Mitogen-Activated Protein Kinase Inhibition and Cardioplegia-Cardiopulmonary Bypass Reduce Coronary Myogenic Tone

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Background—Cardioplegia-cardiopulmonary bypass (C/CPB) is associated with coronary microcirculatory dysfunction. Regulation of the microcirculation includes myogenic tone. Mitogen-activated protein kinases (MAPK) have been implicated in coronary vasomotor function. We hypothesized that vasomotor dysfunction of the coronary microcirculation is mediated in part by alterations in extracellular signal regulated kinase 1/2 (ERK1/2) activity following C/CPB in humans.

Methods and Results—Atrial myocardium was harvested from patients (n=15) before and after blood cardioplegia and short-term reperfusion under conditions of CPB. Myogenic tone of coronary arterioles was measured by videomicroscopy. Microvessel tone was determined post-C/CPB and after PD98059, a MAPK/ERK kinase 1/2 (MEK1/2) inhibitor. MAPK phosphatase-1 (MKP-1) and activated ERK1/2 were measured by Western blot. MKP-1 gene expression was determined by Northern blot. In situ hybridization and immunohistochemistry were used to localize myocardial MKP-1 and activated ERK1/2, respectively. Myogenic tone was reduced in coronary arterioles post-C/CPB (−10.5±0.9%, P<0.01 versus control/pre-C/CPB, n=5). Myogenic tone was decreased in coronary microvessels after 30 µmol/L (n=5) and 50 µmol/L (n=5) PD98059 treatment (−11.0±0.8% and -14.6±2.0%, respectively, both P<0.01 versus control/pre-C/CPB). Myocardial levels of activated ERK1/2 were reduced post-C/CPB (0.6±0.1, post/pre-C/CPB ratio, P<0.05, n=5) while MKP-1 levels increased (4.2±0.6, post/pre-C/CPB ratio, P<0.05, n=5). Myocardial MKP-1 gene expression increased post-C/CPB (3.0±0.8, post/pre-C/CPB ratio, P<0.05, n=5). MKP-1 and activated ERK1/2 localized to coronary arterioles in myocardial sections.

Conclusions—Coronary myogenic tone is dependent on ERK1/2 and decreased after C/CPB. C/CPB reduces levels of activated ERK1/2, potentially by increased levels of MKP-1. The ERK1/2 signal transduction pathway in part mediates coronary microvascular dysfunction after C/CPB in humans. (Circulation. 2003;108[suppl II]:II-348–II-353.)

Key Words: cardioplegia ■ cardiopulmonary bypass ■ microcirculation ■ signal transduction ■ surgery

Vasomotor dysfunction is a significant consequence of the inflammatory response to cardiopulmonary bypass (CPB).1 Altered vascular tone because of vasomotor dysfunction may manifest clinically as hypotension or cause alterations in organ perfusion. Specifically, coronary microcirculatory function has been shown to be impaired due to cardioplegia-cardiopulmonary bypass (C/CPB),2 and microvessels (less than 150 µm internal diameter) serve as the principle regulators of vascular resistance.

The coronary microcirculation is regulated by metabolic, neurohumoral, and myogenic mechanisms. Myogenic tone is an intrinsic property of vascular smooth muscle cells and functions in the autoregulation of the coronary microcirculation.3 In studies using a porcine model of C/CPB from our laboratory, diminished myogenic tone was demonstrated in the coronary as well as peripheral microcirculations.4,5 Thus, a systemic defect in vascular tone post-C/CPB due to decreased myogenic tone has been suggested. Myogenic activity of coronary arterioles has been reported in humans through the study of microvessels from atrial and ventricular myocardial tissue.6 In this study, protein kinase C (PKC) was suggested to regulate the myogenic tone of the coronary microvessels. However, the role of mitogen-activated protein kinases (MAPK) in the regulation of myogenic tone in human coronary microvessels has not been examined. Again, in our porcine model of CPB, alterations in the activity of MAPK, specifically extracellular signal regulated kinases 1/2 (ERK1/2), were shown during CPB and reperfusion.7,8 While the...
protein levels of ERK1/2 were unchanged, the levels of the phosphorylated, activated form of ERK1/2 were diminished after CPB. Accordingly, MAPK/ERK kinase 1/2 (MEK1/2), the kinase upstream of ERK1/2 that phosphorylates and activates ERK1/2, also showed a decrease in the activated form post-CPB. In this study, we assessed the hypothesis that vasomotor dysfunction of the coronary microcirculation is mediated in part by alterations in ERK1/2 activity following blood cardioplegia and short-term reperfusion under conditions of CPB in humans.

