial stunning), loss of myocardocytes following a period of ischemia has important consequences for long-term cardiac function, particularly when multiple cardiac procedures are required for correction of congenital defects.

We therefore tested the hypothesis that, in a model of postischemic contractile dysfunction, treatment with a commonly used catecholamine such as dopamine leads to increased cytosolic calcium and activation of pro-apoptotic signaling cascades, resulting in apoptotic cell death and hence loss of cardiomyocytes. Furthermore, we evaluated the role of alterations in excitation-contraction coupling and contractile protein calcium sensitivity in the development of cardiomyo-
cyte apoptosis by using both calcium-sensitizing and calcium-desensitizing agents.

Materials and Methods

Animal Care
All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol was reviewed and approved by the Investigational Animal Care and Use Committee at Children’s Hospital, Boston, Massachusetts.

Perfusion Protocol
New Zealand white rabbits (2.5 kg) were anesthetized by intravenous injection of ketamine (100 mg/kg) and heparin. The hearts were rapidly excised and perfused in the Langendorff mode at constant perfusion pressure with modified Krebs-Henseleit (KH) buffer as previously described in more detail.6,7 The hearts were not paced. After 30-minute stabilization, coronary perfusion was ceased and the hearts were subjected to a period of global ischemia in a heated chamber filled with humidified room air. In preliminary experiments, hearts were subjected to different periods of ischemia at varying temperature with or without cardioplegic arrest, and contractile function and myocardial necrosis were determined. Based on these results, a protocol resulting in postischemic contractile dysfunction without significant myocardial infarction was established. Figure 1 shows that 45-minute ischemia at 35°C with cardioplegic arrest (KH buffer with 22.5 mmol/L K+) resulted in significant postischemic contractile dysfunction without significant myocardial necrosis (ie, myocardial stunning). All ischemia-reperfusion experiments described were conducted according to this protocol.

Experimental Groups
All hearts were subjected to 45-minute normothermic ischemia with cardioplegic arrest and reperfused for 120 minutes. In control hearts (n=6), modified KH buffer was used. Dopamine-treated hearts (n=6) were reperfused with modified KH buffer in which 20 nM dopamine was added to the perfusate 5 minutes into reperfusion and given throughout the entire reperfusion period. In another group of hearts (n=6), 2,3-butanedione monoxime (BDM) at a concentration of 5 mmol/L was added just prior to the beginning of the dopamine infusion. BDM directly inhibits the myosin MgATPase and hence decreases cross-bridge cycling and myocardial contractility.8 The concentration of BDM was chosen because it had no effect on intracellular calcium handling but resulted in a 30% decrease in developed pressure in dopamine-treated hearts. Another group of hearts was treated with the calcium-sensitizing agent ORG 30029 (250 μmol/L, n=4). ORG was added to the perfusate 5 minutes into reperfusion, and the concentration used resulted in a significant increase in contractility without increased intracellular calcium cycling.7 All drugs were also given to nonischemic hearts (n=4 each group) over a period of 120 minutes. Measurements of cardiac function, calcium handling, and activation of pro-apoptotic signal cascades were performed in all groups of hearts.

Function Measurements
Left ventricular (LV) pressure was measured pre-ischemia and in 30-minute intervals during reperfusion. The LV balloon was filled stepwise in increments of 0.1 mL and diastolic and systolic pressures recorded. These data were used to determine the diastolic and systolic pressure-volume relationships. Coronary flow was measured by timed collection of the coronary effluent. Myocardial oxygen consumption (MvO2) was derived from the arteriovenous difference in O2 tension (Stat Profile Plus 9, Nova Biochemical, Waltham, MA), multiplied by coronary flow and divided by dry heart weight.

