Effects of Cardiopulmonary Bypass on Endothelin-1–Induced Contraction and Signaling in Human Skeletal Muscle Microcirculation

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Background—We investigated the effects of cardiopulmonary bypass (CPB) on the contractile response of human peripheral microvasculature to endothelin-1 (ET-1), examined the role of specific ET receptors and protein kinase C-alpha (PKC-α), and analyzed ET-1–related gene/protein expression in this response.

Methods and Results—Human skeletal muscle arterioles (90 to 180 μm in diameter) were dissected from tissue harvested before and after CPB from 30 patients undergoing cardiac surgery. In vitro contractile response to ET-1 was assessed by videomicroscopy, with and without an endothelin-A (ET-A) receptor antagonist, an endothelin-B (ET-B) antagonist, or a PKC-α inhibitor. The post-CPB contractile response of peripheral arterioles to ET-1 was significantly decreased compared with pre-CPB response. The response to ET-1 was significantly inhibited in the presence of the ET-A antagonist BQ123 but unchanged in the presence of the ET-B receptor antagonist BQ788. Pretreatment with the PKC-α inhibitor safingol reversed ET-1–induced response from contraction to relaxation. The total protein levels of ET-A and ET-B receptors were not altered after CPB. Microarray analysis showed no significant changes in the gene expression of ET receptors, ET–1–related proteins, and protein kinases after CPB.

Conclusions—CPB decreases myogenic contractile function of human peripheral arterioles in response to ET-1. The contractile response to ET-1 is through activation of ET-A receptors and PKC-α. CPB has no effects on ET-1–related gene/protein expression. These results provide novel mechanisms of ET-1–induced contraction in the setting of vasomotor dysfunction after cardiac surgery. (Circulation. 2010;122[suppl 1]:S150–S155.)

Key Words: cardiopulmonary bypass ■ endothelin ■ genes ■ microcirculation ■ vasoconstriction

Cardiopulmonary bypass (CPB) is widely recognized to induce a systemic inflammatory response that leads to various degrees of organ dysfunction in multiple systems. For instance, CPB is associated with reduced vascular resistances in the skeletal muscle and peripheral circulation, which can lead to systemic hypotension and subsequent organ/tissue malperfusion. Specifically, CPB impairs contractile responses of peripheral arterioles to phenylephrine and vasopressin. The molecular mechanisms responsible for this vasomotor dysfunction have been extensively investigated in our laboratory. Several protein kinases, such as mitogen-activated protein kinases, extracellular signal regulated kinases 1/2, p38 kinase, and protein kinase C (PKC) have been suggested to be involved in vasomotor dysfunction. PKC-α has also been found to play an important role in α-adrenergic signaling in human peripheral microvasculature and in vasomotor dysfunction after CPB.

Endothelin-1 (ET-1), a vasoactive biopeptide, elicits potent and prolonged effects on cardiomyocytes and smooth muscle cells. Increases in plasma and interstitial myocardial ET-1 have been reported to occur in the perioperative period after cardiac surgery requiring CPB. The induction and release of ET-1 in the early post–coronary artery bypass graft period have been suggested to be detrimental, whereas decreased ET-1 or blockade of ET receptors during CPB may be beneficial in animals and humans. However, the role of ET-1 in CPB-related microvascular vasomotor dysfunction in human peripheral arterioles and the molecular mechanisms underlying ET-1–induced vasomotor response in the physiological setting and after CPB remain to be elucidated. Therefore, this study was designed to examine the effect of CPB on microvascular responses of human peripheral microvessels to ET-1, ET receptor antagonists, and a PKC-α blocker and to relate these responses to possible alterations in expression/localization of ET receptors and related gene expression in human skeletal muscle tissue.
Methods

Human Subjects and Tissue Harvesting
Samples of skeletal muscle from the left internal mammary artery bed were harvested before and after CPB from 30 patients. The pre-CPB specimen was taken after cannulation and the post-CPB specimen was collected from a different location in the left internal mammary artery bed after removal of the aortic cross-clamp and weaning from CPB. Tissue samples for immunoblot analysis assay were immediately frozen in liquid nitrogen. Tissue for immunofluorescent staining was fixed in 10% formalin-buffered solution for 24 hours followed by paraffin mounting and sectioning into 5-µm slices. Tissue for microvascular studies was placed in cold (5°C to 10°C) Krebs buffer solution. All procedures were approved by the Institutional Review Board of Beth Israel Deaconess Medical Center, Harvard Medical School, and informed consent was obtained from all enrolled patients as required by the institutional review board.

