Aprotinin Preserves Cellular Junctions and Reduces Myocardial Edema After Regional Ischemia and Cardioplegic Arrest

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Background—Cardiac surgery with cardiopulmonary bypass (CPB) and cardioplegic arrest has been associated with myocardial edema attributable to vascular permeability, which is regulated in part by thrombin-induced alterations in cellular junctions. Aprotinin has been demonstrated to prevent activation of the thrombin protease-activated receptor, and we hypothesized that aprotinin preserves myocardial cellular junctions and prevents myocardial edema in a porcine model of regional ischemia and cardioplegic arrest.

Methods and Results—Fourteen pigs were subjected to 30 minutes of regional ischemia, followed by 60 minutes of CPB, with 45 minutes of crystalloid cardioplegia, then 90 minutes of post-CPB reperfusion. The treatment group (n = 7) was administered aprotinin (40 000 kallikrein inhibitor units [KIU]/kg loading dose, 40 000 KIU/kg pump prime, and 10 000 KIU/kg per hour continuous infusion). Control animals (n = 7) received normal saline. Myocardial vascular endothelial (VE)-cadherin, β-catenin and γ-catenin, and associated mitogen-activated protein kinase (MAPK) pathways were assessed by immunoblot and immunoprecipitation. Histologic analysis of the cellular junctions was done by immunofluorescence. Myocardial tissue water content was measured. VE-cadherin, β-catenin, and γ-catenin levels were significantly greater in the aprotinin group (all P < 0.05). Immunofluorescence confirmed that aprotinin prevented loss of coronary endothelial adherens junction continuity. Aprotinin reduced tyrosine phosphorylation in myocardial tissue sections. Phospho-p38 activity was ∼30% lower in the aprotinin group (P = 0.007). The aprotinin group demonstrated decreased myocardial tissue water content (81.2 ± 0.5% versus 83.5 ± 0.3%; P = 0.01) and reduced intravenous fluid requirements (2.9 ± 0.2 L versus 4.0 ± 0.4 L; P = 0.03).

Conclusions—Aprotinin preserves adherens junctions after regional ischemia and cardioplegic arrest through a mechanism potentially involving the p38 MAPK pathway, resulting in preservation of the VE barrier and reduced myocardial tissue edema. (Circulation. 2005;112[suppl I]:I-196–I-201.)

Key Words: cardioplegia ■ cardiopulmonary bypass ■ cell adhesion molecules ■ edema ■ endothelium ■ ischemia ■ signal transduction

Cardiac surgery with cardiopulmonary bypass (CPB) and cardioplegic arrest has been associated with increased vascular permeability leading to myocardial edema and, in severe cases, cardiac dysfunction. Cellular junctions, particularly the adherens junctions, have been demonstrated to be key determinants of vascular permeability and endothelial barrier function. Adherens junctions are critical components in cellular adhesion between endothelial cells and are composed of transmembrane vascular endothelial (VE)-cadherins that form the extracellular connections between VE cells and catenins that link the intracellular portion of the cadherin proteins to the cytoskeleton. Alterations in the VE-cadherin complex have been demonstrated to result in increased vascular permeability and edema formation. Several factors appear to induce vascular permeability through a VE-cadherin–dependent mechanism such as tumor necrosis factor, VE growth factor, histamine, neutrophil-associated proteases, oxygen radicals, and thrombin. Molecular mechanisms that appear to be involved in thrombin-mediated vascular permeability include tyrosine phosphorylation and mitogen-activated protein kinase (MAPK) pathways. In addition, the thrombin protease-activated receptor (PAR) has been shown to mediate increases in endothelial permeability attributable to thrombin. The serine protease inhibitor aprotinin has been demonstrated to prevent the proteolytic activation of the thrombin PAR. Previously, we have shown that in a porcine model, CPB is associated with the degradation of VE-cadherin, β-catenin, and γ-catenin, resulting in the loss of
coronary VE integrity.10 We now hypothesize that aprotinin preserves myocardial cellular junctions and prevents myocardial edema in a clinically relevant porcine model of regional ischemia and cardioplegic arrest.

Methods

Animals

Animals were housed individually and provided with laboratory chow and water ad libitum. All experiments were approved by the Beth Israel Deaconess Medical Center animal care and use committee and the Harvard Medical Area standing committee on animals (institutional animal care and use committee) and conformed to the US National Institutes of Health guidelines regulating the care and use of laboratory animals (NIH publication 5377-3, 1996).