Calcium Measurements
Measurement of beat-to-beat intracellular calcium transients was performed in a separate set of intact perfused hearts, as we have previously described and validated in detail.4,5 Intracellular calcium ([Ca2+]i) was measured at the end of the reperfusion period with the Ca2+-sensitive dye rhod-2-AM. (Molecular Probes, Eugene, OR). The hearts were loaded with the cell-permeable acetoxymethylester (rhod-2-AM; 0.5 mg/0.25 mL DMSO infused over 2 minutes at 37°C without recirculation) after 100-minute reperfusion followed by a 15-minute washout period. In other experiments, rhod-2 was loaded after 15 minutes of reperfusion and [Ca2+]i was recorded during the onset of inotrope infusion. A modified spectrofluorometer (SLM-Aminco, Springfield, IL) provided excitation light at 524 nm and recorded emission light at 589 nm. Tissue absorbance was quantified using the ratio of scattered excitation light at 524 nm (peak rhod-2 absorbance in myocardial tissue) and 589 nm. The change in absorbance over time was then used to account for differences in dye loading or changes in tissue dye concentration. At the end of each experiment 2,2’dithiodipyridine (100 μmol/L) was infused over a
period of 2 minutes to induce calcium release from the sarcoplasmic reticulum. This was immediately followed by bolus injection of calcium ionophore A23187 (calcimycin) in 10 mL 10% calcium solution to maximize calcium entry from the extracellular space. Fluorescence was recorded with a time resolution of 40 ms during the infusion, and maximum fluorescence ($F_{\text{max}}$) was determined to calculate systolic and diastolic calcium concentration using the following equation:

$$[\text{Ca}^{2+}] = \frac{K_d \times (F_i - F_o)}{(A/A_{\text{max}})(F_{\text{max}} - F_o) - F_i}$$

where $[\text{Ca}^{2+}]$ is the free intracellular calcium concentration, $K_d$ is the dissociation constant for rhod-2 with calcium, $F_i$ is fluorescence at a specific time point, $F_o$ is autofluorescence measured before dye loading, $A_i$ and $A_{\text{max}}$ are tissue light absorbance at the specific time point or at the end of the experiment, respectively.

**Western Immunoblotting**

At the end of the respective perfusion protocol, the hearts were snap frozen in liquid nitrogen and stored at −80°C. LV tissue was homogenized in cold buffer containing 20 mmol/L Tris HCl, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 1 mmol/L PMSF, 25 µg/mL leupeptin, 0.3 mmol/L sucrose at pH 7.4, and centrifuged at 1000g for 5 minutes (supernatant=unfractionated tissue extract). For detection of PARP cleavage, nuclear protein extracts were prepared by resuspension of the pellet in buffer containing 420 mmol/L NaCl, 20 mmol/L HEPES-KOH pH 7.9, 25% glycerol, 1.5 mmol/L MgSO4, 0.1 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L PMSF and incubation for 20 minutes (high-salt extraction) followed by sonication for 60 seconds. Debris was then removed by brief centrifugation (supernatant=nuclear protein extract). Protein samples of 30 µg each were separated by SDS-page gel electrophoresis and transferred to nitrocellulose membranes. Coomassie brilliant blue R-250 staining of gels confirmed equal protein loading. The membranes were incubated with the following primary antibodies: anti-human caspase-9 (polyclonal, recognizes pro-caspase-9 and proteolytic fragment; Calbiochem-Novabiochem Corp., San Diego, CA), anti-human caspase-8 (polyclonal, recognizes proteolytic fragment; Upstate, Waltham, MA), anti-human caspase-3 (polyclonal, recognizes pro-caspase-3 and proteolytic fragment; Upstate, Waltham, MA), anti-human Bax (polyclonal; Upstate), anti-rat/mouse Bcl-2 (polyclonal; R&D Systems, Minneapolis, MN), anti-calf PARP (monoclonal, recognizes full length PARP and proteolytic fragment; Oncogene, La Jolla, CA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody and detection using enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). Laser densitometry was performed to quantify the intensity of the respective bands.

**TUNEL Staining**

LV tissue samples were obtained at the end of the respective perfusion protocol, fixed in 5% paraformaldehyde in phosphate-buffered saline (pH 7.4), paraffin-embedded, and sectioned. TUNEL staining was performed in deparaffinized and rehydrated sections with terminal deoxynucleotidyl transferase-mediated digoxigenin-dNTP (1 hour at 37°C), followed by detection using fluorescein-labeled anti-digoxigenin antibody (Apop tag® kit, Intergen, Norcross, GA). Sections were also stained for nuclei (DAPI) and actin. Both total nuclei and TUNEL-positive nuclei were counted electronically in 3 random fields of vision per tissue section using Metamorph® software, and the results were expressed as number of TUNEL-positive nuclei per 1000 total nuclei.