Methods

Myocardial Tissue Collection

Samples of right atrial appendage were harvested from patients (n = 15) undergoing coronary artery bypass graft (CABG) surgery before and after exposure of the heart to blood cardioplegia and short-term reperfusion under conditions of CPB. Double 3-0 polypropylene purse-string sutures (Ethicon) were placed in the atrial appendage. During placement of the venous cannula, the first sample of atrial appendage was harvested (pre-C/CPB). The superior suture was tightened to secure the venous cannula. The inferior suture remained loose to allow this portion of the atrium to be perfused with blood, exposed to CPB and blood cardioplegia, and reperfused after removal of the aortic cross clamp. The cardioplegia consisted of a 4:1 mixture of oxygenated blood with a hyperkalemic crystalloid solution. The second sample of atrial appendage (post-C/CPB) was harvested after C/CPB during removal of the venous cannula. Myocardial tissue was immediately frozen in liquid nitrogen for molecular biology studies or was placed in cold (5°C to 10°C) MOPS buffer solution for microvessel studies. The study was approved by the Clinical Research Committee of the Beth Israel Deaconess Medical Center.

In Vitro Coronary Microvessel Studies

Coronary arterioles were isolated for in vitro organ bath videomicroscopy as previously described. Microvessels dissected from the right atrial appendage (50 to 150 μm internal diameter) were isolated in an organ chamber, cannulated with dual glass micropipettes, and secured with 10-0 nylon monofilament suture (Ethicon, Somerville, NJ). Both micropipettes were connected to a pressure reservoir to allow variation of intraluminal pressure using a burette manometer. MOPS-1% albumin buffer solution (pH 7.4, 37°C) was equilibrated and continuously circulated through the organ chamber and reservoir (total volume, 100 mL). Using an inverted, 40× to 200× microscope (Olympus Optical, Tokyo, Japan) connected to a video camera, the vessel image was projected onto a monitor (Hitachi Densihi, Tokyo, Japan). An electronic dimension analyzer (Living System Instrumentation, Burlington, VT) was used to measure internal lumen diameter.

Microvessel Protocol

Myogenic tone of coronary arterioles was determined by measurement of the microvessel internal diameter at intraluminal pressures of 10 to 100 mm Hg as previously described. Microvessels were analyzed from control/pre-C/CPB (n = 5) and post-C/CPB (n = 5) myocardial tissue. Control/pre-C/CPB microvessels also were treated with papaverine (10⁻⁶ mol/L) to determine the passive tone at intraluminal pressures of 10 to 100 mm Hg. In addition, control/pre-C/CPB microvessels were pretreated with the MEK1/2 inhibitor PD98059 at 30 μmol/L (n = 5) and 50 μmol/L (n = 5) doses for 30 minutes. Microvessels equilibrated for 30 minutes at 50 mm Hg. The internal diameter then was measured at 10 mm Hg, and in increments of 10 mm Hg up to 100 mm Hg. At each pressure, the microvessel was stabilized for 2 to 3 minutes before measurement. Finally, microvessels were washed with MOPS-1% albumin buffer solution and potassium chloride (75 mmol/L) was applied to test microvessel viability and responsiveness. The internal diameter of each vessel was normalized to the microvessel diameter at a pressure of 50 mm Hg after the application of papaverine.

Western Blot Analysis

Myocardial tissue collected pre-C/CPB (n = 5) and post-C/CPB (n = 5) was analyzed for levels of the activated, phosphorylated forms of ERK1/2 by immunoblotting as previously described. Total protein was fractionated from myocardial tissue total lysate by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) using a semi-dry transfer apparatus. Membranes were stained with Ponceau S and incubated with anti-phospho p44/p42 MAPK (New England Biolabs, Beverly, MA) antibody for immunoblotting. The membranes were then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. Peroxidase activity was visualized by means of an enhanced chemiluminescence substrate system (Amersham, Piscataway, NJ) and radiography. Immunoblots were analyzed by digitalization and quantification of the radiographs using a flatbed scanner and NIH Image software (National Institutes of Health, Bethesda, MD).

Northern Blot Analysis

Total RNA from pre-C/CPB (n = 5) and post-C/CPB (n = 5) myocardial samples was isolated by a Trizol-based method (Invitrogen, Carlsbad, CA). Total RNA samples were fractionated in a 1% agarose/formalin gel, and transferred to nitrocellulose membranes. A human MKP-1 cDNA probe (534 base PST-1 fragment) was labeled with α32P-dCTP (PerkinElmer, Boston, MA) using a random-priming labeling kit (Roche, Indianapolis, IN). Blots were hybridized at 68°C for 3 hours in QuikHyb solution (Stratagene, Cedar Creek, TX), washed in SSC, placed in 0.1% SDS for 15 minutes at room temperature followed by SSC wash, and placed in 0.1% SDS for 15 minutes at 60°C. Blots were analyzed by radiography, and digitalized and quantified as described above.