Microvessel Reactivity
Skeletal muscle arterioles (90- to 180-µm internal diameters) from the left internal mammary artery bed were dissected from pre- and post-CPB tissue samples. Microvessel studies were performed by in vitro organ bath videomicroscopy as described previously.13 Microvessel studies were performed on pre- and post-CPB skeletal muscle microvessels as follows: (1) measurement of contraction to increasing doses of ET-1 (10^{-12} to 10^{-7} M) (n=10), (2) measurement of contraction to ET-1 with and without ET-A receptor antagonist BQ123 (10^{-7} M) or ET-B antagonist BQ788 (10^{-7} M) (n=6), and (3) measurement of contraction to ET-1 with and without specific PKC-α blocker safingol (2.5×10^{-5} M) pretreatment for 20 minutes. Internal diameter was defined as the diameter measured after cannulation of the vessel and equilibration in the buffer solution. Internal diameters measured after treatment with ET-1 were calculated as percentage from the baseline diameter. The microvessels were washed with a Krebs buffer solution and allowed to equilibrate 15 to 30 minutes between interventions.

Immunoblot
Small arteries from skeletal muscle from 6 patients were dissected and cleaned of connective tissues and solubilized in SDS-PAGE buffer. Total protein (40 µg) was fractionated on an 8% to 16% SDS-PAGE gel, then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, Mass) as previously described.3 Membranes were incubated for 1 hour at room temperature with 1:200 dilutions of individual rabbit polyclonal primary antibodies to ET-A and ET-B receptors (Santa Cruz Biotechnology, Santa Cruz, Calif). The membranes were then incubated for 1 hour with horseshadish peroxidase–conjugated secondary anti-rabbit Ig, washed 3 times in Tris buffer saline, and processed for chemiluminescent detection (Pierce, Rockford, Ill) on X-ray film (Kodak, Rochester, NY). Band intensity was measured by densitometric analysis of autoradiograph films using NIH Image J 1.33.

Confocal Immunofluorescence Microscopy
Skeletal-muscle tissue sections from 6 patients were deparaffinized in xylene, rehydrated in graded ethanol and phosphate-buffered saline solution (PBS), and antigen-unmasked with sodium citrate (10 mMol/L, pH = 6.0), followed by PBS wash and blocking with 2% bovine serum albumin in PBS at room temperature for 2 hours. After PBS wash, sections were incubated overnight with ET-A and ET-B receptor antibodies (each used at 1:200) (Santa Cruz Biotechnology) at 4°C. Anti-mouse, α-smooth muscle actin (1:1000) (Sigma-Aldrich; St. Louis, Mo) was used to detect microvascular smooth muscle. Sections were then washed in PBS and incubated with the appropriate Alexa-fluor secondary antibody and mounted using fluorescent mounting medium (Vector Labs, Burlingame, Calif). Tissue was visualized using a Zeiss LSM510 confocal microscope system (Carl Zeiss MicroImaging, Inc, Thornwood, NY). Tissue labeling with secondary antibodies (ET-A or ET-B) alone and primary and secondary antibodies for α-smooth muscle actin served as negative controls.

RNA Isolation and Microarray Processing
Total RNA was isolated from ~200 mg of tissue samples with a Trizol-based method, following the manufacturer’s protocol (Gibco BRL; Grand Island, NY). For microarray analysis, after the quantitative and qualitative assessment of extracted total RNA, single-stranded followed by double-stranded cDNA synthesis was performed. Biotin-labeled cRNA was obtained by in vitro transcription of double-stranded cDNA using AFTI kit (Affymetrix, Santa Clara, Calif). cRNA was further purified, fragmented, hybridized overnight onto Affymetrix gene chips, and washed in streptavidin followed by array scanning as previously described.16

Microarray Analysis
Transcriptional profiling was performed on HG-U133 plus 2.0 Affymetrix chips, with matched prebypass samples serving as controls. Quality control, normalization, differential gene expression, and statistical analysis of microarray data were performed using JMP Genomics (SAS, Cary, NC). After quality control analysis, 1 outlier sample was eliminated from the analysis. Chips were then normalized using the Robust Multichip Average statistical method, and gene expression in post-CPB samples were compared with pre-CPB samples using 1-way ANOVA. A post hoc false detection rate algorithm with α of 0.05 was applied to control for false-positives. Fold changes >1 or <−1 were considered real changes, and −log_{10} (probability values) >4.14 were considered significant.

