Bradykinin Preconditioning Improves the Profile of Cell Survival Proteins and Limits Apoptosis After Cardioplegic Arrest

Jun Feng, MD, PhD; Cesario Bianchi, MD, PhD; Jennifer L. Sandmeyer, BS; Frank W. Sellke, MD

Background—We hypothesized that preconditioning the heart with bradykinin (BK) would improve the profile of antiapoptotic proteins and inhibit myocardial apoptosis.

Methods and Results—Eighteen rabbit hearts were retrogradely perfused with Krebs-Henseleit buffer (KHB). Six control hearts were perfused with KHB for 90 minutes without cardioplegia ischemia. Six hearts were arrested for 30 minutes (37°C) with crystalloid cardioplegia (CCP). Six BK preconditioning (BKPC) hearts received a 10-minute coronary infusion of 10^{-8} M BK-enriched KHB followed by a 5-minute recovery period and were then arrested for 30 minutes with CCP. The hearts were reperfused for 30 minutes with KHB. BKPC significantly improved the recovery of left ventricular pressure (73 ± 5 versus 51 ± 4 mm Hg; P<0.05) and reduced the percentage of myocardial apoptosis (3.4 ± 0.3% versus 1.2 ± 0.2%; P<0.05) as compared with CCP. There were no significant differences in total protein levels of caspase 3, Bcl-2, Bad, and Bax, among the groups. Both BKPC and CCP induced phosphorylation of Bad at Ser^{12}, but the BKPC group had higher phosphorylated Bad than CCP (4.4 ± 0.5 versus 2.0 ± 0.3; P<0.05). Both BKPC and CCP alone increased caspase 3 cleavage and activity as compared with controls (P<0.05 and P<0.01, respectively), but BKPC caused less cleavage and activation of caspase 3 than CCP alone (P<0.05).

Conclusions—BKPC increased Bad phosphorylation, inhibited caspase 3 activation, and limited myocardial apoptosis, which were associated with improvement of left-ventricular performance. These results identify novel molecular mechanisms underlying the protective effects of BKPC during cardiac surgery. (Circulation. 2005;112[suppl I]:I-190–I-195.)

Key Words: apoptosis ■ bradykinin ■ cardioplegia ■ ischemia ■ protein

Ischemia preconditioning (IPC) is a phenomenon whereby exposure of the myocardium to a brief period of ischemia and reperfusion (I/R) markedly limits myocardial necrosis induced by subsequent prolonged I/R.3 Recent studies have suggested that IPC prevents myocardial apoptosis.2 Apoptosis and necrosis are two different types of cell death. In contrast to necrosis, apoptosis is a “programmed cell death,” and it is associated with nuclear alterations, such as chromatin condensation and DNA fragmentation, and with the formation of apoptotic bodies.3 Apoptosis has been considered as one of the mechanisms of cell loss during I/R injury.3 Although the mechanism responsible for I/R-induced apoptosis is incompletely understood, it has been demonstrated that I/R-induced apoptosis is mainly regulated by the Bcl-2 family of proteins and cysteine protease family of caspasess.3,4

Several cell survival factors have been shown to improve the profile of antiapoptotic proteins and prevent myocardial apoptosis.4 Bradykinin (BK) has been widely accepted as one of the endogenous protective factors to improve-left ventricular (LV) performance, attenuate myocardial necrosis, and reduce the incidence of arrhythmias after myocardial I/R.5,6 However, it is not known if BK also affects I/R-induced apoptosis-related proteins and myocardial apoptosis. Therefore, we hypothesized that BKPC would improve the profile of antiapoptotic proteins and prevent apoptosis after myocardial I/R.

Methods

Experimental Model

Animals were cared for in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and Harvard Medical Area Standing Committee approved the protocols used in this study.

New Zealand white rabbits (1.5 to 2.5 kg) were used in this study (Millbrook Farm). Rabbits were anesthetized with ketamine (35 mg/kg) and xylazine (2.5 mg/kg IM) and anticoagulated with heparin (2000 U/kg IV), after which the heart was rapidly exposed. The aorta
was cannulated, and the heart was retrogradely perfused in situ to avoid ischemia. The heart was then excised and mounted in an organ chamber on a Langendorff perfusion system. The heart was retrogradely perfused at 70 mm Hg with a modified Krebs-Henseleit buffer (KHB) with the following composition (mmol/L): 118 NaCl, 25 NaHCO3, 1.2 KHP04, 4.7 KCl, 1.2 MgSO4, 1.8 CaCl2, and 11 glucose. 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.). Right ventricular myocardial temperature was measured with a thermistor needle probe (Mallinckrodt, Inc.) and was maintained at 37°C throughout the experiment. Our Langendorff apparatus permits instantaneous change of the perfusion fluids from standard KHB to one containing different pharmacological substances or cardioplegia solution by adjusting an inlet valve to the aortic perfusion cannula.

