Biochemical and Structural Evidence for Pig Myocardium Adherens Junction Disruption by Cardiopulmonary Bypass

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Background—Given that cardiopulmonary bypass (CPB) is associated with edema and heart dysfunction and that adherens junctions may regulate vascular permeability barrier integrity and cardiomyocyte function, we investigated adherens junction protein steady-state levels in a pig model of CPB.

Methods and Results—Pigs were subjected to normothermic CPB for 90 minutes, followed by post-CPB perfusion for 90 minutes. Atrial and ventricular myocardium tissue samples were harvested before institution of bypass (basal levels) and at the end of post-CPB perfusion. Adherens junctions were analyzed by either total lysate or cadherin immunoprecipitates that were immunoblotted for pan-cadherin, VE-cadherin, β-catenin, and γ-catenin. Adherens junction solubility was addressed with Triton X-100 extraction. Frozen tissue sections were labeled with the same antibodies, and adherens junctions were visualized by confocal microscopy. Immunoblotting of total lysates revealed an increase in smaller-molecular-weight fragments of VE-cadherin, β-catenin, and γ-catenin after post-CPB perfusion, indicating partial protein degradation. Smaller-molecular-weight fragments recognized by VE-cadherin and β-catenin antibodies were also obtained from VE-cadherin immunoprecipitation, indicating degradation of endothelial cell adherens junctions. A prominent increase in adherens junction complex solubility was observed in post-CPB perfusion samples. Confocal microscopy of hearts obtained before CPB showed a continuous, homogeneous pattern of cell-cell labeling that contrasted with an irregular, discontinuous, punctuate, or zigzag pattern observed in post-CPB perfusion samples, corroborating biochemical data.

Conclusions—These results indicate that CPB is associated with signs of degradation of endothelial and cardiomyocytes adherens junctions, pointing to a molecular mechanism leading to increased vascular permeability and cardiomyocyte dysfunction. (Circulation. 2001;104[suppl I]:I-319-I-324.)

Key Words: cardiopulmonary bypass ■ endothelium ■ myocardium ■ intercalated disks ■ adherens junctions
noblotting, immunoprecipitation, solubility assay, and confocal microscopy. We documented important changes in AJs by CPB.

Methods

Animal Preparation
Yorkshire pigs (20 to 25 kg) of either sex were premedicated with ketamine (10 mg/kg IM) and anesthetized with α-chloralose and urethane (60 and 300 mg/kg IV initially and then 15 and 60 mg/kg every 60 minutes as needed, respectively). Pigs were intubated, mechanically ventilated (Harvard Apparatus Inc), and subjected to normothermic CPB as described in detail previously.10 Atrial and ventricular tissues were harvested before institution of 90 minutes of CPB and after 90 minutes of post-CPB perfusion.

All animals received humane care in compliance with the Beth Israel Deaconess Medical Center Animal Care and Use Committee and the National Research Council’s Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animals and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Immunoblotting

Total lysate from heart tissue was obtained by 30-second homogenization (PowerGen 125 Homogenizer, Fischer) on ice in lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% 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 Ponceau S,11 digitalized (see below), and then incubated with 5% nonfat dry milk in 50 mmol/L Tris-HCl, pH 8.0 for 1 hour at room temperature with either anti–VE-cadherin (1:1000), –β-catenin (1:2000), or γ-catenin (1:2000). The sections were washed 4 times in PBS and incubated for 30 minutes in 1.5% BSA in PBS containing a 1:300 dilution of mouse monoclonal anti–VE-cadherin (C-19, 4g1), rabbit anti–α-catenin (Amersham Pharmacia Biotech Inc) or rabbit anti-goat IgG (Jackson Immunolabs), all conjugated with Cy3. The slides were then mounted with Vectorshield (Vector Laboratories) and observed on a Bio-Rad MRC-1024ES confocal microscope (Bio Rad Microscience).