**Infarct Size**

Creatine kinase (CK) release from the heart was quantified in coronary effluent collected in 5-minute and 30-minute intervals using Oliver and Rosalki’s enzymatic assay (Sigma-Aldrich, St. Louis, MO). At the end of the respective perfusion protocol, 2-mm thick slices of left ventricular myocardium were prepared and incubated in 1% triphenyltetrazolium chloride (TTC) in phosphate-buffered saline at 37°C for 20 minutes. The stained slices were placed on a flatbed scanner and electronic images obtained. The area of TTC-negative-stained infarct regions was measured using Scion image analysis software (Scion Corp., Frederick, MD). Infarct size was quantified for each slide using the formula negative stained area/positive stained area × 100, and the mean value of all slices obtained from 1 ventricle was calculated.

**Statistical Analysis**

Analysis of calcium recordings was performed using Sigma Plot software (version 4.0, SPSS Inc., Chicago, IL). Data are expressed as the mean±standard error and statistical analysis was performed using the SPSS software package (version 9.0, SPSS Inc.). Differences between the groups were tested for significance by one-way ANOVA using Bonferroni’s correction for multiple comparisons. If normal distribution and equal variance testing was passed, Student t test was used to compare individual data sets. A two-tailed probability value of less than 0.05 was considered statistically significant.

**Results**

**Postischemic Function and Calcium Handling**

As shown in Figure 1, 45 minutes of warm ischemia with high-potassium cardioplegic arrest resulted in significantly impaired contractile function at 30-minute reperfusion. After 120 minutes of reperfusion, LV contractility and relaxation had improved but were still depressed. Coronary flow was lower after 30-minute reperfusion compared with pre-ischemia (44±3 mL/min versus 52±7 mL/min, P<0.05), and not different from control after 120 minutes of unmodified reperfusion (47±5 mL/min versus 51±5 mL/min, P=0.6). Myocardial oxygen consumption ($\text{MvO}_2$) throughout reperfusion was not significantly different from nons ischemic hearts, and the relationship of $\text{MvO}_2$ and LV work (assessed as heart rate × developed pressure) indicated contractile inefficiency and oxygen wasting (data not shown). Diastolic cytosolic $[\text{Ca}^{2+}]$ was significantly elevated at 30-minute reperfusion (0.44±0.09 µmol/L versus 0.21±0.05 µmol/L, P<0.02), and the amplitude of the calcium transients was lower than pre-ischemia. After 120-minute reperfusion, however, intracellular calcium handling had essentially normalized (Table 1).

**Dopamine Treatment**

At the onset of dopamine treatment, both the amplitude of the calcium transient and LV developed pressure increased and diastolic pressure decreased rapidly (Figure 2), associated with an increase in heart rate (173±9 bpm versus 138±12 bpm, P<0.01), $\text{MvO}_2$ (0.94±0.12 mL/min/g dry weight versus 0.81±0.5 versus mL/min/g, P<0.05), and coronary flow (59±8 mL/min versus 49±6 mL/min, P<0.02), (all measured at 30-minute reperfusion). After 120-minute reperfusion, the systolic pressure-volume relationship (Figure 3) as well as the metabolic parameters (the Table) demonstrated the ongoing positive inotropic effect, although the diastolic pressure-volume relationship had shifted to the left, indicating impaired myocardial relaxation. After 120-minute reperfusion, calcium transients were higher in dopamine treated than in untreated postischemic hearts, although diastolic and mean $[\text{Ca}^{2+}]$ was significantly elevated, indicating sustained intracellular calcium overload (Figure 4).
Measurements of Cytosolic Calcium, Coronary Flow, and Oxygen Consumption in Nonischemic and Postischemic Hearts Treated with Different Inotropes for 120 Minutes