**Measurements**

Isovolumetric measurement of LV performance was made using a compliant latex balloon connected to a pressure transducer inserted in the LV across the mitral valve. A calibrated syringe attached to the pressure transducer system was used to fill the balloon with a volume of saline needed to maintain a LV end diastolic pressure (LVEDP, mm Hg) of 5 mm Hg during the measurement of baseline LV performance. This same balloon volume was used for subsequent measurements of LV performance after reperfusion. LV performance was assessed by measurement of LV systolic pressure (LVSP, mm Hg) and LVEDP [mm Hg, LVSP - LVEDP = LV developed pressure (LVDVP)]. Positive and negative first derivatives of LVSP (+dP/dt and -dP/dt, mm Hg/s) were calculated as indices of ventricular contractility and compliance, respectively. Analog pressure data from the LV balloon were amplified and converted to pressure and analog data from the LVSP. A digital signal for on-line data recording and computation (Gould-PONEMAN, Gould). Continuous pressure measurements were sampled at specific time points in each experiment. Coronary flow (CF; mL/min) was measured by timed collection of effluent from the right ventricle exiting the heart from the severed pulmonary artery. Hearts failing to generate a LVDP >80 mm Hg or a CF <25 mL/min during the stabilization phase of the experiment were excluded from additional study.

**Experimental Protocols**

After 30 minutes of equilibration, hearts were divided into the following 3 groups. The control group was additionally buffer-perfused for 60 minutes without cardioplegic ischemia (n = 6). In the crystalloid cardioplegia (CCP) groups, the hearts were arrested for 30-minute ischemia with CCP (n = 6). In the BKPC group, hearts received a 10-minute coronary perfusion of 10–8 M BK-enriched KHB, followed by a 5-minute recovery period, and were then arrested for 30 minutes with CCP (BKPC + CCP; n = 6). The composition of the crystalloid cardioplegic solution was as follows (mmol/L): 121 NaCl, 25 KCl, 12 NaHCO3, 1.5 CaCl2, 16 MgCl2, and 11.1 glucose (pH 7.6). A single dose (50 mL) of cardioplegia solution was infused at 60 mm Hg via a separate perfusion column. After a total of 30 minutes of cardioplegic arrest, the hearts from the CCP and BKPC groups were reperfused for 30 minutes with KHB. At the conclusion of reperfusion, the hearts were then excised, and the LV-free wall was cut into several pieces for apoptosis analysis and molecular comparisons.

**Immunoblotting**

Whole-cell lysates were isolated from the homogenized LV samples (100 mg) with a radioimmunoprecipitation assay (Biotech) and centrifuged at 12,000 × g for 10 minutes at 4°C to separate soluble from insoluble proteins. The supernatant protein concentration was measured spectrophotometrically at a 595-nm wavelength with a DC protein assay kit (Bio-Rad). Forty micrograms of total protein were fractionated on 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation) using a semidry transfer apparatus (Millipore). All of the incubations were at room temper-
Effects of BKPC on Bcl-2-Family Protein Expression and Bad Phosphorylation

The changes in expression of Bcl-2, Bad, and Bax proteins were analyzed by immunobloting (Figure 1a through 1c). Densitometrically, there were no significant differences in expression of the Bcl-2, Bad, and Bax proteins after I/R among the groups. CCP induced phosphorylation of Bad at Ser112 as compared with control, but BKPC caused greater phosphorylation of Bad (4.4-fold versus 2.0-fold; \( P<0.05 \)) (Figure 1d) than CCP.

