INACTIVATION OF THE MEK/ERK PATHWAY IN THE MYOCARDIUM DURING CARDIOPULMONARY BYPASS

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Objectives: A general pro-inflammatory response after cardiopulmonary bypass (CPB) may involve changes in signal transduction and in part be responsible for arrhythmias and myocardial dysfunction after cardiac surgery. The MEK/ERK (mitogen-activated protein kinase kinase/extracellular regulated kinase) pathway is common to many stimuli and may play a pivotal role in morbidity associated with CPB. We investigated the changes in MEK/ERK pathway and related enzymes after CPB in pigs.

Methods: We examined ventricular and atrial tissue from pigs before 90 minutes of normothermic CPB and after 90 minutes of post-CPB perfusion. The activities and protein levels of kinases MEK1/2, ERK1/2, a cellular tyrosine kinase (c-Src), protein kinase B (Akt), and the protein levels of mitogen-activated protein kinase phosphatase (MKP-1) were studied by immunoblotting ventricular and atrial myocardium lysates and labeling sections with antibodies that recognize the activated forms of the kinases and the phosphatase. Control pigs were subjected to sternotomy and heparinization but not CPB.

Results: We found a consistent inactivation of MEK/ERK pathway in both ventricular and atrial myocardium with an increase in MKP-1, a negative regulator of ERK1/2. The activities and protein levels of c-Src and Akt were not significantly modified before or after CPB, suggesting a certain degree of specificity for the MEK/ERK pathway. Such changes were not observed in controls. The decrease of ERK1/2 and MEK1/2 phosphorylation 90 minutes after termination of CPB (as well as the increase of nuclear MKP-1 protein levels) was also apparent by confocal microscopy.

Conclusions: These results collectively reveal a prevalence of inhibitory mechanisms in the MEK/ERK signal transduction machinery in myocardium subjected to CPB. (*J Thorac Cardiovasc Surg* 2001;121:773-81)

Cardiopulmonary bypass (CPB) is an essential component of many cardiac operations. There is, however, significant morbidity associated with CPB includ-

ing microvascular dysfunction (spasm and vasoplegia),¹ increased endothelial permeability (edema),² cardiac arrhythmias, and myocardial contractile dysfunction.³

Protein phosphorylation by kinases is critical in the regulation of enzymes and other cellular proteins. The 44-kDa extracellular regulated kinase (ERK1, p44 mitogen-activated protein [MAP] kinase) and its 42-kDa isoform, ERK2 (p42 MAP kinase), have been determined to be involved in muscular contraction,^{4,5} vascular tone,⁶ and myocardium survival and function.⁷ In addition, inhibition of MEK1/2 (mitogen-activated protein kinase kinase [MAPK kinase]), the kinase that phosphorylates ERK1/2, reduces edema induced by tetradecanoyl phorbol acetate in mice,⁸ pointing to a direct involvement of the MEK/ERK1/2 pathway with acute inflammation. ERK1/2 is activated by many different stimuli, contributing to cellular

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Fig 1. Simplified MEK/ERK pathway and its possible relation to c-Src and Akt (see text for details). *ERK*, Extracellular regulated kinase; *MEK*, mitogen-activated protein kinase [MAPK kinase]; *MKP-1*, mitogen-activated protein kinase phosphatase; *Akt*, protein kinase B; *c-Src*, cellular tyrosine kinase pp60; *PI3k*, phosphatidylinositol 3-kinase.

proliferation and differentiation and cell cycle regulation and survival⁹ (Fig 1).

Deactivation of ERK1/2 can be mediated by MKP-1/CL100, a dual specific phosphatase¹⁰ that dephosphorylates both serine and threonine residues (Fig 1). MKP-1 (MAP kinase phosphatase), a 39-kDa protein, is localized in the nucleus and can be rapidly induced by factors such as oxidative stress.^{11,12} It remains to be investigated whether MKP-1 is involved in the pathophysiologic mechanisms associated with CPB.