Immunohistochemistry

Myocardial tissue was fixed in 10% formalin, embedded in paraffin, and sectioned (5 μm). Sections were deparaffinized in xylene, rehydrated in graded ethanol and PBS, and permeabilized with 0.5% Triton X-100. Endogenous peroxidase activity was eliminated by treatment with 1.5% hydrogen peroxide in PBS, followed by PBS wash, and blocking over night with 3% BSA at 4°C. Incubation with anti-phospho-ERK1/2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was for 2 hours at room temperature. Sections were then washed in PBS and incubated with the appropriate biotin-conjugated secondary antibody and freshly prepared avidin-biotin-peroxidase complex (Santa Cruz Biotechnology). Peroxidase was revealed using the diaminobenzidine-hydrogen method. Sections were then counter stained with methyl green, dehydrated, and mounted with Permount.

In Situ Hybridization

In situ hybridization was performed as previously described, using a digoxigenin (DIG)-labeled anti-sense cRNA probe of human MKP-1 cDNA. Frozen sections (10 μm) were fixed, acetylated, and hybridized at 68°C over 3 nights to the cRNA probe (100 ng/mL). Hybridized probe was visualized using alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Kierkegaard and Perry Laboratories, Gaithersburg, MD). Control sections were incubated with the sense cRNA probe (100 ng/mL).

Statistical Analysis

Values are shown as mean±SEM. Statistical analyses were performed using the Mann-Whitney test and two-way analysis of variance (ANOVA). Statistical significance was accepted at P<0.05.
Results

Patient and Microvessel Characteristics

Atrial tissue was sampled from 15 patients undergoing nonemergent CABG surgery. Samples were not taken from redo or combined cardiac procedures. Mean CPB time was 81±19 minutes, and the average time of cardioplegic arrest was 65±8 minutes. The average age was 66±4 years. Twelve of the patients were male. Mean ejection fraction was 65±8%. Mean coronary microvessel internal diameters after treatment with 30 μmol/L (n=5) and 50 μmol/L (n=5) PD98059 to inhibit ERK1/2 activation by blockade of upstream MEK1/2 resulted in decreased myogenic tone in a dose dependent manner (−11.0±0.8% and −14.6±2.0%, respectively, both P<0.01 versus control/pre-C/CPB). The decreases in myogenic tone with PD98059 treatment were demonstrated by the increases in microvessel internal diameter and significant upward shifts of the pressure-diameter curves in Figure 2.

Decreased Coronary Myogenic Tone After C/CPB

Coronary arteriole myogenic tone at transmural pressures of 10 to 100 mm Hg was demonstrated in control/pre-C/CPB microvessels. Treatment of control/pre-C/CPB microvessels with papaverine resulted in increased internal diameters and a significant upward shift of the pressure-diameter curve because of loss of myogenic tone (−17.0±1.0%; P<0.01, papaverine-treated control/pre-C/CPB versus control/pre-C/CPB, n=5). The result of papaverine treatment represents the passive tone of the vessel without contribution from the vascular smooth muscle. Myogenic tone of coronary arterioles was significantly reduced post-C/CPB compared with control/pre-C/CPB (−10.5±0.9%; P<0.01, post-C/CPB versus control/pre-C/CPB). The result of papaverine treatment represents the passive tone of the vessel without contribution from the vascular smooth muscle. Myogenic tone of coronary arterioles was significantly reduced post-C/CPB compared with control/pre-C/CPB (−10.5±0.9%; P<0.01, post-C/CPB versus control/pre-C/CPB).

MEK1/2 Inhibition Reduced Coronary Myogenic Tone

Treatment of control/pre-C/CPB microvessels with 30 μmol/L (n=5) and 50 μmol/L (n=5) PD98059 to inhibit ERK1/2 activation by blockade of upstream MEK1/2 resulted in decreased myogenic tone in a dose dependent manner (−11.0±0.8% and −14.6±2.0%, respectively, both P<0.01 versus control/pre-C/CPB). The decreases in myogenic tone with PD98059 treatment were demonstrated by the increases in microvessel internal diameter and significant upward shifts of the pressure-diameter curves in Figure 2.

