Myocardial Protection and Vascular Biology

Molecular Indices of Apoptosis After Intermittent Blood and Crystalloid Cardioplegia

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Background—We investigated whether intermittent blood and crystalloid cardioplegia differentially affect myocardial apoptosis and apoptosis gene-related proteins.

Methods and Results—Rabbit hearts were perfused with Krebs-Henseleit buffer on a Langendorff apparatus. Control hearts (n=6) were perfused for 120 minutes without cardioplegic ischemia. Hearts were arrested for 60 minutes with warm (37°C) crystalloid cardioplegia (iW-CCP) (n=8) or with warm blood cardioplegia (iW-BCP) (n=8) administered intermittently. In cold (0 to 4°C) groups, hearts were arrested for 60 minutes with cold crystalloid cardioplegia (iC-CCP; n=8) or with cold blood cardioplegia (iC-BCP; n=6) administered intermittently. The hearts were reperfused for 30 minutes with Krebs-Henseleit buffer. iC-BCP significantly preserved the recovery of left ventricular and microvascular function compared with the other 3 experimental groups. There were no significant differences in total protein levels of caspase 3, Bcl-2, Bad, and Bax among the groups. iC-BCP significantly induced greater phosphorylation of Bad (5.6±0.8-fold) as compared with the other 3 groups (3.4±0.6-fold in iC-CCP, P<0.05; 2.5±0.3 in iW-BCP, P<0.05; and 1.4±0.2 in iW-CCP, P<0.01). iC-BCP induced less caspase 3 activation and apoptosis than the other 3 groups.

Conclusions—iC-BCP is superior to the other cardioplegic solutions in increasing the phosphorylation of Bad, inhibiting the activation of caspase 3, and preventing apoptosis. These effects of iC-BCP were associated with preserved left ventricular function and endothelium-dependent relaxation of coronary microvessels. (Circulation. 2005;112[suppl I]:I-184–I-189.)

Key Words: apoptosis ■ cardioplegia ■ microcirculation ■ reperfusion ■ transplantation

Apoptosis and necrosis are two different forms of cell death. In contrast to necrosis, apoptosis is a “programmed cell death” and is associated with nuclear alterations, such as chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies. Apoptosis has been considered as one mechanism of cell loss during ischemia/reperfusion (I/R) injury. However, the mechanism underlying I/R-induced apoptosis remains incompletely elucidated. It has been shown that I/R-induced apoptosis is mainly regulated by the Bcl-2 family of proteins and cysteine protease family of caspases. Bad and Bax are proapoptotic Bcl-2-family proteins, whereas, phosphorylation of Bad inhibits its binding to and inactivation of antiapoptotic Bcl-2. Thus, increases in phosphorylation of Bad and/or expression of Bcl-2 would prevent myocardial apoptosis.

Recent studies have also demonstrated that hyperkalemic cardioplegic ischemia during open heart surgery was associated with the induction of endothelial cell and cardiomyocyte apoptosis in animals and humans. Cardioplegia arrest induced apoptosis signal cascades in endothelial cells and cardiac myocytes in the human myocardium. These findings suggest that apoptosis may play an important role in myocardial stunning after open heart surgery. Continuous blood cardioplegia has been reported to be superior to continuous crystalloid cardioplegia in inhibiting myocardial apoptosis during ischemic arrest. Intermittent crystalloid cardioplegia or intermittent blood cardioplegia has been commonly used for myocardial preservation during open heart surgery. We have shown previously that intermittent blood cardioplegia is better than intermittent crystalloid cardioplegia in preserving myocardial function and endothelium-dependent microvascular response. However, it has not been investigated whether intermittent blood or crystalloid cardioplegia may also affect molecular indices of apoptosis. Thus, the objective of this study was to determine the possible differential effects among intermittent warm crystalloid cardioplegia (iW-CCP), intermittent warm blood cardioplegia (iW-BCP), intermittent cold crystalloid cardioplegia (iC-CCP), and intermittent cold blood cardioplegia (iC-BCP) on the expression of Bcl-2-family proteins, the activation of caspase 3, and apoptosis. In addition, we also investigated the effects of these cardioplegic protocols on the recovery of LV and microvascular endothelium function.
Methods
All of the 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 conforms to the National Institutes of Health guidelines regulating the care and use of laboratory animals.