More recently, Akt (protein kinase B) (Fig 1), a 57-kDa protein serine/threonine kinase,¹³⁻¹⁵ has been shown to improve survival of cardiomyocytes in culture deprived of serum, as well as to protect murine hearts against ischemia/reperfusion injuries in vivo.¹⁶ Thus, Akt could possibly play a role in myocardial protection during CPB. It has also been demonstrated in vivo that activation of Akt is associated with vasodilatation through a nitric oxide-dependent mechanism,¹⁷ suggesting that Akt could regulate the edema that follows CPB.

Activation of ERK1/2 may be dependent on another enzyme, c-Src, a tyrosine kinase (Fig 1). For example, c-Src plays a pivotal role in ERK1/2 activation by hydrogen peroxide in cultured cardiomyocytes.¹⁸ Furthermore, a c-Src inhibitor decreases Akt activity stimulated by ischemia/reperfusion in cultures of cardiomyocytes,¹⁹ pointing to a possible role of c-Src in post-CPB injury.

Given that the aforementioned signal transduction pathways are related to cardiac function and edema formation, we studied the protein levels and activity of selected enzymes in the pathway (Fig 1) in ventricular and atrial myocardium from pigs before and after CPB. Using specific antibodies against the phosphorylated forms of Akt, ERK1/2, MEK1/2, activated c-Src, and MKP-1, we examined the state of their activation and protein steady state levels in myocardial tissue.

Material and methods

Animal preparation. Yorkshire pigs (20-25 kg) of either sex were premedicated with ketamine (10 mg/kg, intramuscularly) and anesthetized with α -chloralose and urethane (60 mg/kg and 300 mg/kg intravenously initially and then 15 mg/kg and 60 mg/kg every 60 minutes as needed, respectively). Pigs were intubated and mechanically ventilated (Harvard Apparatus, Inc, S Natick, Mass). After the induction of anesthesia and tracheal intubation, a fluid-filled catheter was introduced into the femoral artery for the measurement of peripheral arterial pressure. After a sternotomy was performed, pigs were given heparin (500 U/kg) and cannulated via the distal ascending aorta and the right atrium through the purse-string sutures. Normothermic CPB was instituted with an oxygenator (Bentley, Bio-2; Baxter Healthcare Corp, Irvine, Calif) and standard roller pump. Hemodynamic parameters were maintained as described in detail previously.²⁰ In brief, blood flow was maintained from 2.0 to 3.0 L $\cdot$ min⁻¹ (2.6-4.2 L $\cdot$ min⁻¹ $\cdot$ m⁻²) to assure a mean pressure of 40 to 70 mm Hg. Arterial blood gases were adjusted by ventilatory

Fig 2. Anti-phospho-ERK1/2 (A), anti-phospho-MEK1/2 (B), anti-MKP-1 (C), and respective control (D, E, and F) immunoblottings of total protein lysate from pig ventricle before (b) and 90 minutes after (a) post-CPB perfusion or 180 minutes after start of sham operations in controls as described in "Material and methods." ERK1/2 phosphorylation significantly decreases after 90 minutes of CPB and 90 minutes of post-CPB perfusion ($0.39\text{-fold} \pm 0.15$ as compared with basal levels, $P = .006$, $n = 5$), as well as MEK1/2 phosphorylation ($0.36\text{-fold} \pm 0.22$ as compared with basal levels, $P = .009$, $n = 5$), whereas MKP-1 levels increase perfusion ($5.11\text{-fold} \pm 2.2$ as compared with basal levels, $P = .03$, $n = 5$). No significant changes are observed in controls. The figure shows the result of 2 different experiments representative of 5 pigs studied (CPB) or 1 experiment representative of 4 controls. Ponceau S or total protein was used to ascertain proper protein fractionation and transfer.

rate and tidal volume to maintain Po_2 more than 80 mm Hg, PCO_2 more than 30 and less than 45 mm Hg, and pH between 7.30 and 7.45. After pigs were weaned from CPB, the mean post-CPB perfusion pressure was maintained between 40 and 70 mm Hg. Atrial ($n = 7$) and ventricular ($n = 5$) tissue samples were harvested before institution of 90 minutes of CPB and after 90 minutes of post-CPB perfusion. In control animals ($n = 4$), the same surgical procedure described above was used, except that after sternotomy, catheter placement, and heparinization, CPB was not instituted. Samples of atrial and ventricular myocardium were harvested after catheter placement (equivalent to before CPB), after 90 minutes, and at the end of 180 minutes (equivalent to 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 Animal Resources, National Research Council, and published by the National Academy Press, revised 1996.