Experimental Design

Pigs (30 to 35 kg) were divided randomly into control (n=7) and aprotinin treatment (n=7) groups. Groups were subjected to regional left ventricular (LV) ischemia by left anterior descending (LAD) occlusion distal to the first diagonal branch for 30 minutes before CPB. Animals underwent CPB with cardioplegic arrest for 60 minutes. After 5 minutes of CPB, a period of 45 minutes of hyperkalemic cardioplegic arrest followed. The aortic cross-clamp was removed and the LAD occlusion released to reperfuse the myocardium for 10 minutes on CPB, after which the animal was weaned from CPB. The myocardium was reperfused for a total of 90 minutes after CPB. Intravenous fluids were administered post-CPB to maintain a mean arterial pressure (MAP) of 60 mm Hg. The treatment group received aprotinin systemically (Bayer Pharmaceuticals Corporation) as follows: a 40 000 KIU/kg IV loading dose, 40 000 KIU/kg CPB circuit prime, and 10 000 KIU/kg per hour IV continuous infusion. The control group was administered intravenous crystalloid solution. Arterial blood gas (ABG), arterial blood pressure, hematocrit (Hct), LV pressure, coronary blood flow, heart rate (HR), EKG, O2 saturation, temperature, and intravenous fluid requirements were measured and recorded. At the completion of the protocol, the heart was excised, and tissue samples from the ischemic-reperfused, distal LAD territory were collected for molecular analyses and measurement of tissue water content as described below.

Surgical Procedure

Pigs were anesthetized with intramuscular ketamine hydrochloride (20 mg/kg) and xylazine (15 mg/kg). General anesthesia with isoflurane gas was maintained by endotracheal intubation and mechanical ventilation, which was held during CPB. The right internal jugular (IJ) and carotid artery were cannulated for monitoring. Through a median sternotomy, a 2-mm ultrasonic coronary flow probe (Transonic Systems Inc.) was placed distal to the site of LAD occlusion. Pigs were given intravenous heparin (300 U/kg) and cannulated via the distal ascending aorta and right atrium. A vessel loop was passed around the LAD distal to the first diagonal branch for occlusion. Regional ischemia was confirmed by cyanosis of the distal LAD territory and cessation of coronary blood flow as measured by the flow probe. CPB was initiated with a kaolin-activated clotting time of >480 s, which was maintained with repeat administrations of intravenous heparin. The proximal aorta was cross-clamped, and cold crystalloid cardioplegia was infused into the aortic root. An initial 300 mL of cold, high-potassium cardioplegia (0°C to 4°C; 25 mmol/L K+), was administered, followed by 150 mL of cold, low-potassium cardioplegia (0°C to 4°C; 12 mmol/L K+) each 15 minutes. The composition of the crystalloid cardioplegic solution was (in mmol/L): 121 NaCl, 25 or 12 KCl, 12 NaHCO3, and 11 glucose.

Immunoblotting

Total lysate was obtained from myocardial tissue samples previously snap-frozen in liquid nitrogen by 30-s homogenization (PowerGen 125 Homogenizer; Fischer) on ice in lysis buffer containing 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitors (Complete; Roche) and centrifuged at 12 000g for 10 minutes at 4°C to separate soluble from insoluble proteins. The supernatant protein concentration was measured spectrophotometrically at 595-nm wavelength (DU640; Beckman) with a BCA protein assay kit (Pierce). Total protein was fractionated on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) with a semidry transfer apparatus (Millipore). Membranes were stained with Pronase S and then incubated with 5% nonfat dry milk in 50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, and 0.1% Tween 20 (TBST) buffer for 1 hour at room temperature (RT) to block nonspecific binding. Membranes were incubated with antibodies (in 2.5% nonfat dry milk in TBST) anti–VE-cadherin (Santa Cruz Biotechnology), anti–β-catenin, or anti–γ-catenin (Transduction Laboratories), anti–phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), and anti–ERK1/2 (Cell Signaling), or anti–phospho-p38 and anti-p38 (Cell Signaling) for 2 hours. After washing with TBST, membranes were incubated for 1 hour at RT in 2.5% nonfat dry milk in TBST diluted with the appropriate secondary antibody conjugated to horseradish peroxidase. Peroxidase activity was visualized with enhanced chemiluminescence and exposed to x-ray films (Amer sham). Immunoblots were analyzed after digitalization of x-ray films with a flat-bed scanner (Hewlett Packard) and NIH Image 1.62 software (National Institutes of Health).