Increased MKP-1 and Decreased Activated ERK1/2 After C/CPB

In myocardial tissue sampled post-C/CPB, levels of the phosphorylated, activated form of ERK1/2 were significantly decreased after C/CPB (0.6±0.1, post/pre-C/CPB ratio, P<0.05, n=5; C/CPB, cardioplegia-cardiopulmonary bypass).
reperfusion under conditions of CPB, which may have significantly decreased after blood cardioplegia and short-term.

The principle finding of this study is that coronary microvasculature is dependent on ERK1/2 and significantly decreased post-C/CPB. When considering the passive tone of control/pre-C/CPB microvessels after papaverine treatment as complete loss of myogenic tone, the extent of the reduced myogenic tone post-C/CPB approached the level of passive tone. Any preservation of myogenic tone post-C/CPB may have been from the use of blood cardioplegia as opposed to crystalloid cardioplegia. In our porcine model of C/CPB, we found that the reduction in myogenic tone was significantly less with blood cardioplegia compared with crystalloid cardioplegia.10

MAPK are serine-threonine protein kinases that function in signal transduction cascades. The three major types of MAPK are ERK1/2, c-Jun NH2-terminal protein kinase (JNK), and p38 kinase. ERK1/2 has been implicated in several physiological processes including cytokine production11 and vascular endothelial cell permeability.12 Specifically, the ERK1/2 pathway has been shown to regulate coronary vascular smooth muscle cell contraction.13,14 In our study, treatment of coronary arterioles with PD98059, the MEK1/2 inhibitor, resulted in decreased myogenic tone. Inhibition of MEK1/2, the kinase upstream of ERK1/2, prevents the phosphorylation and activation of ERK1/2. The reduction of myogenic tone in coronary arterioles due to PD98059 suggested a dose dependent response with a trend of greater loss of myogenic tone with increasing doses of PD98059. Compared with the attenuation of myogenic tone post-C/CPB, the defect in myogenic tone because of pharmacologic inhibition of the ERK1/2 pathway caused a reduction in myogenic tone that more closely resembled the passive tone, suggesting near complete loss of myogenic tone. The dependence of coronary microvascular myogenic tone on the ERK1/2 pathway was consistent with our findings that myogenic tone is reduced post-C/CPB and that levels of activated ERK1/2 also are decreased post-C/CPB. While levels of activated ERK1/2 were diminished after C/CPB, MKP-1 protein levels and gene expression were increased, consistent with our previous findings in a porcine model of CPB.7,8 These observations provide a potential mechanism for the deactivation of ERK1/2. MKP-1 functions to dephosphorylate and deactivate ERK1/2.15 In addition, both the decreased levels of activated ERK1/2 and increased MKP-1 localized to coronary arterioles in myocardial sections, providing further support for the role of the ERK1/2 pathway in vasomotor dysfunction of the coronary microcirculation after C/CPB.

**Activated ERK1/2 and MKP-1 Localized to Coronary Arterioles**

Immunohistochemical analysis of myocardial sections revealed decreased activated ERK1/2 staining of coronary arterioles and cardiomyocytes post-C/CPB compared with pre-C/CPB sections (Figure 5). Specifically, the decreased staining of activated ERK1/2 was observed in the vascular smooth muscle layer of the coronary arterioles. Evaluation of MKP-1 in myocardial sections by in situ hybridization showed increased staining of MKP-1 mRNA in post-C/CPB sections compared with pre-C/CPB (Figure 6). Similarly, MKP-1 mRNA staining of myocardial sections localized to coronary arterioles and cardiomyocytes.

**Discussion**

The principle finding of this study is that coronary microvascular myogenic tone is dependent on ERK1/2 and significantly decreased after blood cardioplegia and short-term reperfusion under conditions of CPB, which may have significant implications regarding post-operative regulation of myocardial perfusion. Myogenic tone was demonstrated at baseline in control/pre-C/CPB microvessels, and significantly decreased post-C/CPB. When considering the passive tone of control/pre-C/CPB microvessels after papaverine treatment as complete loss of myogenic tone, the extent of the reduced myogenic tone post-C/CPB approached the level of passive tone.

**Figure 4.** Representative Western blot (top) and Northern blot (bottom) showing MKP-1 protein and mRNA levels in myocardial tissue. Both levels of MKP-1 protein and gene expression were increased after C/CPB (4.2 ± 0.6 and 3.0 ± 0.8, post/pre-C/CPB ratio, respectively, both P < 0.05, n = 5; C/CPB, cardioplegia-cardiopulmonary bypass; B, before/pre-C/CPB; A, after/post-C/CPB).