Immunoblotting. Total lysate from atrial and ventricular tissue was obtained by homogenization (PowerGen 125 Homogenizer; Fischer, Pittsburg, Pa) for 30 seconds on ice in lysis buffer containing 1% NP-40 surfactant, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) and by centrifugation at 12,000g for 10 minutes at 4°C to separate solubilized from unsolubilized protein. The supernatant protein concentration was measured spectrophotometrically at a 595-nm wave length (DU640; Beckman Coulter, Inc, Fullerton, Calif) using a BCA protein assay kit (Pierce Chemical Company, Rockford, Ill). Total protein was fractionated on 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, Mass) using a semi-dry transfer apparatus (Millipore). Membranes were stained with Ponceau S,²¹ digitized (see below), 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 to block nonspecific binding. Membranes were incubated with rabbit anti-phospho p44/42 MAPK (New England Biolabs, Beverly, Mass)

Fig 3. Anti-phospho-ERK1/2 (A), anti-phospho-MEK1/2 (B), anti-MKP-1 (C), and respective control (D, E, and F) immunoblottings of total protein lysate from pig atria before (b) and 90 minutes after (a) post-CPB perfusion or 180 minutes after start of sham operations in controls as described in "Material and methods." ERK1/2 phosphorylation significantly decreases after 90 minutes of CPB and 90 minutes of post-CPB perfusion tissue (0.47-fold $\pm$ 0.10 as compared with basal levels, $P = .007$, $n = 7$), as well as MEK1/2 phosphorylation (0.49-fold $\pm$ 0.03 as compared with basal levels, $P = .008$, $n = 7$), whereas MKP-1 levels increased (4.35-fold $\pm$ 1.15 as compared with basal levels, $P = .02$, $n = 4$). The figure shows the result of 2 different experiments representative of 7 pigs studied (CPB) or 1 experiment representative of 4 controls. Ponceau S or total protein was used to ascertain proper protein fractionation and transfer.

1:2500 (v/v) dilution in 2.5% nonfat dry milk, rabbit anti p44/42 MAPK (New England Biolabs) 1:2500 (v/v) dilution, rabbit anti-phospho MEK1/2 (New England Biolabs) 1:1000 (v/v) dilution, mouse anti-MEK-1 (Zymed Laboratories, San Francisco, Calif) 1:1000 (v/v) dilution, rabbit anti-phospho-Akt (New England Biolabs) 1:1000 (v/v) dilution, goat anti-MKP-1 (V-15) (Santa Cruz Biotechnology, Santa Cruz, Calif) 1:1000 (v/v) dilution, rabbit anti-c-Src (kindly provided by Dr Joan Brugges), and activated mouse anti-c-Src²² (generously provided by Dr Kawakatsu) 1/1000 (v/v) for 2 hours for immunoblotting. After being washed with TBST, the membranes were incubated for 1 hour in 2.5% nonfat dry milk in TBST containing 1:3000 diluted with the appropriated secondary antibody either a sheep anti-mouse (Amersham Pharmacia Biotech Inc, Piscataway, NJ), rabbit anti-goat immunoglobulin G, or sheep anti-rabbit immunoglobulin G (Jackson Immunolabs, West Grove, Pa), at 1:5000 (v/v) dilution conjugated to horseradish peroxidase. Peroxidase activity was visualized by means of an enhanced chemiluminescence substrate system as previously described²³ and exposed to x-ray films (Amersham, Arlington Heights, Ill).