Immunoprecipitation

Myocardial tissues were lysed as described above in 1 mL immunoprecipitation buffer (62.5 mmol/L Tris-HCl, 100 mmol/L NaCl, 1% NP-40, 0.1% Tween 20, 1 mmol/L NaVO4, pH 8.0, and protease inhibitors) for 30 minutes on ice. Antibody against VE-cadherin (C-19; 4 μg) was added to the lysate and incubated for 2 hours at 4°C.agarose (50 μL)-coupled anti-mouse or anti-rabbit antibody (Sigma) was added for an additional 30 minutes, and then the agarose beads were sedimented by brief centrifugation and washed by resuspending and pelleting 3× with 1 mL immunoprecipitation buffer. Then, 100 μL of 2× Laemmli buffer was added to the agarose pellet and boiled for 5 minutes, and 10 μL was fractionated on 10% SDS-PAGE and processed for immunoblotting by use of anti–VE-cadherin, anti–β-catenin, or anti–γ-catenin.

Triton X-100 Solubility

Tissue extracts were separated in Triton X-100–soluble and –insoluble fractions according to a protocol modified from Lampugnani et al.2 Samples were initially homogenized (PowerGen 125 Homogenizer) for 30 s at 0°C in extraction buffer I containing 1% Triton X-100, 1% NP-40, 10 mmol/L Tris-HCl, and 150 mmol/L NaCl (TBS) with 2 mmol/L CaCl2, pH 7.5, and protease inhibitors. Extracts were centrifuged at 12 000g for 5 minutes at 4°C to separate Triton X-100–soluble from –insoluble fractions. This supernatant was consider the Triton X-100–soluble fraction. After the first extraction, the pellets were gently washed 3× in TBS plus protease inhibitors and then resuspended in the same volume of the Triton X-100 supernatant and homogenized in extraction buffer II containing 0.5% SDS, 1% NP-40, and TBS with protease inhibitors. Extracts were centrifuged at 12 000g for 5 minutes at 4°C; this supernatant was considered the Triton X-100–insoluble fraction. The Triton X-100–soluble and –insoluble fractions then were used for immunoblotting for VE-cadherin, β-catenin, and γ-catenin as described above.

Immunofluorescence

LV myocardial sections were fixed in 10% buffered formalin (overnight at RT), paraffin embedded, sectioned (5-μm thick), deparaffinized in xylene, and rehydrated in graded ethanol. Antigen retrieval was obtained by bringing the slides to boiling twice in 10 mmol/L sodium citrate buffer, pH 6.0, and cooling to RT. After antigen retrieval, slides were equilibrated in PBS (Boston Bioproducts), and sections were incubated overnight at 4°C with 1.5% BSA.
to block nonspecific binding. Several sections from the control and aprotinin-treated tissues were then incubated for 1 hour at RT with anti–β-catenin, anti–γ-catenin, or anti-phosphotyrosine (Santa Cruz Biotechnology) antibodies. Sections were washed 4× in PBS and incubated for 30 minutes in 1.5% BSA in PBS containing the appropriated secondary antibody, Cy3 conjugated. Slides were then mounted with Vectashield (Vector Laboratories) and observed on a Bio-Rad MRC-1024ES confocal microscope (Bio-Rad).

Tissue Water Content
Myocardial tissues were weighed, incubated at 110°C for 12 hours and then weighed again. Percent tissue water was calculated as (wet weight/dry weight)/wet weight×100.

Data Analysis
Data are shown as mean±SEM. Comparisons between samples were analyzed by 2-tailed t test or Mann–Whitney test as indicated. Statistical significance was accepted at P<0.05.

Results

VE-Cadherin, β-Catenin, and γ-Catenin Levels Are Greater With Aprotinin Treatment
Myocardial tissue lysates showed greater levels of VE-cadherin with aprotinin treatment by >60% compared with controls (P=0.02; Figure 1). Although the total levels of β-catenin and γ-catenin were not changed in the tissue lysate, aprotinin treatment appeared to prevent the degradation of β-catenin, shown by the additional lower molecular weight bands representing degradation products of β-catenin in the control group that were not observed in the aprotinin treatment group (Figure 1). Immunoprecipitation of VE-cadherin followed by immunoblotting revealed that the β-catenin and γ-catenin associated with VE-cadherin forming the adherens junctions were significantly greater in the aprotinin group by 18% (P=0.007) and 47% (P<0.0001), respectively, compared with controls (Figure 2).