BKPC Inhibits Caspase 3 Cleavage and Activation

There were no alterations in total caspase 3 proteins among control, BKPC, and CCP (Figure 2a). Both BKPC and CCP induced caspase 3 cleavage, a prerequisite for caspase 3 activation, but CCP caused more caspase 3 cleavage than BKPC (6.2±0.4-fold versus 2.7±0.3-fold, respectively; \( P<0.05 \)) (Figure 2b). Caspase 3-like activity in the myocardium was significantly increased after CCP-induced I/R compared with the control. BKPC significantly inhibited the CCP-induced activation of caspase 3 (Figure 2c).

BKPC Reduces Myocardium Apoptosis

Figure 3a through 3c illustrates the TUNEL-positive myocyte nuclei photographed at a magnification of \( \times200 \). 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 the CCP group compared with the control group (\( P<0.05 \)). The number of TUNEL-positive myocyte nuclei was significantly reduced by BKPC as compared with CCP (\( P<0.05 \)).

Discussion

BK has been considered as one of the mediators or triggers for early and delayed IPC.\(^7\) Pretreating the heart with BK before crystalloid cardioplegic ischemia significantly im-

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<th>Hemodynamic Parameters in Control, CCP, and BKPC Groups</th>
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<td>Variables</td>
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Values are mean±SEM, \( n=6 \), for each group. RP indicates reperfusion.

\( ^*P<0.05 \) vs initial; \( ^†P<0.05 \); \( ^‡P<0.05 \); \( ^§P<0.05 \) vs CCP.

Figure 1. ImmunobLOTS showing total Bcl-2 (a), Bax (b), Bad (c), and phosphorylated Bad (d) protein levels from control hearts, hearts after CCP, and hearts after BKPC. Bar graph showing the fold-increase in Bcl-2 (a), Bax (b), Bad (c), and phosphorylated Bad (d) in the 3 different groups. \( n=6 \) for each group. Values are mean±SEM. \( ^*P<0.01 \), \( ^**P<0.05 \) vs control, \( ^†P<0.05 \) vs CCP.
proves postischemic LV and microvascular function, suggesting that pharmacological BKPC may be an important addition to our current methods of myocardial and microvascular protection with hyperkalemic cardioplegia.8–10 Recent clinical studies have confirmed those animal studies and have shown that BKPC improves ischemia tolerance in patients undergoing angioplasty and coronary artery bypass surgery.11–12 Consistent with previous studies, the present study used a pharmacologic preconditioning regimen with 10 minutes of \(10^{-4}\) M BK infusion and 5 minutes of KHB before crystalloid cardioplegic ischemia indicating that BK preconditioned the rabbit heart against postischemic LV dysfunction.

Recent studies have demonstrated that cardioplegia infusion and cardiopulmonary bypass trigger apoptosis signal pathways and induce cardiomyocyte apoptosis in animals and humans.13–15 These studies suggest that apoptosis may play an important role in myocardial stunning after open heart surgery.3,13–15 The present study investigated the effects of BKPC on I/R-induced myocardial apoptosis in rabbits. Our results show that cardioplegia I/R significantly induces myocardial apoptosis. In contrast, BKPC significantly inhibits cardioplegia I/R-related myocardial apoptosis. The antiapoptotic effects of BKPC may be associated with its improving postischemic LV performance.

**Figure 2.** (a) Representative blots and graph showing the expressions of proform caspase 3 from control hearts, hearts after CCP, and hearts after BKPC. (b) Blots showing cleaved caspase-3 protein and bar graph showing the fold-increase of cleaved p17-subunit of caspase 3 protein levels in the 3 different groups; n=6 for each group. (c) Caspase 3-like activity of cellular protein was measured with a fluorimetric assay. Values are mean±SEM. *\(P<0.05\), **\(P<0.01\) vs control, †\(P<0.05\) vs CCP.

**Figure 3.** Paraffin sections stained with TUNEL. TUNEL-positive nuclei stain brown (arrows) and TUNEL-negative nuclei stain green (magnification, ×200). (a) Tissue section from buffer perfused control; (b) CCP; (c) BKPC.
The Bcl-2 family of proteins controls apoptosis by modifying mitochondrial permeability and the release of cytochrome c. 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 and activation of death-effector caspases, such as caspase 3. Phosphorylation and inactivation of the proapoptotic protein Bad prevent the release of mitochondrial cytochrome c, thereby preventing apoptosis. Our studies demonstrated that cardioplegic I/R did not increase the total Bcl-2 family proteins, but induced phosphorylation of Bad. The discrepancies between the other studies and this one may be because of the different animal models used, such as a long period of I/R versus a short period of I/R. Moreover, BKPC did not change the expression of the Bcl-2 family proteins but caused greater phosphorylation of Bad at Ser112 than cardioplegia alone. The enhanced phosphorylation of Bad by BKPC may contribute to its antiapoptotic effects (Figure 5).