Confocal microscopy. Frozen tissue samples were sectioned (5 μ m thickness) in a cryostat (Tissue Tek; Miles Laboratories, Elkhart, Ind) and fixed for 20 minutes in 4% (w/v)

paraformaldehyde solution in phosphate-buffered saline solution (PBS), followed by permeabilization with 1% (w/v) SDS in PBS for 5 minutes.²⁴ After being washed with PBS, sections were blocked overnight at 4°C with 1.5% bovine serum albumin against nonspecific binding. Different sections from the same tissues were then incubated for 1 hour at room temperature with rabbit anti-phospho p44/42 MAPK (New England Biolabs) 1:100 (v/v) dilution in 1.5% (w/v) bovine serum albumin in PBS, rabbit anti-phospho MEK1/2 (New England Biolabs) 1:100 (v/v) dilution, or goat anti-MKP-1 (V-15) (Santa Cruz Biotechnology) 1:100 (v/v) dilution. The sections were washed 4 times in PBS and incubated for 30 minutes in 1.5% bovine serum albumin in PBS containing a 1:300 dilution of the appropriated secondary antibody, a sheep anti-mouse (Sigma Chemical Co, St Louis, Mo), donkey anti-goat immunoglobulin G (Jackson Immunolabs), or sheep anti-mouse immunoglobulin G (Jackson Immunolabs), all conjugated with Cy3. The slides were then mounted with Vectashield (Vector Laboratories, Inc, Burlingame, Calif) and observed on a Bio-Rad MRC-1024ES confocal microscope (Bio-Rad Microscience, Hercules, Calif).

Data acquisition and statistical analysis. Immunoblottings were analyzed after digitalization of x-ray films using a flat-bed scanner (ScanJet 4c; Hewlett-Packard Company, Palo Alto,

Fig 4. Immunoblotting of total protein lysate from pig atria before (*b*) and 90 minutes after (*a*) post-CPB perfusion as described in "Material and methods." **A**, The lane with total c-Src was probed with a c-Src antibody 327. The lane with activated c-Src was probed with antibody that only recognizes c-Src in its active form (antibody 29). **B**, Akt phosphorylation was not significantly different between before and after post-CPB perfusion. The figure shows the results of 1 or 2 different experiments representative of 6 pigs studied. Ponceau S was used to ascertain proper protein fractionation and transfer.

Calif) and NIH Image 1.62 software (National Institutes of Health, Bethesda, Md). Comparisons between samples were analyzed by 1-way analysis of variance followed by a 2-tailed *t* test using Microsoft Excel software (Microsoft Corporation, Seattle, Wash). Values are expressed as mean fold change $\pm$ standard deviation of the mean. Ponceau S staining was used to determine proper protein fractionation and equivalent loading. Only samples with similar protein fractionation and protein loading less than 20% differences were analyzed further. The optical density ratio of the bands to that of Ponceau S was used to correct for small uneven loading (<20%).

Results

Phosphorylation levels of ERK1/2 decrease after CPB. The presence of an *in vivo* phosphorylation of ERK1/2 in basal levels was observed in both ventricular (Fig 2, A and D) and atrial (Fig 3, A and D) myocardium. Whereas levels of phosphorylated ERK1/2 decreased in ventricular myocardium (0.39-fold $\pm$ 0.15 as compared with basal levels, *P* = .006, *n* = 5) after 90 minutes of post-CPB perfusion (Fig 2, A), levels did not decrease (1.07-fold $\pm$ 0.18 as compared with basal levels, *P* > .2, *n* = 4) after 180 minutes in controls (Fig 2, D).

A similar picture was observed in atrial myocardium. After 90 minutes of post-CPB perfusion, a decrease in phosphorylated levels of ERK1/2 in atrial tissue (0.47-fold $\pm$ 0.10 as compared with basal levels, *P* = .007, *n* = 7) was consistently observed, as shown in Fig 3, A. However, protein levels of ERK1/2 were not modified after 90 minutes of post-CPB perfusion (0.96-fold $\pm$ 0.01 as compared with basal levels, *P* = .06, *n* = 7). In controls (Fig 3, D), both phosphorylated (1.12-fold $\pm$ 0.28 as compared with basal levels, *P* = .15, *n* = 4) and total ERK1/2 (1.02-fold $\pm$

0.09 as compared with basal levels, *P* > .2, *n* = 4) levels did not significantly change after 180 minutes of sham operations not involving CPB.