Experimental Model
New Zealand white rabbits (1.5 to 2.5 kg) were used in this study (Millbrook Farm). Rabbis were anesthetized with ketamine (35 mg/kg) and xylazine (2.5 mg/kg. IM) and anticoagulated with heparin (2000 U/kg, IV); the heart was rapidly exposed and excised; the aorta was cannulated and then mounted in an organ chamber on a Langendorff perfusion system. The heart was retrogradely perfused at 70 mm Hg with a modified Krebs-Henselet buffer (KHB) with the following composition (mmol/L): NaCl 118, NaHCO3 25, KHPO4 1.2, KCl 4.7, MgSO4 1.2, CaCl2 1.8, and glucose 11.0. The KHB was equilibrated with 95% O2 and 5% CO2, adjusted to a pH of 7.35 to 7.4 at 37°C and filtered with a 5-μm filter (Gilman Scientific, Inc.).

Measurements
Isovolumetric measurement of left ventricular (LV) performance was made using a compliant latex balloon connected to a pressure transducer, inserted in the LV across the mitral valve. The LV balloon was filled with saline. The volume of the balloon was kept constant during the entire experiment. LV performance was assessed by measurement of LV systolic pressure (LVSP, mm Hg) and LV end-diastolic pressure (mm Hg; LVSP – LV end-diastolic pressure=LV-developed pressure (LVDP)). Positive and negative pressure equilibrated with 95% O2 and 5% CO2, adjusted to a pH of 7.35 to 7.4 at 37°C and filtered with a 5-μm filter (Gilman Scientific, Inc.).

Experimental Protocols
After 30 minutes of equilibration, hearts were divided into the following 3 groups. The control group was additionally buffer-infused with either iW-CCP (n=8) or iW-BCP (n=8) every 20 minutes during 60 minutes of hypothermic ischemia. The cold cardioplegic groups, hearts were initially infused with 50 mL of 0 to 4°C cardioplegic infusion by 30% to 50% of the baseline diameter. Once the steady-state tone was reached, the dose responses to nitroprusside (SNP, 1 nmol/L to 100 nmol/L) and adenosine 5’-diphosphate (ADP; 1 nmol/L to 100 μmol/L) were applied extraluminally. SNP, a nitric oxide (NO) donor, directly relaxes vascular smooth muscle via an endothelium-independent cGMP-mediated pathway. ADP, an endothelium-dependent vasodilator, relaxes vascular smooth muscle via stimulation of endothelial NO synthase, which releases NO. One or 2 interventions were performed on each vessel. The vessels were washed 3 times with KHB and allowed to equilibrate drug free for 15 to 30 minutes between interventions.

Immunoblotting
Total protein was fractionated on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore; Millipore Corporation). Membranes were incubated with rabbit polyclonal anti-Bcl-2, Bad, and Bax (Cell Signaling Tech, Inc.); mouse monoclonal anti-phospho-Bad (Cell Signaling Tech, Inc.); and rabbit polyclonal anti-caspase 3 (Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. The membranes were incubated with the appropriate secondary antibody either a sheep antirabbit or sheep antimouse IgG (Jackson Immunolabs) dilution conjugated to horseradish peroxidase. Immune complexes were visualized using the enhanced chemiluminescence detection system, and bands were measured by densitometric analysis of autoradiographic films.

In Vitro Coronary Microvessel Studies
Coronary artery microvessels (100 to 180 μm in internal diameter) from the LV free wall were dissected under a ×10 to ×60 microscope (Olympus Optical). Microvessels were placed in a microvessel chamber, cannulated with dual glass micropipettes measuring 40 to 80 μm in diameter, and secured with 10-0 nylon monofilament sutures (Ethicon, Inc.). Oxygenated (95% O2 and 5% CO2), warmed KHB (37°C) was continuously circulated through the microvessel chamber and a reservoir containing 100 mL. The vessels were pressurized to 40 mm Hg in a no-flow state by using a burette manometer filled with a KHB. With an inverted microscope (×40 to ×200, Olympus CK2, Olympus Optical) connected to a video camera, the vessel image was projected onto a television monitor. An electronic dimension analyzer (Living System Instrumentation) was used to measure the internal lumen diameter and wall thickness. Vessels were allowed to bathe in the organ chamber for at least 30 minutes before a pharmacologic intervention.