**Figure 5.** Representative myocardial sections showing activated ERK1/2 pre-C/CPB (left) and post-C/CPB (right) by immunohistochemistry. Staining of activated ERK1/2 localized to coronary arterioles (solid arrows) and cardiomyocytes (open arrows). The dark staining of coronary arterioles pre-C/CPB was reduced in post-C/CPB myocardial sections showing decreased levels of activated ERK1/2 (C/CPB, cardioplegia-cardiopulmonary bypass).
The finding of diminished coronary myogenic tone after C/CPB is consistent with previous studies from our laboratory. In our porcine model, coronary myogenic tone was reduced following CPB alone. Considering the defect in myogenic tone was shown to occur after CPB without cardioplegic arrest, these results suggest that the effect of C/CPB on myogenic tone may be a systemic phenomenon, possibly exacerbated in the heart by suboptimal myocardial protection or exposure of the vascular tissue to a hyperkalemic environment. Consistent with this concept of systemic myogenic dysfunction due to C/CPB, myogenic tone was similarly reduced in peripheral microvessels from skeletal muscle in our pig model of C/CPB. Systemic loss of myogenic tone may contribute to hypotension due to vasomotor dysfunction and decreased systemic vascular resistance. Hypotension because of impaired myocardial and systemic myogenic tone potentially may cause reductions in organ perfusion in critical circulations such as the coronary, cerebral, renal, and mesenteric systems. This mechanism of vasomotor deficiency leading to hypotension is consistent with the findings of increased myogenic tone in skeletal muscle arterioles from spontaneously hypertensive rats and in coronary arterioles from patients with hypertension. In addition, a reduction of myogenic tone may also impair the regulatory function of the coronary circulation. The loss of coronary myogenic tone potentially decreases the capacity of the coronary vasculature to prevent large, sudden increases in intraluminal pressure. The observation of myogenic tone without myogenic contraction may be related to the common use of calcium channel blockers in our patient population, considering the significant role of voltage-gated calcium channels in the arteriolar myogenic response. Alternatively, the lack of myogenic contraction may be a result of the examination of human atrial microvessels, in contrast to ventricular microvessels that demonstrated myogenic tone and contraction in our porcine model of C/CPB. The presence of myogenic contraction in porcine ventricular microvessels allowed for the observation that while CPB alone caused reduced myogenic tone, exposure to crystalloid cardioplegia resulted in attenuated myogenic tone and contraction. Certainly, atrial and ventricular arterioles in vivo are subject to different influences of transmural pressure, neurohumoral agents, flow-induced responses, and metabolic regulation. Our in vitro microvessel technique provides for isolated, no-flow investigation of microvessel reactivity at specific intraluminal pressures, thus removing these factors that affect vasomotor regulation differently in the atrial and ventricular myocardium. However, differences in intrinsic characteristics of atrial and ventricular microvessels such as the myogenic function would be apparent.

Myogenic function has been shown in human coronary arterioles from ventricular myocardium, so further study is needed to determine if C/CPB causes a defect in myogenic tone and possibly myogenic contraction in human ventricular arterioles. Based on our previous work that demonstrated reduced myogenic tone and contraction because of C/CPB in porcine ventricular arterioles, one may suspect that human ventricular arterioles may be subject to diminished myogenic function in a similar fashion. In addition, we have shown in our pig model of CPB that alterations in ERK1/2, MEK1/2, and MKP-1 were similar in atrial and ventricular tissue. The potential effects of the volatile anesthetic used intraoperatively on microvascular myogenic tone also were considered. The agent used during CABG surgery in these patients was
isoflurane, which has been shown to preserve the myogenic tone of coronary arterioles.22

In this study, we demonstrated that (1) coronary myogenic tone is dependent on ERK1/2 and significantly decreased after C/CPB, (2) C/CPB reduces levels of activated ERK1/2 in myocardial tissue in humans, potentially by increased dephosphorylation due to elevated levels of MKP-1, and (3) C/CPB decreases ERK1/2 activity and increases MKP-1 in coronary arterioles. Therefore, we conclude that the ERK1/2 signal transduction pathway in part mediates coronary microvascular dysfunction after blood cardioplegia and short-term reperfusion under conditions of CPB. Considering the potential role of MKP-1 in the deactivation of ERK1/2, the use of phosphatase inhibitors in the prevention of coronary myogenic dysfunction due to C/CPB may be warranted. Continued investigation of the molecular mechanisms of coronary vasomotor dysfunction may further the development of methods to decrease the morbidity associated with cardiac surgery.

References

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