Data Acquisition and Statistical Analysis

Immunoblots were analyzed after digitalization of x-ray films with a flat-bed scanner (ScanJet 4c, Hewlett Packard) and NIH Image 1.62 software (National Institutes of Health). Comparisons between samples were analyzed by 1-way ANOVA, followed by a 2-tailed t test using Microsoft Excel (Microsoft Corporation). Values are expressed as mean fold change ± SEM. Ponceau S staining was used to determine proper protein fractionation and equivalent loading. The optical density ratio of the bands to Ponceau S staining was used to correct small uneven loading. Only samples with similar protein fractionation and protein loading with <20% differences were analyzed further.

Results

Detection of Smaller-Molecular-Weight Fragments of VE-Cadherin, β-Catenin, and γ-Catenin After Post-CPB Perfusion

The levels of immunoreactive VE-cadherin (120 kDa) did not change significantly before and after post-CPB perfusion (1.02 ± 0.05-fold change compared with before, P > 0.05, n = 7); there was, however, an increase in immunoreactivity in smaller-molecular-weight species, particularly an ~100-kDa fragment (2.93 ± 0.82-fold change compared with before, P < 0.001, n = 7) (Figure 1A). When VE-cadherin was immunoprecipitated, a fragment of similar size was also observed (Figure 1B).

β-Catenin did not change significantly after CPB (1.05 ± 0.01-fold change compared with before, P > 0.05, n = 7) in total lysates (Figure 1A). Nevertheless, small-molecular-weight species of β-catenin, 2 fragments of ~75 kDa (2.18 ± 0.74 compared with before, P < 0.05, n = 7) and 70 kDa, were increased after post-CPB perfusion (Figure
In addition, β-catenin immunoblotting after VE-cadherin immunoprecipitation showed that the 75- and 70-kDa fragments were also associated with VE-cadherin (Figure 1B).

Likewise, changes in total lysates γ-catenin before and after 90 minutes of post-CPB perfusion (Figure 1A) were not significantly different (1.03 ± 0.01-fold change compared with before, \( P > 0.05, n = 7 \)). As shown for β-catenin, small-molecular-weight species of γ-catenin, 2 fragments of \( \approx 75 \) kDa (1.72 ± 0.25-fold change compared with before, \( P < 0.002, n = 7 \)) and 55 kDa, were increased after post-CPB perfusion (Figure 1B). VE-cadherin immunoprecipitation followed by γ-catenin blotting showed that the 75- and 55-kDa fragments of γ-catenin were associated with VE-cadherin (Figure 1B).

**VE-Cadherin and β-Catenin Increases in Triton X-100–Soluble Fraction After Post-CPB Perfusion**

We used Triton X-100–soluble and –insoluble fractions to determine the solubility of endothelial cell AJ proteins before and after CPB (Figure 2) because Triton X-100 is used as a criterion to determine the extent of AJ association to the cytoskeleton. Immunoprecipitation with VE-cadherin and immunoblotting showed that VE-cadherin became almost totally soluble after CPB compared with tissues before CPB (Figure 2A). In addition, VE-cadherin–associated β-catenin showed degradation fragments (Figure 2B), indicating both AJ complex–increased solubility and degradation.

**Endothelial Cell AJ Morphology Is Altered After Post-CPB Perfusion**

Morphological changes in the AJIs were apparent by confocal microscopy (Figure 3). Before CPB, VE-cadherin, localized to the endothelial cell contacts, showed a regular and continuous line along the capillary network in both atrial and ventricular myocardium sections. In contrast, at the end of post-CPB perfusion, loss of continuity became apparent through the observation of gaps on atrial capillary walls, as well as in ventricular capillaries, where VE-cadherin redistributed into a heterogeneous, zigzag pattern. In larger vessels, particularly arterioles, differences in VE-cadherin distribution pattern between before and after CPB samples were noticeable but less obvious (data not shown).
Cardiomyocyte AJ Morphology Is Also Altered After Post-CPB Perfusion