<table>
<thead>
<tr>
<th></th>
<th>Diastolic [Ca(^{2+})]</th>
<th>[Ca(^{2+})] Amplitude</th>
<th>Heart Rate</th>
<th>CF (mL/min)</th>
<th>MvO(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postischemic hearts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.25±0.08</td>
<td>0.61±0.09</td>
<td>138±12</td>
<td>47±4</td>
<td>0.85±0.06</td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.54±0.17(^*)</td>
<td>0.80±0.07(^*)</td>
<td>164±9(^*)</td>
<td>56±8(^*)</td>
<td>0.92±0.1(^*)</td>
</tr>
<tr>
<td>BDM + Dopamine</td>
<td>0.52±0.16(^*)</td>
<td>0.78±0.06(^*)</td>
<td>169±17(^*)</td>
<td>61±7(^*)</td>
<td>0.84±0.07</td>
</tr>
<tr>
<td>ORG 30029</td>
<td>0.28±0.1</td>
<td>0.58±0.09</td>
<td>141±10</td>
<td>54±5</td>
<td>0.89±0.04</td>
</tr>
<tr>
<td>Nonischemic hearts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.21±0.05</td>
<td>0.53±0.1</td>
<td>144±8</td>
<td>51±5</td>
<td>0.69±0.06</td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.27±0.08</td>
<td>0.71±0.13(†)</td>
<td>171±11(†)</td>
<td>64±7</td>
<td>0.77±0.04(†)</td>
</tr>
<tr>
<td>BDM + Dopamine</td>
<td>0.24±0.06</td>
<td>0.74±0.06(†)</td>
<td>168±4(†)</td>
<td>67±11</td>
<td>0.72±0.03</td>
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<tr>
<td>ORG 30029</td>
<td>0.28±0.02</td>
<td>0.55±0.09</td>
<td>153±7</td>
<td>55±4</td>
<td>0.79±0.07(†)</td>
</tr>
</tbody>
</table>

\([\text{Ca}^{2+}]\) indicates cytosolic calcium (µM); CF, coronary flow (mL/min); MvO\(_2\), myocardial oxygen consumption (mL/min/g dry weight).

\(^*P<0.05\) vs control, \(†P<0.05\) vs nonischemic control.

BDM + Dopamine Treatment
2,3-Butanedione monoxime (5 mmol/L) given after 5-minute reperfusion decreased LV developed pressure by approximately 80% without any measurable effect on intracellular calcium handling (Figure 2). Once dopamine was added to the perfusate, systolic [Ca\(^{2+}\)] rose similar to dopamine-only treatment, and contractility partially recovered. After 120-minute reperfusion, both the systolic and diastolic pressure-volume relationship were shifted to the right, indicating the inhibiting effect of BDM on contractile protein interaction, but diastolic [Ca\(^{2+}\)], was elevated indicating intracellular calcium overload similar to hearts treated with dopamine only (Figure 4). Heart rate and coronary flow were higher than in untreated post-ischemic hearts, but MvO\(_2\) was not increased (Table 1).

ORG 30029 Treatment
ORG 30029 (25 mmol/L) added to the perfusate increased LV developed pressure with slightly longer delay than dopamine, without increasing systolic [Ca\(^{2+}\)]. After 120-minute reperfusion with ORG 30029, the systolic pressure-volume relationship was shifted to the right, indicating a sustained increase in contractility, but neither diastolic [Ca\(^{2+}\)], nor the amplitude of the calcium transient were significantly different from untreated postischemic hearts (Figure 4). The diastolic pressure-volume relationship was shifted to the left, indicating impaired myocardial relaxation, most likely due to the increase in calcium sensitivity of the contractile proteins in the presence of post-ischemic calcium overload. The increase in contractility was not associated with significantly higher heart rate of oxygen consumption, but coronary flow was higher (Table 1).