Phosphorylation of MEK1/2 correlates with phosphorylation of ERK1/2. Since the steady state of ERK1/2 phosphorylation is altered after CPB, it is possible that similar changes would be observed in the phosphorylation steady state of MEK1/2, the upstream kinases that phosphorylate ERK1/2. As illustrated in Fig 2, B, phosphorylated levels of MEK1/2 significantly decreased in ventricular tissue (0.36-fold $\pm$ 0.22 as compared with basal levels, *P* = .009, *n* = 5) after 90 minutes of CPB perfusion. However, they did not decrease (1.07-fold $\pm$ 0.18 as compared with basal levels, *P* = .10, *n* = 4) after a total of 180 minutes in controls (Fig 2, E). Such findings correlate well with ERK1/2 dephosphorylation.

As observed in ventricular myocardium, there was a prominent dephosphorylation of MEK1/2 in atrial myocardium (0.49-fold $\pm$ 0.03 as compared with basal levels, *P* = .008, *n* = 7) (Fig 3, B) without significant changes in MEK1/2 protein levels (0.96-fold $\pm$ 0.01 as compared with basal levels, *P* > .2, *n* = 7). In controls (Fig 3, E), neither phosphorylated MEK1/2 levels (1.12-fold $\pm$ 0.28 as compared with basal levels, *P* > .2, *n* = 4) nor total MEK-1 levels (1.08-fold $\pm$ 0.1 as compared with basal levels, *P* > .2, *n* = 4) were significantly changed after 180 minutes of sham operations not involving CPB.

Induction of MKP-1 expression after CPB. A robust induction of MKP-1 immunoreactivity was observed in ventricular myocardium after 90 minutes of CPB and 90 minutes of post-CPB perfusion (5.11-fold $\pm$ 2.2 as compared with basal levels, *P* = .03, *n* = 5), as shown in Fig 2, C. At the same time point in

Phospho-ERK1/2

A

Before CPB

Post-CPB perfusion

MKP-1

B

Before CPB

Post-CPB perfusion

Fig 5. A, Confocal microscopy images from pig atria before (*left*) and 90 minutes after (*right*) post-CPB perfusion as described in "Material and methods." Notice the intensity decrease of phospho-ERK1/2 signal post-CPB perfusion, confirming immunoblotting observations. Images were obtained from similar fields and same magnification and image adjustments. **B,** Confocal microscopy images from pig atria before (*left*) and 90 minutes after (*right*) post-CPB perfusion as described in "Material and methods." MKP-1 is induced at the end of post-CPB perfusion, localizing mainly in cardiomyocyte nuclei. Images were obtained from similar fields, magnifications, and adjustments.

controls (Fig 2, *F*), however, MKP-1 was not induced (1.07-fold $\pm$ 0.13 as compared with basal levels, $P = .11$, $n = 4$). It is interesting to note that MKP-1 can be detectable in samples before CPB, indicating basal expression in porcine myocardium.

Conspicuous induction of MKP-1 was also observed in atrial tissue 90 minutes after initiation of post-CPB perfusion (Fig 3, *C*) (4.35-fold $\pm$ 1.15 as compared with

basal levels, $P = .02$, $n = 4$). In controls, however, MKP-1 expression (Fig 3, *F*) was not significantly altered (1.13-fold $\pm$ 0.35 as compared with basal levels, $P = .08$, $n = 4$).

Active forms of c-Src and phospho-Akt did not change after CPB. In the same atrial tissues as analyzed above, neither c-Src protein levels (1.01-fold $\pm$ 0.01 as compared with basal levels, $P = .09$, $n = 6$) nor

c-Src activity ($0.99\text{-fold} \pm 0.05$ as compared with basal levels, $P > .2$, $n = 6$) were modified (Fig 4, A). Similar results were obtained for activity of Akt ($0.98\text{-fold} \pm 0.1$ as compared with basal levels, $P = .17$, $n = 7$) and Akt protein levels ($0.96\text{-fold} \pm 0.01$ as compared with basal levels, $P > .2$, $n = 7$) (Fig 4, B).