VE-Cadherin Levels Are Preserved in Triton X-100 Insoluble Fraction
Triton X-100 soluble and insoluble fractions of the tissue lysate were performed to determine the extent of VE-cadherin associated with the cytoskeleton (insoluble fraction) and the amount dissociated from the cytoskeleton (soluble fraction). Although the levels of VE-cadherin were not changed in the Triton X-100–soluble fraction, aprotinin treatment resulted in higher levels of VE-cadherin in the Triton X-100–insoluble fraction by more than twice that of controls (P=0.02), suggesting that aprotinin preserves VE-cadherin and the adherens junctions associated with the cytoskeleton (Figure 3).

Aprotinin Preserves Coronary Endothelial Adherens Junctions as Shown by Immunofluorescence
Evaluation of LV myocardial tissue sections by immunofluorescence confirmed that the higher levels of the adherens junction proteins β-catenin and γ-catenin in the aprotinin-treated animals were primarily within the coronary endothelial cells (Figure 4).

Aprotinin Reduces Tyrosine Phosphorylation
Tyrosine phosphorylation, a marker of VE-cadherin dissociation,5 was measured in LV myocardial tissue sections. Aprotinin resulted in decreased tyrosine phosphorylation
associated with coronary endothelial cells compared with controls (Figure 5).

p38 MAPK Pathway Activity Is Reduced by Aprotinin

MAPK pathways were examined in myocardial tissue lysate. Activated, phospho-p38 was decreased by >30% in the aprotinin group compared with controls ($P<0.007$), whereas total p38 levels were unchanged. Both activated, phospho-ERK1/2 and total ERK1/2 were not different in aprotinin and control groups (Figure 6).

Intravenous Fluid Requirements Are Lowered by Aprotinin

No significant differences in HR, MAP, ABG parameters, Hct, or temperature were observed between groups. Yet, to maintain these parameters, particularly a MAP of 60 mm Hg, the aprotinin-treated animals required significantly less total intravenous fluid than the control animals ($2.9\pm0.2$ L versus $4.0\pm0.4$ L; aprotinin versus control; $P=0.03$; Figure 7).

Aprotinin Decreases Myocardial Tissue Water Content

Tissue edema as measured by LV tissue percent water content was reduced in the aprotinin group compared with controls ($81.2\pm0.5\%$ versus $83.5\pm0.3\%$; aprotinin versus control; $P=0.01$; Figure 8).

Discussion

In our study using a porcine model of regional myocardial ischemia and cardioplegic arrest, the main findings are that aprotinin: (1) prevents the reduction of VE-cadherin levels in LV tissue, (2) prevents the reduction of $\gamma$-catenin and $\beta$-catenin associated with VE-cadherin, (3) prevents the degradation of VE-cadherin primarily associated with the cytoskeleton, (4) prevents the activation of the p38 MAPK pathway, and (5) reduces myocardial tissue edema. VE-cadherin levels were greater in the total myocardial tissue lysate and the VE-cadherin immunoprecipitate after aprotinin therapy. Although $\beta$-catenin and $\gamma$-catenin levels were unchanged in the total myocardial tissue lysate, $\beta$-catenin degradation, suggested by additional lower molecular weight bands representing degradation products, $^{10}$ appeared reduced by aprotinin. Furthermore, as components of the adherens junctions, $\beta$-catenin and $\gamma$-catenin were greater in the VE-cadherin immunoprecipitate compared with controls. These findings are consistent with aprotinin, preventing dissociation

Figure 3. VE-cadherin levels are higher in the Triton X-100–insoluble fraction with aprotinin treatment. Left, Immunoblots of Triton X-100–soluble and –insoluble fractions showing that the higher levels of VE-cadherin with aprotinin treatment are in the insoluble fraction, indicating association with the cytoskeleton. Right, Histogram showing quantification of the immunoblots. Cx indicates control; Rx, aprotinin. *$P<0.01$.

Figure 4. Immunofluorescence demonstrates preservation of adherens junctions in the aprotinin-treated group. Confocal microscopy immunofluorescence images demonstrating the high-intensity and more continuous signals of the $\beta$-catenin– and $\gamma$-catenin–labeled endothelial cells after aprotinin treatment compared with the lower-intensity and irregular signals of labeled endothelial cells from the control group.