Gene ontology and canonical pathway analyses were carried out using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, Calif).

Chemicals
ET-1, BQ123, and BQ788 were obtained from Sigma-Aldrich. Safingol was purchased from Avanti Polar Lipids (Alabaster, Ala). ET-1 was dissolved in ultrapure distilled water and prepared on the day of the study. BQ123, BQ788, and safingol were dissolved in dimethylsulfoxide to make a stock solution. All stock solutions were stored at 4°C or −20°C. All dilutions were prepared daily.

Data Analysis
Data are presented as the mean and standard error of the mean (SEM). Microvessel responses are expressed as the percentage of contraction of baseline. Microvascular reactivity data were analyzed using repeated-measures ANOVA followed by Student t test. Paired
t tests were used to compare the Western blot data before and after CPB. Probability values <0.05 were considered significant.

## Results

### Patient Characteristics

The patient characteristics are listed in Table 1. All patients with preoperative hypertension were receiving antihypertensive medication (β-blocker, aspirin, calcium channel blocker, or angiotensin-converting enzyme inhibitor).

### Microvascular Reactivity

The post-CPB contractile responses of skeletal muscle arterioles to ET-1 were significantly decreased compared with pre-CPB responses (25±4% versus 46±5%, P<0.05, Figure 1A). Pretreatment of pre-CPB peripheral arterioles with ET-A receptor antagonist BQ123 significantly inhibited ET-1–induced vasoconstriction (8±1% versus 46±5%, P<0.05, Figure 1B). Incubation of post-CPB peripheral arterioles with BQ123 before addition of ET-1 also resulted in an inhibition of vasoconstriction (5±0.6% versus 25±4%, P<0.05). In contrast, inclusion of the ET-B receptor antagonist BQ788 in pre- or post-CPB arterioles failed to affect ET-1–induced vasoconstriction (42±3% versus 46±5%; 29±3% versus 25±4%, P>0.05, respectively, Figure 2A and 2B). In addition, pretreatment with the PKC-α inhibitor safingol in pre- and post-CPB arterioles significantly reversed ET-1 response from contraction into a slight relaxation, respectively (from 46±5% to −15±2%; from 25±4% to −11±2.5%, P<0.05, Figure 3A and 3B).

### Effect of CPB on the Gene Expression of ET-1, ET-A, and ET-B Receptors and Related Protein Kinases

The data obtained from microarray analysis are summarized in Figure 4A and Table 2. The post-CPB fold changes of gene expression of ET-1, ET-A, and ET-B receptors were less than 1-fold, suggesting there were no significant changes in ET-1, ET-A, and ET-B receptor gene expression (Figure 4A). The ET-related protein kinases, such as mitogen-activated protein kinases, and PKC also were not significantly altered after CPB (Table 2).

### Effect of CPB on Levels of ETA and ETB Receptor Polypeptides

Pre-CPB skeletal muscle levels of the ET-A and ET-B receptor polypeptides were unchanged in the post-CPB period, as detected by immunoblot (Figure 4B).

### Effect of CPB on Microvessel Distribution of ET-A and ET-B Receptor Polypeptides

Immunofluorescent staining of coronary microvessels displayed a strong signal for ET-A receptors localized to the microvasculature and a relatively weak signal of post-CPB vessels compared with pre-CPB vessels (Figure 5A). ET-B receptors were mainly localized to endothelial cells but were seldom detected after CPB (Figure 5B). Negative controls documented a low level of background fluorescence (red) and a strong signal of α-actin stained on smooth muscle (green, Figure 5A and 5B).

## Discussion

There are several new findings in the present study. First, ET-1 induced dose-dependent vasoconstriction of human skeletal muscle arterioles, and the contractile response to ET-1 was significantly reduced after CPB. Second, the responses to ET-1 were significantly inhibited in the presence of ET-A receptor antagonist BQ123 but unchanged in the pretreatment with ET-B receptor antagonist BQ788. Third, the presence of ET-A and ET-B receptor polypeptides in...
human skeletal muscle microvasculature was documented by immunoblot and by immunofluorescence microscopy. ET-A was predominant in both smooth muscle and endothelial cells in skeletal muscle microvasculature, whereas ET-B appeared less abundant. Fourth, CPB did not change total polypeptide levels of either ET-A or ET-B. Finally, the contractile responses to ET-1 were significantly reversed to relaxation in the presence of the PKC-\(\alpha\) inhibitor safingol.