Signaling through tyrosine kinase (TK) has been demonstrated to confer protection against I/R injury through its activation of phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and endothelial nitric oxide synthase (eNOS). Several studies have shown that BKPC activates these prosurvival signaling kinase cascades. BKPC-induced phosphorylation and inactivation of Bad may contribute to its antiapoptotic effects (Figure 5).

Akt has been reported to phosphorylate and activate eNOS, producing nitric oxide (NO), which has been implicated in myocardial protection. NO, in turn, has been shown to inhibit opening of the mitochondrial permeability transition pore and caspase 3 activation. BK has been found to induce phosphorylation and activation of eNOS via Akt and subsequently enhance NO release. The beneficial effects of BKPC have been shown to be abrogated in the presence of

**Figure 4.** Bar graph illustrates the percentage of TUNEL-positive nuclei from control hearts, hearts after CCP, and hearts after BKPC. BKPC significantly reduced the percentage of TUNEL-positive nuclei. Values are mean±SEM. *P<0.05 vs control; †P<0.05 vs CCP.

**Figure 5.** Proposed molecular mechanisms responsible for I/R-induced apoptosis. I/R stress induces the translocation of proapoptotic Bcl-2 proteins, such as Bad, from cytosol to mitochondria. The Bad in mitochondrial then forms a proapoptotic complex with antiapoptotic Bcl-xl, which, in turn, leads to the release of cytochrome c from mitochondria to cytosol. The released cytochrome c, together with dATP, apoptotic protein activating factor-1 (apaf-1), and caspase 9, form the apoptosome resulting in the subsequent processing and activation of death-effector caspases, such as caspase 3. The terminal caspase 3 finally attacks the nucleate, resulting in nuclei fragmentation. This translocation is inhibited by survival factors that induce phosphorylation of Bad, leading to its cytosolic sequestration. BK, a survival factor, activates the signal transduction cascades via BK-B2 receptor. Infusion of BK induces the phosphorylation of TK. TK then activates PI3K and Akt cascades, which, in turn, phosphorylates proapoptotic Bad. Akt also phosphorylates and activates eNOS, thereby preventing apoptosis.
N-ω-nitro-L-arginine methyl ester, a nonselective eNOS inhibitor. Thus, eNOS may also participate in the antiapoptotic effects of BKPC.

In the present study, we observed that terminal caspase 3 was cleaved into the active p17-subunit of caspase 3 after cardioprotective pretreatment. We additionally found that the cleaved caspase 3 was activated and increased after cardioprotective arrest and reperfusion. BKPC significantly inhibited the cleavage and activation of caspase 3. The BKPC-induced inactivation of terminal caspase 3 may lead to its inhibiting myocardial apoptosis after cardioprotective arrest. To the best of our knowledge, this is the first report showing that BK is capable of improving Bad phosphorylation, inhibiting caspase 3 activation, and limiting apoptosis after myocardial arrest.

Angiotensin converting enzyme (ACE) inhibitors have been widely used to limit I/R injury, which appears to be dependent on inhibition of BK degradation. The cardioprotective effects of ACE inhibitors could be abolished in the presence of HOE 140 (icatibant), a specific BK-B2 receptor antagonist. Thus, our data support the use of ACE inhibitors in the treatment of myocardial infarction, hypertrophy, and heart failure, because cell apoptosis often occurs during these pathophysiological processes. The isolated buffer-perfused heart has been widely used in the investigation of cardioprotective I/R. However, there are some advantages and disadvantages of this experimental preparation. For example, the advantage of isolated buffer-perfused heart is the elimination of extrinsic neural input and hormonal factors. The disadvantage of the isolated heart perfused with low viscosity media lacking red cells is the abnormally high-coronary flow rate as compared with the blood-perfused heart.

In conclusion, we demonstrate that BKPC improves LV performance and limits myocardial apoptosis with concomitant phosphorylation of Bad and inactivation of caspase 3. These results may provide novel molecular mechanisms responsible for the protective effects of BKPC and open new avenues for research into the clinical limitations of I/R injury after open-heart surgery.

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References


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