Because the pan-cadherin antibody used (see Methods) did not recognize VE-cadherin (Figure 4A through D) in pig myocardium, we could study separately the cardiomyocyte cadherins and endothelial cadherin. Before CPB, there was evident cadherin labeling at the intercalated disks and a less intense, homogenous distribution at the lateral sarcolemma (Figure 5A). After 90 minutes of post-CPB perfusion, however, the distribution was quite irregular, particularly in the lateral cell-cell contacts and intercalated disks, with an uneven distribution and discontinuity forming gaps (Figure 5B).

Before CPB, β-catenin was present predominantly in the intercalated disks in a continuous homogeneous pattern with some lateral labeling of cardiomyocytes in a dotlike pattern (Figure 5C). After 90 minutes of post-CPB perfusion, β-catenin labeling along the sarcolemmas appeared as a punctuated and irregular pattern (Figure 5D). The γ-catenin labeling pattern had distribution changes similar to those described above for β-catenin (Figure 5E and F).

Discussion

Because one of the major problems associated with CPB is tissue edema and arrhythmias, a better understanding of the molecular mechanism involved in this process may have great clinical significance. To start addressing some of the possible molecular mechanisms leading to an increase in vascular leakiness and myofibrillar dysfunction after CPB, we investigated the steady-state levels of molecules of the AJ complex in heart total lysates (Figure 1A) and after VE-cadherin immunoprecipitation (Figure 1B). We report the presence of AJ protein breakdown and increases in Triton X-100 solubility in a CPB model, pointing to possible molecular mechanism of edema formation after CPB.

AJ disruption has been related to vascular permeability increase. For example, anti–VE-cadherin antibody led to increased permeability when used in mice in vivo, suggesting that VE-cadherin is an important determinant of vascular integrity.14 Thus, it is possible that the VE-cadherin degradation (Figure 1), solubility (Figure 2), and distribution changes (Figure 3) observed are related to CPB-associated permeability changes.

Our immunoblotting and immunoprecipitation data indicate that there is a cleavage of endothelial cell AJ proteins after 90 minutes of CPB perfusion (Figure 1) and detected a basal level of protein degradation, a fact not frequently reported in cell culture experiments. Despite such differences, there are similarities between the in vitro and in vivo data described here. For instance, the degradation of VE-cadherin, β-catenin, and γ-catenin has been recorded in human umbilical vein endothelial cells after removal of growth factors from media (an apoptotic model).15 Other studies show that shear stress induces a decrease in total VE-cadherin, β-catenin, and γ-catenin levels16 and that 4 hours of ATP
Despite that total VE-cadherin, β-catenin, and γ-catenin did not significantly change after post-CPB perfusion, the present data show that smaller-molecular-weight forms in total lysates and associated with VE-cadherin increased (Figure 2), indicating that CPB is associated with AJ complex degradation with loss of cell-cell contact integrity.

Another important point to be considered is the difference in Triton X-100 solubility of AJ proteins observed after post-CPB perfusion, as suggested by our experiments, indicates endothelial cell-cell contact disassembly, resulting in vascular permeability changes.

The vascular changes observed in the myocardium after post-CPB perfusion were more evident in the capillary network than in larger vessels in which VE-cadherin labeling showed a discontinuous pattern, with gaps visible along the endothelial wall (Figure 3B), in contrast to a continuous, regular distribution before CPB (Figure 3A). These observations were similar to those recorded in previous studies in vitro.9,12,14,16 VE-cadherin staining changed from continuous to discontinuous after 3 to 6 hours of a wound to the endothelial cell monolayer12 and after VEGF addition to endothelial cells in culture.9 2 hours after an injection of anti-VE-cadherin antibody in vivo,14 and after 8.5 hours of shear stress.16

The previous conditions, although diverse, led to a permeability increase and may mimic situations likely to take place during extracorporeal circulation, corroborating our in vivo results.