Microvessel Study Protocols
After equilibration in the organ chamber, the coronary microvessels were constricted with the thromboxane A2, analog U46619 (1 μmol/L) by 30% to 50% of the baseline diameter. Once the steady-state tone was reached, the dose responses to nitroprusside (SNP, 1 nmol/L to 100 μmol/L) and adenosine 5’-diphosphate (ADP; 1 nmol/L to 100 μmol/L) were applied extraluminally. SNP, a nitric oxide (NO) donor, directly relaxes vascular smooth muscle via an endothelium-independent cGMP-mediated pathway. ADP, an endothelium-dependent vasodilator, relaxes vascular smooth muscle via stimulation of endothelial NO synthase, which releases NO. One or 2 interventions were performed on each vessel. The vessels were washed 3 times with KHB and allowed to equilibrate drug free for 15 to 30 minutes between interventions.

Caspase 3 Activity
Caspase 3 activity in the LV tissue was measured with a caspase 3-like activity assay kit (BioVision) according to the manufacturer’s directions. LV tissue (50 mg) was homogenized in lysis buffer followed by centrifugation (16,000×g, 4°C, 10 minutes). The supernatants were incubated with reaction buffer and DEVD-7-amino-4-trifluoromethyl coumarin substrate at 37°C for 60 minutes. Caspase 3-like activity was detected in a luminescence spectrometer (Perkin Elmer, LS50B, EG&G). The fold-increase in caspase 3 activity was determined by comparing these results with the level of the uninhibited controls.

TUNEL Staining
Heart ventricular tissues were fixed in formalin for 24 hours, embedded in paraffin, and sectioned. The apoptotic cells were identified by TUNEL using an apoptosis detection kit according to the manufacturer’s protocol (Chemicon Inc.). Ten photographs (magnification, ×200) of each tissue section were taken. The number of cardiomyocytes with clear nuclear staining, indicating apoptosis, was expressed as the number of TUNEL-positive cells per total nuclei, respectively.

Drugs
U46619, SNP, and ADP were obtained from Sigma Chemical. All of the drugs were dissolved in ultrapure distilled water. All of the solutions were prepared on the day of the study.
The recovery of LVDP, greater in iW-BCP-perfused hearts than in iW-CCP-perfused hearts (Figure 1A). The mean concentrations required to obtain 10% of contraction after the application of the thromboxane A2 analog U46619 was 37 ± 5 mol/L in the iC-CCP group, and 41 ± 10 mol/L in the control group, 146 ± 8 mol/L in the iW-CCP group, and 142 ± 11 mol/L in the iW-BCP group. The percentage of contraction after the application of the thromoxane A2 analog U46619 was 37 ± 5% in the control group, 41 ± 5% in the iW-CCP group, 33 ± 4% in the iW-BCP group, 40 ± 6% in the iC-CCP group, and 31 ± 4% in the iC-BCP group. The mean concentrations required to obtain 10% of contractions were 5 × 10^{-5} mol/L in the control group, 1 × 10^{-5} mol/L in the iW-CCP group, 2 × 10^{-5} mol/L in the iW-BCP group, 3 × 10^{-5} mol/L in the iC-CCP group, and 4 × 10^{-5} mol/L in the iC-BCP group.

**Endothelium-Independent and Dependent Relaxation**

The endothelium-independent relaxations of coronary microvessels to SNP were similar in the control, iW-CCP, iW-BCP, iC-CCP, and iC-BCP groups, indicating no changes in efficacy of smooth-muscle responsiveness (Figure 1A). The endothelium-dependent relaxation of microvessels to ADP was significantly impaired in iC-CCP as compared with the control response (P<0.05) (Figure 1B). The relaxation of microvessels to ADP was slightly impaired in iC-BCP compared with the control response (Figure 1B).
Bcl-2-Family Protein Expression and Bad Phosphorylation

The changes in expression of Bcl-2, Bax, Bad, and phosphorylated Bad proteins were analyzed by Western blot assay (Figure 2A–2D). There were no significant differences among the groups in the expressions of the Bcl-2, Bax, and Bad proteins after cardioplegic I/R (Figure 2A–2C). All of the experimental cardioplegic solutions induced significant phosphorylation of Bad at Ser112 compared with the control (P<0.05) (Figure 2D). The phosphorylated Bad levels in iW-CCP (1.4-fold) and iW-BCP (2.5-fold) were significantly less than those in iC-CCP (3.4-fold; P<0.05) and iC-BCP (5.6-fold; P<0.05). Furthermore, iC-BCP caused significantly greater phosphorylation of Bad than iC-CCP (5.6-fold versus 3.4-fold; P<0.05) (Figure 2D).