Postischemic Apoptosis/Necrosis
In hearts subjected to 45-minute ischemia and 120-minute reperfusion, no evidence of cardiomyocyte apoptosis was detected. The average number of TUNEL-positive nuclei was 5.5±1.6/1000 compared with 4.8±0.9/1000 in nonischemic control hearts (\(P=0.7\)) (Figure 5). There was no evidence of PARP cleavage in nuclear protein extracts, and neither caspase-3, caspase-8, or caspase-9 fragmentation was detected (Figure 6). Bcl-2 and Bax expression or ratio was not different from nonischemic control hearts (Figure 6). TTC staining was negative in all hearts, and CK release during reperfusion was not different from untreated postischemic hearts. However, postischemic dopamine treatment was associated with a significantly higher number of TUNEL-positive nuclei (32.5±9/1000, \(P<0.01\)), and PARP fragmen-

Figure 2. Postischemic cytosolic calcium ([Ca\(^{2+}\)]) and left ventricular pressure (LVP) recorded for 120 seconds during the onset of inotrope infusion. Dopamine (20 nM) added to the perfusion buffer; ORG 30029, the calcium sensitizer ORG 30029 (250 µmol/L) added to the perfusate; Dopamine + BDM, 2,3-butanedione monoxime (5 mmol/L) followed by dopamine (20 nM) added to the perfusate. The arrows mark the onset of infusion of the respective drug.
Caspase-3 cleavage was consistently detected in all dopamine-treated postischemic hearts. A caspase-9 cleavage/activation product was clearly identified in all dopamine-treated postischemic hearts, together with a decrease in intensity of the band representing the caspase-9 pro-form. The expression of pro-caspase-8 was not different from that in untreated hearts. A mild increase in optical density of the band representing the caspase-8 cleavage product was noted only in 3 out of 6 dopamine-treated hearts. Bcl-2 expression was decreased, and Bax expression was increased, indicating a shift toward pro-apoptotic events in dopamine-treated myocardium. Neither TTC staining nor CK release showed any evidence of significant myocardial infarction in post-ischemic dopamine-treated hearts. In postischemic hearts treated with dopamine in the presence of BDM, the frequency of TUNEL-positive nuclei was similar to that seen in hearts treated with dopamine alone (38±4/1000, P<0.01 versus Control). PARP cleavage was also detected in the majority of BDM + dopamine-treated hearts. Furthermore, the pattern of caspase-3, caspase-8, and caspase-9 activation/fragmentation was virtually identical to the one seen in hearts treated with dopamine only, and the Bcl-2/Bax expression pattern also indicated a pro-apoptotic state. Again, there was no indication of significant myocardial necrosis as evidenced by negative TTC stains and negligible CK release. In hearts treated with ORG 30029 during reperfusion, TUNEL-positive nuclei were not more frequent than in untreated postischemic hearts (3.4±1.7/1000, P=0.8), and no evidence of PARP fragmentation was detected. No activation or cleavage of caspase-3, caspase-8, or caspase-9 was evident in any of the ORG 30029-treated hearts, and the Bcl-2/Bax expression pattern was consistent with the absence of pro-apoptotic factors. TTC staining was negative throughout, and CK release during reperfusion was not different from controls.

**Nonischemic Hearts**

Untreated control hearts perfused for 150 minutes did not show any decline in LV contractility over time. Mean developed pressure at a diastolic pressure of 5 mmHg was 98±7 mmHg after 30-minute perfusion and 95±10 mmHg after 150-minute perfusion. Coronary flow, MvO₂, and intracellular calcium handling did not significantly change, either. There was no detectable fragmentation of caspase-3, caspase-8, caspase-9, or PARP, and the Bcl-2/Bax expression balance clearly indicated an anti-apoptotic state. There were 4.2±1.3/1000 TUNEL-positive nuclei in untreated, nonischemic hearts. Treatment with dopamine immediately increased the amplitude of the calcium transient, associated with the expected increase in contractility and relaxation. In contrast to the postischemic hearts, there were no signs of intracellular calcium overload after 120-minute perfusion with dopamine, and relaxation was not impaired. The average number of TUNEL-positive nuclei was 5.3±1.1/1000 (P=0.5 versus untreated hearts). Neither caspase cleavage nor PARP fragmentation were detectable, and the expression of Bcl-2 exceeded that of Bax. Concomitant treatment with BDM abolished the positive inotropic effect of dopamine without altering the dopamine-induced changes in calcium handling.
similar to the effect seen in postischemic hearts. Again, there was no evidence for activation of pro-apoptotic signaling cascades. In nonischemic hearts treated with ORG 30029, contractility increased without concomitant lusitropic effect, intracellular calcium handling remained unchanged, and there was no evidence for cardiomyocyte apoptosis.

