Ventricular Dysfunction After Cardioplegic Arrest Is Improved After Myocardial Gene Transfer of a β-Adrenergic Receptor Kinase Inhibitor

Hendrik T. Tevaearai, MD; Andrea D. Eckhart, PhD; Kyle F. Shotwell, BS; Katrina Wilson, BS; Walter J. Koch, PhD

Background—Acute cardiac contractile dysfunction is common after cardiopulmonary bypass (CPB). A potential molecular mechanism is enhanced β-adrenergic receptor kinase (βARK1) activity, because β-adrenergic receptor (βAR) signaling is altered in cardiomyocytes after cardioplegia. Therefore, we examined whether adenovirus-mediated intracoronary delivery of a βARK1 inhibitor (Adv-βARKct) could prevent post-CPB dysfunction.

Methods and Results—Rabbits were randomized to receive 5 × 10¹¹ total viral particles of Adv-βARKct or PBS. After 5 days, hearts were arrested with University of Wisconsin solution, excised, and stored at 4°C for 15 minutes or 4 hours before reperfusion on a Langendorff apparatus. Left ventricular (LV) function measured by end-diastolic pressure response to preload augmentation, contractility (LV dp/dtₘₐₓ), and relaxation (LV dp/dtₘᵦᵣₜ) was assessed by use of increasing doses of isoproterenol and compared with a control group of nonarrested hearts acutely perfused on the Langendorff apparatus. In the PBS-treated hearts, LV function decreased in a temporal manner and was significantly impaired compared with control hearts after 4 hours of cardioplegic arrest. LV function in Adv-βARKct-treated hearts, however, was significantly enhanced compared with PBS treatment and was similar to control nonarrested hearts even after 4 hours of cardioplegia. Biochemically, several aspects of βAR signaling were dysfunctional in PBS-treated hearts, whereas they were normalized in βARKct-overexpressing hearts.

Conclusions—Myocardial gene transfer of Adv-βARKct stabilizes βAR signaling and prevents LV dysfunction induced by prolonged cardioplegic arrest. Thus, βARK1 inhibition may represent a novel target in limiting depressed ventricular function after CPB. (Circulation. 2001;104:2069-2074.)

Key Words: ischemia ■ reperfusion ■ cardiopulmonary bypass ■ gene therapy ■ signal transduction

Cold cardioplegic cardiac arrest, either during cardiopulmonary bypass (CPB) procedures or during harvest and transport of a donor heart for transplantation, induces a degree of cardiac dysfunction by the time the heart is reperfused. Left ventricular (LV) compliance as well as LV contractility and relaxation are often impaired, which necessitates the use of isotropic agents for postoperative hemodynamic support. 1 At a molecular level, the myocardial β-adrenergic receptor (βAR) signaling system is crippled during the cold ischemic period and is not immediately restored by warm blood reperfusion. 2-6 Importantly, cardiac βARs are the most powerful means to support myocardial contractile function, and thus, the isotropic reserve of post-surgery hearts is lost.

Both β₁- and β₂-ARs are desensitized during CPB, which might be a direct result of a large local release of catecholamines. 2-4 In addition, although total βAR density does not change in human atrial samples taken before, during, or after CPB, β-agonist–stimulated adenylyl cyclase activity is decreased, suggesting an important role for βAR uncoupling. 2-6 The β-adrenergic receptor kinase (βARK1 or GRK2), a member of the G protein–coupled receptor kinase family (GRK), is responsible for phosphorylating and uncoupling agonist-occupied βARs. 7 Interestingly, βARK1 expression and activity are increased in different forms of heart disease, including heart failure 8 and myocardial ischemia. 9 Consistent with this is the finding that cardiac βARK1 expression can also be increased after acute catecholamine exposure. 10 Thus, βARK1 may be the key molecule in initiating βAR uncoupling early after cardioplegic arrest.

βARK1 activity has been shown to be elevated in models of ischemia-reperfusion, 9,11 and we recently demonstrated that myocardial recovery after ischemia-reperfusion injury is significantly impaired in transgenic mice overexpressing βARK1. 12 Although cold cardioplegic solutions are supposed to protect the heart, some similarities may exist between
ischemia-reperfusion and cardioplegic cardiac arrest followed by warm blood reperfusion. Accordingly, we hypothesized that inhibition of βARK1 may represent a new strategy to prevent myocardial dysfunction after reperfusion of cardioplegia-arrested hearts. We previously developed a peptide that inhibits βARK1 activity (βARKct).\(^{13-15}\) The βARKct is a 194-amino-acid peptide corresponding to the carboxyl terminus end of βARK1, and it includes the sequence responsible for binding to the βγ-subunit of activated heterotrimeric G proteins (G\(\beta\gamma\)), a process required for βARK1 activity.\(^{13-15}\) In the present study, we demonstrate that intracoronary adenovirus-mediated gene transfer of the βARKct (Adv-βARKct) before cardioplegic arrest prevents LV dysfunction by the time the heart is reperfused.