Changes in AJ steady state may not be the sole mechanism responsible for increased permeability after CPB. In fact, it has been reported that endothelial cells in culture exposed to an organ preservation solution, occludin, a component of tight junctions, decrease and correlate with a permeability increase, whereas there was no significant decrease in VE-cadherin.18 The same report, however, shows that gaps and irregularities are observed in both occludin and VE-cadherin staining. Further investigation may determine the involvement of both AJs and tight junctions in CPB-associated permeability increase.

Because the pan-cadherin antibody used (see Methods) did not recognize VE-cadherin (Figure 4 B through D), we were able to study separately endothelial cell AJ cadherins from other cell-cell contact cadherins in the myocardium. Pan-cadherin labeled not only the end-to-end junctions of the cardiomyocytes (intercalated disks) but also the lateral cell-cell contacts, albeit less intense (Figure 4 E and F), indicating a pattern similar to heart N-cadherin distribution. Although no previous reports showed pig myocardium AJ labeling with a cadherin antibody, the pattern we described is similar to the findings in cultured chicken cardiomyocytes in which N-cadherin is found in lateral borders in a periodic staining pattern associated with β- and α-catenin.19 Interestingly, in a chronic aortic stenosis guinea pig model, β-catenin at intercalated disks was redistributed, although its total quantity remained unchanged, as demonstrated by immunoblotting.20

It was observed after 90 minutes of CPB perfusion that the dotlike aspect of catenin distribution, particularly β-catenin, changed into a more punctuate pattern, suggesting an early redistribution of catenins and disorganization of cardiomyocyte intercalated disks. β-Catenin and γ-catenin showed smaller molecular fragments when immunoprecipitated with pan-cadherin, corroborating the redistribution of catenins along the cardiomyocyte sarcolemma at post-CPB heart samples. Because perturbation of the N-cadherin/catenin complex is associated with the myofibril disorganization,19 catenin redistribution could be related to myocardium dysfunction. Indeed, a recent report identified a homozygous mutation in the γ-catenin gene only in individuals with arrhythmogenic right ventricular cardiomyopathy, indicating the involvement of this AJ protein in myocardium function.21
It has been recently suggested that AJ protein cleavage, as recorded in our results and under different conditions, is related to apoptosis. There is recent evidence that in culture cells, β-catenin and γ-catenin are cleaved by caspase-3 during apoptosis. A time course of β-catenin cleavage was established as follows. First, there is cleavage at the C-terminus, generating a fragment of 90 kDa; second, consecutive cleavages at the N-terminus result in fragments of 85, 76, and 72 kDa; finally, another C-terminus cleavage generates a 70-kDa fragment. Interestingly, the fragments we observed in total lysates and VE-cadherin immunoprecipitation blotted for β-catenin were similar in size (Figure 2), although the experimental conditions were different. Also, in human umbilical vein endothelial cell–induced apoptosis, there is 1 γ-catenin degradation fragment detectable as early as 2 hours and 3 fragments after 4 hours of induction, similar to our protocol time course. Although apoptosis is involved in AJ protein cleavage in cultured cells, it is not necessarily the major or only mechanism of CPB-associated AJ disruption, given that the onset of an irreversible process like apoptosis causing most of the permeability increase would possibly determine higher CPB-related morbidity and mortality in patients than what is actually reported.

In summary, our results show that endothelial cell AJ$s and intercalated disks are partially degraded and biochemically modified (increase in solubility) after 90 minutes of post-CPB perfusion. These results are corroborated by confocal microscopy localization of AJ complexes in cell-cell contacts. Those changes in the steady state of AJ proteins may partially explain the increased permeability associated with CPB and myocardium dysfunction. Further investigation will determine whether disruption of other cell-cell contacts, such as tight and gap junctions, and their involvement in cell viability are modified by CPB.

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References
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