**Discussion**

In our model of contractile dysfunction following global ischemia with cardioplegic arrest, there was no detectable induction of pro-apoptotic signaling cascades within the first 2 hours of reperfusion. However, when dopamine was used to treat postischemic contractile dysfunction, caspase-9 and caspase-3 fragmentation/activation occurred and Bax expression increased, resulting in nuclear protein (PARP) cleavage and cardiomyocyte apoptosis. This pro-apoptotic state was associated with elevated cytosolic calcium concentration, and occurred even when the dopamine-induced positive inotropy and increased myocardial oxygen consumption were suppressed (BDM). On the other hand, improving left ventricular contractility by increasing contractile protein calcium sensitivity without further elevating cytosolic calcium appeared to prevent caspase activation and nuclear protein breakdown or DNA fragmentation.

There is some evidence that myocardial ischemia-reperfusion may be associated with activation of pro-apoptotic signaling cascades.\textsuperscript{8–11} These studies, however, were performed in models of unmodified ischemia in intact

![Figure 5](image-url)
Figure 6. A: Representative Western immunoblots for caspase-3, caspase-8, caspase-9, Bax, Bcl-2 (all in cytoplasmic protein extracts), and PARP (nuclear protein extract). Postischemic hearts (I/R) and nonischemic hearts (I/R –) were perfused with unmodified perfusate (Control), 20 nM dopamine (Dopamine), 20 nM dopamine and 5 mmol/L BDM (BDM + Dopa), or 250 μmol/L ORG 30029 (ORG 30029). B: Laser densitometry data of apoptosis-related proteins in postischemic hearts. The Y axis indicates arbitrary densitometry units. Solid black bars, control; shaded bars, dopamine; solid gray bars, ORG 30029; dotted bars, dopamine + BDM. Data are mean ± SEM. *P<0.05 versus Control.
hearts or hypoxia in isolated cells. Such protocols are of limited relevance to cardiac surgical procedures. They may also be associated with significant necrosis, which can lead to an overestimation of the extent of cardiomyocyte apoptosis. Furthermore, the functional significance of apoptotic cell death in myocardium that already contains a large number of necrotic cardiomyocytes is uncertain. In this study, we used a model of postischemic contractile dysfunction after cardioplegic cardiac arrest that is not associated with significant cardiomyocyte necrosis or detectable apoptosis induction. Treatment of contractile dysfunction with dopamine, however, clearly led to activation of pro-apoptotic signaling cascades within less than 2 hours, and this effect was also observed even when the positive inotropy was suppressed by direct myosin Mg-ATPase inhibition. Together with the finding that increasing contractility produced by calcium sensitization of the contractile apparatus did not induce apoptotic cell death, this indicates a putative role for sustained elevation of free intracellular calcium for caspase activation followed by apoptotic cell death. This hypothesis is further supported by the evidence suggesting that primarily mitochondrial cytochrome c-activated caspase-9 is activated, not death receptor-associated caspase-8.

Several studies have demonstrated that adrenergic stimulation with high concentrations of catecholamines induces apoptosis in cardiomyocyte cultures. Communal et al demonstrated that norepinephrine induced significant cardiomyocyte apoptosis via protein kinase A activation and increased calcium influx. Saito et al have shown that beta-adrenergic stimulation with isoproterenol also induced cardiomyocyte apoptosis through an increase in intracellular calcium. They demonstrated that isoproterenol modules phosphorylation of the pro-apoptotic Bcl-2 family protein Bad as well as cytochrome c release from mitochondria via calcineurin activation. Inhibition of calcium influx with nifedipine as well as pharmacologic or transgenic calcineurin ablation effectively prevented isoproterenol-induced apoptosis. These results on the role of calcium are consistent with our findings in post-ischemic intact hearts. Xi et al suggested that dystrophin sensitization of the contractile apparatus did not induce apoptotic cell death, this indicates a putative role for sustained elevation of free intracellular calcium for caspase activation followed by apoptotic cell death. This hypothesis is further supported by the evidence suggesting that primarily mitochondrial cytochrome c-activated caspase-9 is activated, not death receptor-associated caspase-8.