In ventricular myocardium, Akt results were similar to those observed in atrial tissue. After 90 minutes of post-CPB perfusion ($0.92\text{-fold} \pm 0.14$ as compared with basal levels, $P > .2$, $n = 5$) and in control pigs ($1.02\text{-fold} \pm 0.07$ as compared with basal levels, $P > .2$, $n = 4$), significant changes were not observed (data not shown).

Confocal microscopy of atrial tissue corroborate immunoblotting results. The decrease of ERK1/2 phosphorylation after 90 minutes of CPB and 90 minutes of post-CPB perfusion was also observed by confocal microscopy (Fig 5, A). Despite the difference in intensity, the phosphorylated form of ERK1/2 localized to the cardiomyocyte surfaces and nuclei before CPB and after 90 minutes of post-CPB perfusion.

Phosphorylated MEK1/2 was mainly observed in cardiomyocyte nuclei, with discrete cytoplasmic staining. Phosphorylated MEK1/2 was slightly decreased 90 minutes after CPB termination (data not shown).

MKP-1 immunostaining, almost exclusively observed on cardiomyocyte nuclei (Fig 5, B), confirmed the evident induction of expression of this dual phosphatase. Despite similar distribution at both time points, a more intense staining was observed after 90 minutes of post-CPB perfusion, and this correlated with immunoblotting results.

Discussion

The main findings of this study are that the levels of phosphorylated ERK1/2 were consistently lower than baseline after 90 minutes of normothermic CPB and 90 minutes of post-CPB perfusion. These decreases were accompanied by a decrease in MEK1/2 phosphorylation, indicating a decrease in upstream activation. MKP-1 protein levels were considerably increased, adding a downstream component to ERK1/2 inactivation. Because we were unable to detect any significant change in c-Src activity or phospho-Akt after CPB, a certain level of specificity to the MEK/ERK pathway is revealed. These results collectively point to a general decrease in the MEK/ERK signal transduction module after CPB.

The mechanisms regulating signal transduction have been studied mainly in cell culture models. Since cell cultures are kept "quiescent" before experimentation, inactivation of signal transduction from a basal level is frequently overlooked. In vivo, however, it appears that

signal transduction pathways have a high basal level of activity. It is important to consider, as shown by immunoblotting and immunostaining, that basal levels of phosphorylated (active) ERK1/2 and MEK1/2, as well as MKP-1 expression, are detected in pig hearts. Because atrial and ventricular myocardium respond similarly to CPB, it is suggested that atrial sampling (a more convenient approach in patients) may well reflect the changes occurring in ventricular myocardium.

It is interesting to note that the MEK/ERK pathway is inactivated by CPB. In fact, different models of ischemia/reperfusion using either cardiomyocyte cultures⁷ or isolated hearts²⁵⁻²⁸ showed that ERK1/2 is activated after reperfusion. However, ERK1/2 phosphorylation shows a characteristic trend in these studies: rapid induction after initiation of reperfusion, reaching a peak and decreasing at the end of the reperfusion period. Consequently, it cannot be ruled out that during the 90 minutes of CPB and 90 minutes of post-CPB perfusion, variations in the activity did not occur, nor can it be assumed that different results would not be obtained at later time points. Because in our protocol we harvested the samples after 90 minutes of post-CPB perfusion, it is possible that ERK1/2 activation might have occurred before we collected our samples. In relation to our findings, it has been reported²⁹ that ERK1/2 phosphorylation in human atrial tissue increases during cardioplegia; ERK1/2 activity was higher after ischemia and showed a further increase after reperfusion. The difference could be related to the fact that the present study does not use an ischemia/reperfusion model, because cardioplegia was not used. In addition, in 6 of 8 patients from the study by Talmor and colleagues,²⁹ the surgical procedure was shorter than our experimental design (ischemia, 44.3 ± 7.7 minutes; reperfusion, 36.3 ± 15.7), an important issue considering the possibility that the activation might not be sustained during the postoperative period.