Figure 5. Aprotinin reduces tyrosine phosphorylation in coronary endothelial cells in myocardial tissue sections. Confocal microscopy immunofluorescence images demonstrating lower-intensity signals of the phospho-tyrosine–labeled coronary endothelial cells after aprotinin treatment compared with the higher-intensity signals of labeled endothelial cells from the control group, with tyrosine phosphorylation being a marker of VE-cadherin dissociation.
of the adherens junction complex and reducing degradation of VE-cadherin and likely also β-catenin because endocytosis and lysosomal degradation are mechanisms by which cadherins are downregulated. In addition, the preservation of VE-cadherin by aprotinin appeared to mainly involve the Triton X-100–insoluble fraction that represents the VE-cadherin associated with the cytoskeleton, providing further evidence to suggest that aprotinin prevents the dissociation of intact adherens junctions complexes, thus maintaining endothelial permeability. These molecular findings were corroborated by our observations using confocal microscopy that β-catenin and γ-catenin demonstrated more intense and continuous signals by immunofluorescence in the aprotinin-treated group compared with controls, consistent with preservation of intact adherens junctions. Furthermore, because tyrosine phosphorylation has been shown to be a key step in VE-cadherin dissociation, we also observed by immunofluorescence that aprotinin reduced levels of tyrosine phosphorylation of coronary endothelial cells. Finally, we were able to correlate these findings clinically with reduced myocardial edema in aprotinin-treated animals as shown by decreased tissue water content.

The serine protease inhibitor aprotinin is well known for blood conservation in cardiac surgery and has been suggested to reduce perioperative stroke. Another promising aspect of aprotinin is an anti-inflammatory effect that has manifest as a reduction of reperfusion injury in experimental models. Although the underlying mechanisms of these processes likely are multifactorial, aprotinin has been shown to specifically inhibit the activation of the thrombin PAR. There have been described 4 members (PAR1 to PAR4) of the PAR family, a subclass of G-protein–coupled receptors, with PAR1 being the predominant receptor in endothelial cells. Inhibition of platelet PAR1 activity has been shown by in vitro studies using platelets from healthy humans and in vivo studies of cardiac surgical patients treated with aprotinin. In addition to the role of PARs in platelet function, PARs also are expressed on endothelial cells and have been shown to mediate thrombin-induced vascular permeability. Stimulation of human umbilical vein endothelial cells with a PAR1 agonist peptide increased endothelial permeability, whereas a PAR2 agonist peptide failed to induce the alteration in permeability. Thus, prevention of thrombin-mediated vascular permeability through

**Figure 7.** Aprotinin treatment resulted in lower intravenous fluid requirements. Intravenous fluid requirements were significantly lower in the aprotinin group compared with the control group to maintain similar hemodynamics (2.9±0.2 L vs 4.0±0.4 L; aprotinin vs control; *P<0.03).

**Figure 8.** Myocardial tissue water content was significantly reduced by aprotinin. Myocardial tissue edema was significantly reduced in the aprotinin group compared with controls as measured by percent tissue water content (81.2±0.5% vs 83.5±0.3%; aprotinin vs control; *P<0.05).
PAR1 inhibition is a potential mechanism by which aprotinin reduces endothelial permeability, likely also involving preservation of adherens junctions.

Although the mechanism by which aprotinin preserves the endothelial barrier potentially involves the inhibition of PAR1 activation, several other molecular mechanisms have been implicated in thrombin-induced endothelial cell permeability such as the Rho GTPases and members of the MAPK family. Inhibition of p38 activity by a selective inhibitor (SB203580) or by a dominant-negative p38 adenoviral vector significantly attenuated thrombin-induced endothelial cell permeability. The results of this study are consistent with our findings that the preservation of myocardial adherens junctions by aprotinin was associated with inhibition of p38 activation. Although these molecular pathways likely contribute to the mechanism of aprotinin in preserving cellular junctions, other effects of aprotinin are also likely involved, such as the reduction of neutrophil extravasation or prevention of oxygen radical production. A couple limitations of our study are worth mentioning. First, although we have provided evidence that aprotinin treatment preserves cellular junctions and have suggested that the p38 pathway may be involved, as is the case with many of the effects of aprotinin, a clear mechanism of action is lacking. Second, the surgeon was not blinded to the therapy, which may have introduced bias during the experimental procedure, such as with the administration of intravenous fluids after CPB despite following a standard protocol to maintain a MAP of 60 mm Hg.

Overall, we demonstrated that aprotinin preserves coronary endothelial adherens junctions and reduces myocardial tissue edema in a porcine model of regional ischemia and cardioplegic arrest. The findings of our study have important implications in the development of molecular strategies to prevent tissue edema and myocardial dysfunction, particularly in the setting of cardiac surgery requiring myocardial protection.

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