**Methods**

**Adenoviral Constructs**

The adenoviral backbone for Adv-βARKct is a second-generation replication-deficient serotype 2 adenovirus with deletions of the E1 and E4 (except for ORF6), as previously described.\(^{16,17}\) Aliquots of 5\(\times\)10\(^{11}\) total viral particles (TVP) were thawed and mixed in PBS for a final volume of 2 mL immediately before intracoronary delivery.

**In Vivo Intracoronary Gene Delivery**

Adult male New Zealand White rabbits (3 kg) were operated on as previously described.\(^{15,17}\) Procedures were humanely performed in accordance with the regulations adopted by the National Institutes of Health and approved by the Animal Care and Use Committee of Duke University. To control for βARKct expression, a group of animals randomly received 2 mL of PBS by use of the same delivery technique.

**Isolated Heart Preparation and LV Function Assessment**

Five days after delivery of either the transgene or PBS, animals were reanesthetized and mechanically ventilated. A clamshell thoracic incision was performed before 3000 IU of heparin was injected intravenously. The inferior vena cava was transected, and animals were partially exsanguinated to unload the LV before the aorta was cross-clamped and 30 mL of University of Wisconsin cardioplegic solution was injected into the LV cavity, allowing cardiac arrest within 2 seconds. The great vessels, pulmonary veins, and superior vena cava were transected, and the heart was transferred into 0.9% saline solution at 4°C. After 15 minutes or 4 hours, hearts were hung on a modified Langendorff apparatus and perfused as previously described.\(^{19,20}\) An LV latex balloon was positioned and connected to a pressure transducer (Millar Instruments), and its volume was adjusted to assess a baseline condition of 0 mm Hg end-diastolic pressure (LVEDP). After 30 minutes of perfusion, baseline LV pressures, responses to standard increases of end-diastolic volume, and pressure transducer responses to standard doses of isoproterenol (Iso) were recorded. After termination of functional measurements, hearts were kept perfused for 30 minutes before samples of the ventricles were frozen in liquid nitrogen for biochemical analysis. A control group included hearts isolated from rabbits that had not undergone surgery, quickly harvested without being arrested, and immediately reperfused on the Langendorff apparatus.

**Myocardial βAR Density and Signaling**

Membranes were prepared as previously described.\(^{15,17,21}\) Total βAR density was determined by radioligand binding with a saturating concentration (300 pmol/L) of \(^{3}H\)-labeled cyanopindolol at 37°C for 1 hour as described.\(^{17}\) For adenyl cyclase activity, 20 μg of myocardial membrane protein was incubated with 0.1 μmol/L [\(\alpha\)-\(^{32}P\)]ATP for 15 minutes at 37°C under basal conditions or in the presence of 1 mmol/L Iso or 10 mmol/L sodium fluoride (NaF).

**Results**

**LV Physiology After Cardioplegia**

To examine the effect of βARK1 inhibition on postcardioplegic LV dysfunction, we delivered either the βARKct transgene (Adv-βARKct) or PBS into normal rabbit hearts by use of an intracoronary in vivo delivery technique we recently developed.\(^{14}\) Transgene expression was confirmed by Northern blot analysis 5 days after delivery of 5\(\times\)10\(^{11}\) TVP of Adv-βARKct (n=9) (Figure 1). LV contractility (LV dP/dt\(_{\text{max}}\)), LV relaxation (LV dP/dt\(_{\text{min}}\)), and LVEDP were all progressively altered by prolonged cardioplegic arrest in hearts that received only PBS compared with nonarrested hearts (Table and Figure 2). In particular, the response to Iso was significantly decreased after 4 hours of cardioplegic arrest compared with control nonarrested hearts (Figure 2C and 2D). LV contractility and relaxation in hearts treated with Adv-βARKct 5 days before surgery, however, were both significantly increased during the reperfusion period after a 15-minute cardioplegic arrest compared with PBS-treated arrested hearts (Figure 2A and 2B). LV function was also improved after 4 hours of cardioplegic arrest in hearts that received intracoronary Adv-βARKct delivery 5 days earlier as opposed to hearts that received PBS only (Figure 2C and 2D). In fact, in the more immediate cardioplegic setting (15 minutes), βARKct expression even enhanced the LV function of hearts significantly above that of the nonarrested control.
Evolution of Hemodynamic Measurements After a Cold Cardioplegic Arrest of 15 Minutes or 4 Hours and Comparison With Control Nonarrested Hearts