Our data reveal not only that CPB induced the expression of MKP-1 in the heart, but also that MKP-1 expression is localized to the nuclei of the cardiomyocytes. This was not surprising considering that ERK1/2 dephosphorylation can be mediated by MKP-1.¹⁰ For instance, angiotensin II, an activator of ERK1/2 in smooth muscle cells,³⁰ induces MKP-1 protein synthesis within 1 hour of stimulation. More important, when protein synthesis is blocked and MKP-1 expression is inhibited, ERK1/2-stimulated phosphorylation is not reversed.³¹ Nevertheless, MKP-1 cannot be solely responsible for ERK1/2 dephosphorylation in our CPB model, because MEK1/2 dephosphorylation clearly points to the involvement of upstream signals. Thus, CPB is likely to generate dif-

ferent levels of ERK inactivation, one from upstream signals (MEK) and the other from parallel, inhibitory molecules (MKP-1).

To which degree the changes observed in the present study relate to the well-established morbidity associated with CPB is not clear at this time. However, our studies support an inactivation of MEK/ERK pathways that could contribute to myocardial depression observed after CPB. In fact, *in vitro*³² and *ex vivo*⁵ studies have revealed that PD98059, a MEK1/2 chemical inhibitor, prevented the increase in muscular contraction-induced ERK1/2 phosphorylation. Also, in rat aorta vascular smooth muscle cells subjected to mechanical stretch, ERK1/2 was phosphorylated to a peak of activity at 20 minutes, returning to basal levels after 30 minutes of stimulation.³³ These observations suggest that, in our model, a certain degree of cardiomyocyte contraction dysfunction can be either a cause or effect of ERK1/2 dephosphorylation. Also, ERK1/2 inactivation can be a cause of the systemic and coronary vascular relaxation observed after CPB.

Another point to be considered is the role of ERK1/2 in preconditioning. In preconditioning, where the heart is subjected to short periods of ischemia and reperfusion before a sustained ischemia/reperfusion period,³⁴ ERK activity increases up to 89% and translocates to the nucleus.³⁵ Given that in our study ERK1/2 is dephosphorylated, it is likely that the hearts of the pigs subjected to CPB lacked the presumptive protective effect of preconditioning. Indeed, a recent pig model study shows that specific MEK1/2 inhibitors reduce ERK activity and may block the beneficial effects of preconditioning.³⁶ This suggests that the inactivation of ERK during CPB may contribute to myocardial injury.

In vitro studies show that the addition of MEK1/2 inhibitor PD98059 to cardiomyocytes subjected to ischemia for 2 hours, followed by reoxygenation for 24 hours, induced apoptosis while inhibiting ERK phosphorylation completely.⁷ Consequently, the relationship between ERK dephosphorylation (also demonstrated in our data) and cardiomyocyte apoptosis may be clinically relevant in CPB. Likewise, the induction of MKP-1 that was observed after post-CPB perfusion should be considered detrimental to cardiomyocyte survival.

There is also *ex vivo* evidence that decreased ERK1/2 activity may be an important feature in cardiac dysfunction, because MEK1/2 inhibition (by pretreatment with PD98059) in isolated rat hearts subjected to ischemia and reperfusion reduced ERK activation by 71% and aggravated cardiac function injury.⁷ Thus, it appears that the ERK1/2 dephosphorylation in the myocardium after

post-CPB perfusion may have clinical significance with regard to preservation of cardiac function.

In conclusion, the MEK/ERK pathway is partially inactivated after 90 minutes of normothermic CPB followed by 90 minutes of post-CPB reperfusion in pigs. Further experiments need to determine the exact time course of ERK/MEK inactivation, as well as the role of the MEK/ERK pathway, in causing detrimental effects in the myocardium and the coronary and peripheral vasculature.

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