There is also evidence supporting a protective role of catecholamines in the development of cardiomyocyte apoptosis. Henaff et al have demonstrated that a low dose of epinephrine protects cardiomyocytes from extracellular signal-regulated kinase (ERK)-induced apoptosis. A 100-fold higher epinephrine concentration, however, induced marked apoptosis even in the absence of ERK activation. Shneyvays et al showed suppression of adenosine A3 receptor-induced cardiomyocyte apoptosis by isoproterenol, probably through a cAMP-independent mechanism. Some of the apparent discrepancies regarding catecholamine stimulation and cardiomyocyte apoptosis may be explained by differential effects of alpha- and beta-adrenoceptors. Iwai-Kanai et al have demonstrated apoptosis-induction by specific beta-adrenoceptor stimulation or direct cAMP elevation, whereas alpha-adrenergic stimulation inhibited the cAMP-induced cardiomyocyte apoptosis. Zaugg et al further defined the responsible adrenoreceptor and showed that cardiomyocyte apoptosis is selectively mediated by beta-1-adrenoceptors. Few studies have addressed catecholamine-induced cardiomyocyte apoptosis in intact hearts or in vivo models. Shizukuda et al treated rats with isoproterenol for 24 hours and found an increased number of TUNEL-positive cardiomyocytes independent from tachycardia or ventricular hypertrophy. Vatner et al described a transgenic mouse model overexpressing cardiac G(s alpha), resulting in chronically increased adenyl cyclase activation, and these mice also developed dilated cardiomyopathy with significant cardiomyocyte apoptosis. In addition to cardiomyocytes, other cardiac cell types such as coronary endothelial cells are also susceptible to catecholamine-induced apoptosis.

We chose to test the apoptosis-inducing effects of dopamine because it is routinely used clinically to treat early postoperative moderate or severe contractile dysfunction, as well as to improve renal blood flow and diuresis. Dopamine stimulates a variety of receptors expressed in the heart, including beta-1-receptors, alpha-1-receptors, and dopamine-1-receptors. We demonstrated that dopamine significantly increased cytosolic calcium in association with induction of pro-apoptotic signaling cascades, particularly those requiring mitochondrial damage. Intracellular calcium overload and increased energy requirement for calcium extrusion may eventually result in mitochondrial calcium accumulation and opening of the permeability transition pore with release of cytochrome c leading to caspase activation. These events are difficult to demonstrate in intact heart models but the link between intracellular calcium, mitochondria, and activation of pro-apoptotic signaling cascades has been readily demonstrated by others. Interestingly, dopamine is also known to specifically induce apoptosis in other cell types. In fact, one current hypothesis regarding the development of Parkinson’s disease suggests that neuronal apoptosis is due to excessive oxidative stress generated by oxidation of endogenous dopamine; 6-hydroxydopamine is a neurotoxin commonly used in the induction of experimental Parkinson’s disease.

Protein kinase C (PKC) is a key regulator of mitochondrial function, and its activation by phosphorylation is involved in the control of mitochondrial membrane potential and calcium leak. In this study, we show that PKC inhibition with staurosporine or chelerythrine significantly reduced pro-apoptotic signaling cascades and caspase activation in response to dopamine, suggesting a role for PKC in the regulation of mitochondrial calcium accumulation and caspase activation. Furthermore, the findings that increasing contractility produced by calcium sensitization of the contractile apparatus did not induce apoptotic cell death, while dopamine induced apoptosis, indicate that dopamine-induced apoptosis is mediated by a distinct signaling pathway involving PKC activation.

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References
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