<table>
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<tr>
<th></th>
<th>LVEDP</th>
<th>LV dP/dt(_{\text{max}})</th>
<th>LV dP/dt(_{\text{min}})</th>
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<tr>
<td></td>
<td>iso=0</td>
<td>iso 0.33 μg/h</td>
<td>iso=0</td>
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<tr>
<td>Control</td>
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<tr>
<td>Baseline</td>
<td>935±26</td>
<td>-855±90</td>
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<tr>
<td>+ 0.1 mL</td>
<td>1080±60</td>
<td>-944±104</td>
<td>-1418±126</td>
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<tr>
<td>+ 0.3 mL</td>
<td>1116±81</td>
<td>-964±94</td>
<td>-1417±90</td>
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<td>PBS 15 min</td>
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<tr>
<td>Baseline</td>
<td>863±132</td>
<td>-847±111†</td>
<td>-1038±158</td>
</tr>
<tr>
<td>+ 0.1 mL</td>
<td>956±129</td>
<td>-920±102†</td>
<td>-1132±142</td>
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<tr>
<td>+ 0.3 mL</td>
<td>998±114*</td>
<td>-950±84‡</td>
<td>-1150±133*</td>
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<tr>
<td>PBS 4 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>705±72*</td>
<td>-642±100‡</td>
<td>-953±109*</td>
</tr>
<tr>
<td>+ 0.1 mL</td>
<td>783±60*</td>
<td>-709±91‡</td>
<td>-1012±94*</td>
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<tr>
<td>+ 0.3 mL</td>
<td>800±63*</td>
<td>-711±91‡</td>
<td>-961±106*</td>
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Baseline condition corresponds to the LVEDV that gives an LVEDP of 0 mm Hg, which was used to standardize LVEDV at the initiation of each experiment.

*P<0.01, †P<0.001, ‡P<0.05 vs control (2-way ANOVA).

A sign of cardiac dysfunction. We found significantly increased heart rate at 4 hours after CPB (Figure 4). Importantly, all groups that had previously been treated with intracoronary βARKct had normal heart rates compared with control nonarrested hearts (Figure 4).

BAR Signaling After Cardioplegic Arrest

We analyzed biochemical BAR signaling in myocardium after reperfusion of arrested hearts. This was done 30 minutes after termination of functional measurements to allow washout of residual Iso. After 15 minutes of cardioplegic expo-
sure, myocardial βAR density was already decreased in LV membranes prepared from hearts that received PBS 5 days earlier (Figure 5A). A similar loss of βAR density was also evident in hearts arrested for 4 hours (Figure 5A). Hearts that received Adv-βARKct 5 days previously, however, had significantly higher βAR density at either 15 minutes or 4 hours, and these values were in the normal range compared with control hearts not exposed to cardioplegia (Figure 5A).

These acute changes in βAR density may reflect internalization, and that may include hyperactive desensitization mechanisms. Therefore, we measured βARK1 (GRK2) levels and activity. We have previously shown that cytosolic myocardial GRK activity is almost exclusively a result of βARK1.22 We found GRK activity to be significantly increased after 4 hours of cardioplegia in hearts previously treated with PBS (Figure 5B). GRK activity, however, was unaltered even after prolonged cardioplegic arrest in hearts treated with Adv-βARKct (Figure 5B). Because active βARK1 resides in the membrane fraction of intact cells after Gβγ-dependent translocation, however, we also examined the levels of membrane βARK1 by immunoblotting. BARK1 levels were elevated in membranes from PBS-treated arrested hearts (Figure 5C). In contrast, less βARK1 was found in the membrane of βARKct-expressing hearts in both the short and long term (Figure 5C).

We also examined βAR signaling in these hearts. Basal and Iso-stimulated adenylyl cyclase activity were decreased after 15 minutes of cold cardioplegic arrest compared with normal nonarrested hearts, whereas they remained unchanged in Adv-βARKct hearts (Figure 5D). NaF-stimulated adenylyl cyclase activity was unchanged at 15 minutes but decreased after 4 hours of cardioplegic exposure (Figure 5D). This may indicate postreceptor defects after cardiac arrest. Interestingly, in LV membrane prepared from hearts that received Adv-βARKct 5 days earlier, NaF-stimulated adenylyl cyclase activity was unchanged at both 15 minutes and 4 hours (Figure 5D). Thus, βARKct expression was capable of restoring the myocardial βAR signaling system in cardioplegia-arrested hearts.