Caspase 3 Cleavage and Activity

There were no alterations in total caspase 3 proteins among control, iW-CCP, iW-BCP, iC-CCP, and iC-BCP (Figure 3A). The active p17 subunit of caspase 3 was detected and significantly increased after iW-CCP, iW-BCP, and iC-CCP as compared with that in control (P<0.05) (Figure 3B). However, a cleavage form of caspase 3 was not detected in iC-BCP. The activated caspase 3 in iW-CCP (7.25-fold) was significantly higher than those in iW-BCP (4.8-fold; P<0.05) and iC-CCP (2.7-fold; P<0.05). The activated caspase 3 in iC-CCP (2.7-fold) was higher in iC-BCP (1.1-fold; P<0.05) (Figure 3C).

TUNEL Staining

Figure 4A–4E shows the TUNEL-positive myocyte nuclei photographed at a magnification of ×200. Figure 4 shows the percentage of TUNEL-positive nuclei in LV sections in the 3 groups. The percentage of TUNEL-positive myocyte nuclei was significantly increased in iW-CCP (Figure 4B) and iW-BCP (Figure 4C) groups compared with the control group (P<0.05 and P<0.05, respectively). iW-BCP (Figure 4D) induced less apoptosis than iC-CCP (Figure 4B). There were no TUNEL-positive cells in the iC-BCP (Figure 4D) and iC-CCP (Figure 4E) groups. The number of TUNEL-positive myocyte nuclei was significantly reduced in iC-BCP and iC-CCP as compared with iW-BCP and iW-CCP (P<0.05) (Figure 4F).

Discussion

Previous studies have demonstrated that microvascular endothelium and LV function are impaired after exposure to cardioplegia and cardiopulmonary bypass.15 The present data are consistent with those previous findings, indicating that hyperkalemic cardioplegia severely reduces the recoveries of microvascular endothelium-dependent relaxation and LV function after ischemic arrest and reperfusion. Although iC-CCP induced better recoveries of LV and vascular function than iW-CCP, iC-CCP is inferior to iC-BCP. Furthermore, the present study also shows that iC-BCP is superior to iW-BCP in preserving the recoveries of microvascular endothelium-dependent relaxation and myocardial function. This study simulates the inadequate cardiac protection conferred by iC-CCP or iW-CCP leading to postischemic LV and microvascular dysfunction, suggesting that intermittent hyperkalemic cardioplegia has a direct and severe effect on the recoveries of microvascular function.

Recent studies indicate that apoptosis is induced in the animal and human cardioplegic/reperfused heart, suggesting the apoptosis may play a key role in myocardial dysfunction after open heart surgery.10–13 These studies have demonstrated that CCP infusion in pigs and humans during cardio-

Figure 2. Immunoblots and graphs showing the protein levels and fold increases in total Bcl-2 (A), Bax (B), Bad (C), and phosphorylated Bad (D) from control, iW-CCP, iW-BCP, iC-CCP, and iC-BCP groups, respectively. Values are mean±SEM; n=6 for each group: *P<0.05 vs control; †P<0.05 vs iW-CCP; ‡P<0.05 vs iW-BCP; § P<0.05 vs iC-CCP.
pulmonary bypass impairs the morphological integrity of the coronary endothelium and induces cardiomyocyte apoptosis.\textsuperscript{10–13} In the present study, we also found that iW-CCP and iW-BCP infusion results in the induction of myocytes apoptosis. The prevention of caspase-3 activation and apoptosis by iC-BCP will possibly contribute to improved short-term LV and long-term microvascular function after surgery. However, we did not find TUNEL-positive cells in iC-CCP- and iC-BCP-treated hearts.