Endothelin-1 is synthesized from the precursor “big endothelin.” The diverse physiological and pathophysiologic effects of ET-1 are mediated through 2 subtypes of receptors, the ET-A and ET-B receptors. Various cell types such as endothelial cells, smooth muscle cells, cardiac myocytes, and macrophages may contribute to the increased release of ET-1 after CPB. The present work demonstrates that ET-A receptors are the predominant subtype found within the human peripheral microvasculature, whereas ET-B receptors appear less abundant. Vasoconstriction was inhibited in the presence of ET-A receptor antagonist but not the ET-B antagonist, further suggesting that ET-A receptors are responsible for ET-1–induced vasoconstriction and not ET-B receptors. Interestingly, ET-A and ET-B receptors were primarily detected in the microvasculature of pre-CPB tissue but almost disappeared after CPB, suggesting that ET receptor internalization may occur after CPB. These findings are consistent with those of previous studies on human radial artery smooth muscle. ET-A and ET-B receptor polypeptides were detected by immunoblot of extracts from human peripheral arteries, and CPB did not alter expression of either polypeptide, suggesting that it may modify the functional state or intracellular distribution of receptor protein rather than the steady-state levels of protein. The data from microarray analysis were consistent with the findings from protein analysis, indicating there are no significant changes in ET-A and ET B receptors gene expression after CPB.

We and several others have previously shown that CPB results in vasomotor dysfunction with reduced contractile responses to norepinephrine and vasopressin in the peripheral microvasculature of animals and humans. The present study demonstrates a similarly reduced contractile response of human peripheral microvessels to ET-1 after CPB. The mechanism responsible for this dysfunction may be related in part to the ET-1 receptor responses to CPB. The endogenous stress response to CPB results in an enhanced release of vasoactive ET-1, which acts predominately on ET-A. The sustained increase in circulating levels of ET-1 in vivo, or prolonged exposure to ET-1 in vitro, may cause subsequent loss of ET-1–mediated vascular smooth muscle cell contraction. In addition, the inflammatory response to CPB can induce activation or release of oxygen free radicals, prostaglandins, nitric oxide, complement, and proinflammatory cytokines, all of which can contribute to vasomotor dysfunction through vasodilatation/vasoconstriction and increased vascular permeability.
PKC-α has been found to be the predominant conventional PKC isoform present in human microcirculation.\textsuperscript{1,4,5} The α-adrenergic–induced vasoconstriction in the human peripheral microvasculature is partially mediated by PKC-α, and CPB results in reduced PKC-α activity in human skeletal microcirculation. The present study indicates that ET-1–induced vasoconstriction in the human peripheral arterioles also acts in part via PKC-α activation. The upstream molec-

**Table 2. Data From Microarray Analysis**

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GRK indicates G-protein–coupled receptor kinase; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; PKC: CaM PK, calmodulin phosphatase kinase; RhoGEF, ρ-guanine nucleotide exchange factor; GSK, glycogen synthase kinase; MTOR, mammalian target of rapamycin; RhoA, Ras homolog gene family, member A; and MAPK, mitogen-activated protein kinase.

**Figure 5.** Immunolocalization of ET-A and ET-B receptor polypeptides in human skeletal microvessels (n=6). Vessels were costained for smooth muscle actin and either ET-AR (A), or ET-BR (B). Matched negative controls are displayed below each row of primary antibody.
ular mechanism contributing to this dysfunction may be diminished by inositol 1,4,5-triphosphate turnover, which results in decreased concentrations of diacylglycerol and calcium, both of which are required for conventional PKC activation. This effect of diminished inositol phospholipid metabolism and blunted vascular smooth muscle cell contraction in response to ET receptor stimulation can also be produced with prolonged exposure to phorbol ester, a highly potent PKC activator.

In conclusion, CPB decreases myogenic contractile function of human peripheral microvessels in response to ET-1. ET-A receptors are predominantly present in human peripheral microcirculation, whereas ET-B receptors are less abundant. The contractile responses to ET-1 are through activation of ET-A receptors and PKC-α. In addition, CPB changes neither total protein expression nor gene expression of ET-A and ET-B. These results provide novel mechanisms of ET-1–induced contraction in vasomotor dysfunction after cardiac surgery.

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Disclosures
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References
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