**Discussion**

Inhibition of βARK1 activity by adenovirus-mediated gene transfer of the βARKct 5 days before a cold cardioplegic cardiac arrest prevents myocardial dysfunction when the heart is reperfused. Compared with normal nonarrested hearts, LV function in βARKct-expressing hearts is improved after a short cold ischemic period, and although function progressively decreases with prolonged cardiac arrest, βARKct expression maintains function within normal limits and delays development of the functional consequences of cardioplegic injury. Previous studies examining biochemical abnormalities of cold cardioplegia or warm blood reperfusion suggested that defects in βAR signaling were associated with this treatment.2–6 Notably, early changes in βAR coupling with its effector adenylyl cyclase occur, similar to what is observed during evolution of chronic cardiac failure.2–5 Our present results demonstrate that gene transfer of a βARK1 inhibitor peptide prevents alterations in βAR signaling and therefore confirm a role of βARK1 in altering ventricular function after cardioplegic arrest.

We have previously demonstrated the positive effect of βARKct overexpression on myocardial function in vivo in transgenic mice15,23,24 or ex vivo in transplantation studies in which transgenes were delivered immediately after graft harvest and before transplantation of the graft into the recipient animal.20 In addition, we recently delivered Adv-βARKct in rabbits simultaneously with the creation of an LV myocardial infarction and observed improved LV function compared with animals that received an empty virus or PBS only.17 In this study, βAR signaling remained normal in animals treated with the βARK inhibitor transgene, whereas it was dramatically impaired in nontreated animals, with a reduction in βAR density, a decrease in adenylyl cyclase activity in response to Iso stimulation, and increased expression and activity of βARK1.17 In fact, the development of

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**Figure 3.** LV compliance as measured by LVEDP in response to increasing LVEDV was measured in hearts exposed to cardioplegic solution and arrested for 4 hours before being reperfused on a Langendorff apparatus. Measures were taken 5 days after intracoronary delivery of either $5 \times 10^{11}$ TVP Adv-βARKct (○, n = 4) or PBS (€, n = 4). Nonarrested control hearts were directly hung and perfused on Langendorff apparatus (×, n = 4). Basal LVEDP was standardized by adjusting intraventricular balloon volume to an LVEDP of 0 mm Hg (basal). *$P < 0.05$ (PBS vs Adv-βARKct), †$P < 0.05$ (PBS vs control), ‡$P = 0.05$ (βARKct vs control).

**Figure 4.** Heart rate during reperfusion on a Langendorff apparatus after 15 minutes or 4 hours of cold cardioplegic arrest. Hearts received 5 days earlier; intracoronary delivery of either $5 \times 10^{11}$ TVP Adv-βARKct (βARKct 15 minutes, n = 5; βARKct 4 hours, n = 4) or PBS (PBS 15 minutes, n = 4; PBS 4 hours, n = 4). Nonarrested control hearts were directly perfused on Langendorff apparatus (control, n = 4). *$P < 0.05$ (PBS 4 hours vs PBS 15 minutes), †$P < 0.05$ (PBS 4 hours vs Adv-βARKct 4 hours).
heart failure that initially is associated with myocardial infarction in this model was significantly delayed in the presence of the βARKct transgene, demonstrating that βARK1 is critically involved in the pathogenesis of ischemic cardiomyopathy.17

The design of our present study offers insight into the acute functional and biochemical changes that occur during the reperfusion period after cardioplegic arrest. Modifications of βAR signaling were dependent on the exposure time to cardioplegic solution in previously untreated (PBS) animals, whereas no significant changes were observed in animals that were pretreated with Adv-βARKct. This effect was seen in hearts exposed short-term or for as long as 4 hours. The immediate loss in βAR density, which persisted after prolonged exposure to cardioplegic solution, was abolished in hearts expressing the βARKct. In fact, βAR density was significantly higher than control counterparts in right ventricles of βARKct-treated hearts, whereas it was unchanged in PBS-treated hearts (data not shown). This suggests that downregulation as opposed to desensitization of βARs is probably not a critical event in the sequence of cardioprotective adaptive mechanisms. The observation that chronic βAR antagonist administration in patients before CPB with cardioplegic arrest did not prevent acute desensitization supports this hypothesis.3,4