The Bcl-2 family of proteins regulates apoptosis by controlling mitochondrial permeability and the release of cytochrome c.\textsuperscript{3,16–19} The antiapoptotic proteins of Bcl-2 reside in the outer mitochondrial wall and inhibit cytochrome c release. The proapoptotic Bcl-2 proteins, such as Bad and Bax, reside in the cytosol but translocate to mitochondria and form a proapoptotic complex with Bcl-xl or Bcl-2. This translocation is inhibited by survival factors that induce phosphorylation of Bad, leading to its cytosolic sequestration.\textsuperscript{7,8} Thus, phosphorylation of Bad may promote cell survival.\textsuperscript{5–9} Kuwahara et al\textsuperscript{6} reported that cardiotrophin-1, a novel member of the interleukin-6 family of cytokines, phosphorylated Bad and prolonged cell survival. Jonassen et al\textsuperscript{7} reported that cardioprotection by insulin is possibly mediated, in part, via Bad phosphorylation. Baines et al\textsuperscript{8} claimed that overexpression of protein kinase C-\(\gamma\) is cardioprotective and leads to increased phosphorylation of Ser112 on Bad. Steenbergen et al\textsuperscript{18} recently found a significant decrease in phospho-Bad (Ser112) in failing hearts, and this action was also consistent with a proapoptotic shift in heart failure. Moreover, the present data indicate that Bad was phosphorylated at Ser112 after intermittent cardioplegic ischemia and subsequent reperfusion. iC-BCP caused greater phosphorylation of Bad at Ser112 than the other 3 cardiologic solutions. The enhanced phosphorylation of Bad by iC-BCP may contribute to its antiapoptotic effects.

The ratio of Bcl-2:Bax protein has been also suggested to determine survival or death after I/R. It has been reported that I/R significantly decreased the expression of Bcl-2 and increased the expression of Bax in the myocardium.\textsuperscript{17,19} In addition, overexpression of Bcl-2 attenuated apoptosis and protected against myocardial I/R injury.\textsuperscript{5} In contrast, we did not find any change in the expression of Bcl-2, Bad, and Bax proteins in the cardioplegic I/R heart. These discrepancies between the others and the present study may be because of differences in the animal models used, such as in vitro versus in vivo, global versus regional ischemia, cardioplegia versus noncardioplegic ischemia, or short-term (30 minutes) versus long-term reperfusion (several hours).

I/R stress may induce the formation of proapoptotic Bcl-2-family protein complexes in mitochondria, which, in turn, leads to the release of cytochrome c.\textsuperscript{17,19} The released cytochrome c, together with dATP, apoptotic protein activating factor-1, and caspase 9, forms the apoptosome resulting in the subsequent processing and activation of death-effector caspases, such as caspase 3.\textsuperscript{17} The active p17-subunit of caspase 3 was detected after iW-CCP, iW-BCP, and iC-CCP, indicating the proteolytical activation of caspase 3. In contrast, the cleaved form of caspase 3 was not detected after iC-BCP. In the present study, we also observed that terminal
caspase 3 was activated after cardioplegic arrest and reperfusion.

In conclusion, intermittent cardioplegic ischemia does not alter the expression of Bcl-2-family proteins but induces the phosphorylation of Bad and activation of caspase 3. These results suggest that changes in phosphorylation or translocation of the Bcl-2-family proteins, rather than its total protein amount, may be the primary indicators of apoptosis induction. iC-BCP is superior to the other 3 cardioplegic solutions in increasing phosphorylated Bad and in inhibiting the activation of caspase 3, which were associated with an improved LV function and endothelium-dependent relaxation of coronary microvessels. All of the functional deterioration is not totally related to apoptosis; rather, necrosis and other I/R-induced changes likely contribute to most of the observed functional change. The changes in apoptosis may not only contribute to short-term functional deterioration but, more importantly, may also contribute to the long-term beneficial effects. Thus, this may affect clinical practice, mainly, by the prevention of apoptosis. This approach may be a novel method to improve both short-term and long-term myocardial preservation after cardioplegia. However, the cardioplegic solution used in the present study was different from that routinely used in clinical applications. Thus, the comparison to other more clinically applicable cardioplegia formulations should be made with caution.

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References
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