With respect to desensitization, we found increased activated βARK1 levels in arrested hearts. Importantly, in the βARKct-treated hearts, less βARK1 appeared to be actively translocated from the cytosol to the membrane after cardioplegia and reperfusion. Thus, it appears that βARK1 plays a critical role in the βAR changes associated with cardioplegic arrest and thus acts as

Figure 5. βAR density (A), cytosolic GRK activity (B), membrane βARK1 levels (C), and adenylyl cyclase activity (D) after 15 minutes or 4 hours of cold cardioplegic cardiac arrest and reperfusion on a Langendorff apparatus. Hearts received 5 days earlier; intracoronary delivery of either $5 \times 10^{11}$ TVP Adv-βARKct (15 minutes, n=5; 4 hours, n=4) or PBS (15 minutes, n=4; 4 hours, n=4). Nonarrested control hearts (Ctl) were directly perfused on Langendorff apparatus (gray bars; n=4). A, βAR density. *P<0.05 (PBS 15 minutes vs βARKct 15 minutes), †P<0.05 (PBS 15 minutes vs control), ‡P<0.05 (PBS 4 hours vs βARKct 4 hours), §P<0.05 (PBS 4 hours vs control). B, GRK activity expressed as percent of values of control hearts. *P<0.05 (PBS 4 hours vs PBS 15 minutes), †P<0.05 (βARKct 4 hours vs PBS 4 hours). C, βARK1 level (expressed as percent values of control hearts) and Western blot in membrane fractions of representative hearts of different groups. + indicates positive control=heart from a βARK1-overexpressing transgenic mouse. *P<0.05 (βARKct 15 minutes vs PBS 15 minutes), †P<0.05 (βARKct 4 hours vs βARKct 15 minutes). D, *P<0.05, †P<0.01, ‡P<0.005 (PBS vs control). §P<0.05 (βARKct vs PBS), ||P<0.05 (PBS 4 hours vs PBS 15 minutes).
the primary target for βARKct action. Therefore, dynamic changes that occur progressively during cold myocardial ischemia certainly explain, at least partially, the progressive degradation in LV function with prolonged cardioplegic cardiac arrest. Adenovirus-mediated gene transfer of βARKct appears to stabilize βAR signaling during cardioplegia and reperfusion and consequently improves LV contractility and relaxation, as well as LV compliance and heart rate.

Adenyl cyclase activity in response to β-agonist stimulation is clearly affected by cold cardioplegic exposure, as demonstrated by animal studies2 as well as results obtained from human right atrial samples taken during CPB procedures.3–6 Few studies have analyzed the adenyl cyclase activity in response to NaF stimulation, and results are controversial. Some studies show no changes in direct G protein–stimulated adenyl cyclase activity,5 whereas others demonstrate a decreased activity in animals6 or in human samples.3 Our results demonstrate a time-dependent variation in NaF-stimulated adenyl cyclase activity. Although Iso-stimulated adenyl cyclase activity was already decreased after a short period of cardioplegic arrest, direct adenyl cyclase stimulation via G proteins (NaF) showed normal activity after 15 minutes of cardioplegia, whereas it was significantly decreased after prolonged exposure to cardioplegia. Thus, with prolonged cardiac arrest, there were both receptor and postreceptor defects in this system. Gene transfer of βARKct before cardioplegic cardiac arrest, however, preserved the adenyl cyclase pathway at all levels. This is probably due to the overall improved function before cardioplegic arrest.

As opposed to progressive modifications in βAR signaling during development of chronic heart failure, CPB represents a clinically relevant situation in which early alterations in βAR signaling take place as a consequence of changes in the extra-cellular milieu during both the cardioplegia and reperfusion periods. Cold cardioplegic solutions are used daily, not only for preservation of a donor heart during harvesting and transport but also primarily for myocardial protection during CPB and cardiac arrest. Ventricular dysfunction that occurs at the time of reperfusion prolongs any hospital stay and favors postcardiotomy morbidity. Therefore, there is a clinical need for different cardioplegic methods that not only provide myocardial protection during the cold ischemic period but also mainly ensure adequate ventricular function during reperfusion. In this regard, adenovirus-mediated gene transfer of an inhibitor of βARK1 before cold cardioplegic arrest may constitute a new strategy to prevent postoperative LV dysfunction. Moreover, it is possible that small molecules could be developed to inhibit βARK1 activity in a pharmacological manner, which may represent a novel therapeutic approach for acute cardiac dysfunction.

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References

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