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Endothelin-1 Induced Contractile Responses of Human Coronary Arterioles via Endothelin-A Receptors and PKC- α Signaling Pathways

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Abstract

Background—We investigated the contractile function in responses to endothelin-1 (ET-1) in the human coronary microvasculature and the roles of endothelin receptors and protein kinase C- α (PKC- α) in these responses.

Methods—Human atrial tissue was harvested from patients undergoing cardiac surgery pre- and post-cardioplegia/cardiopulmonary bypass (CP/CPB). Microvascular constriction was assessed in pre- and post-CP/CPB samples in responses to ET-1, in the presence and absence of an endothelin-A (ET-A), or an endothelin-B (ET-B) receptor antagonist, or a PKC- α inhibitor, respectively. The expression and localization of ET-A and ET-B receptors were also examined using immunoblot and immunofluorescence photomicroscopy.

Results—The post-CP/CPB contractile response of coronary arterioles to ET-1 was significantly decreased compared with the pre-CP/CPB responses. The response to ET-1 was significantly inhibited in the presence of the ET-A antagonist BQ123 (10^{-7} M), but unchanged with the ET-B receptor antagonist BQ788 (10^{-7} M). Pretreatment with PKC- α inhibitor safinolol (2.5×10^{-5} M) reversed the ET-1 responses from contraction into relaxation. The total polypeptide levels of ET-A and ET-B receptors were not altered post-CP/CPB. Immunoblot and immunofluorescent staining displayed strong signals for ET-A receptors and relatively weak signals for ET-B receptors localized on coronary microvasculature.

Conclusion—CP/CPB decreases the contractile function of human coronary microvessels in responses to ET-1. ET-A receptors are predominantly localized in the human coronary microcirculation, whereas ET-B receptors appear to be less abundant. The contractile response to ET-1 is in part through activation of ET-A receptors and PKC- α . These results suggest a role of ET-1-induced contraction in the vasomotor dysfunction after cardiac surgery.

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Keywords

Endothelins; Receptors; protein kinase C; Cardioplegia; Cardiopulmonary bypass; vasomotor dysfunction; Microcirculation

Introduction

Cardioplegia and cardiopulmonary bypass (CP/CPB) elicits complex, multifactorial vasomotor disturbances that vary according to the affected organ beds.¹⁻⁴ Specifically, CP/CPB is associated with an impaired contractile response of coronary and peripheral arterioles to phenylephrine and vasopressin.¹⁻³ This dysfunction may be due to diminished mitogen-activated protein kinases (MAPK), extracellular signal regulated kinases 1/2 (ERK1/2), and protein kinase C alpha (PKC- α) activity, whereas attenuated contraction to vasopressin may result from reduced p38 activity.¹⁻⁴ PKC- α has been found to play an important role in α -adrenergic signaling in human coronary microvasculature and in the vasomotor dysfunction after CP/CPB.⁴⁻⁵

Endothelin-1 (ET-1), a vasoactive biopeptide, exerts potent and prolonged effects on the human vasculature.⁶ Increases in plasma and interstitial myocardial ET-1 have been reported to occur in the perioperative period after cardiac surgery requiring CP/CPB.⁷⁻¹⁰ The induction and release of ET-1 in the early post-CABG period have been felt to be detrimental, whereas, decreases of ET-1 or blockade of ET receptors during CP/CPB have been reported to be beneficial in animal and humans.¹⁰⁻¹³ The role of ET-1 in CP/CPB-related microvascular vasomotor dysfunction in human coronary arterioles and the molecular mechanisms underlying ET-1-induced vasomotor response in the normal physiologic setting and clinically after CP/CPB remain to be elucidated. This study was, therefore, designed to examine the effect of CP/CPB on responses of human coronary microvessels to ET-1, ET-receptor antagonists and PKC- α blocker and to relate these responses to possible alterations in expression and localization of ET receptors and in the human atrial tissue.

Materials and Methods

Human Subjects and Tissue Harvesting

Samples of right atrial appendage were harvested from patients undergoing coronary artery bypass graft (CABG) surgery before and after exposure of the heart to blood CP and short-term reperfusion under conditions of CPB. Samples were handled in a nontraumatic fashion. Double 3-0 polypropylene purse-string sutures (Ethicon, Somerville, NJ) were placed in the atrial appendage. During placement of the venous cannula, the first sample of atrial appendage was harvested pre-CP/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 blood cardioplegia, and reperfused (post-CP/CPB) after removal of the aortic cross-clamp. An initial 600 to 800 mL of cold-blood (0°C to 4°C) hyperkalemic (15 mmol/L K⁺) cardioplegic solution was delivered antegrade into the aortic root. This was followed at 8- to 15-minute intervals with 250 to 300 mL of cold, cardioplegic solution (15 mmol/L K⁺). The cardioplegic solutions consisted of a mixture of oxygenated blood with crystalloid solution of the following final composition (in mmol/L): 15 KCl, 3.5 MgSO₄, 135NaCl, 1.0 CaCl₂, 11 Glucose, 11 Mannitol, 4 Tromethamine.

The second sample of atrial appendage (post-CP/CPB) was harvested after CP/CPB during removal of the venous cannula. 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 h followed by paraffin mounting and sectioning into 5- μ m

slices. Tissue for microvascular studies was placed in cold (5° to 10°C) Krebs-Henseleit buffer (KHB). 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 required by the Institutional Review Board.

Microvessel Reactivity

Microvessel studies were performed by in vitro organ bath videomicroscopy as described previously.^{5,14,15} Coronary microvessels (90-150 µm internal diameter) were dissected from harvested atrial tissue by use of a dissecting microscope (Olympus Optical, Tokyo, Japan). Microvessels were placed in an organ bath (University of Iowa Medical Instrumentation, Iowa City, IA), and cannulated with 10-0 nylon monofilament suture (Ethicon). Oxygenated (95% O₂/5% CO₂) KHB solution warmed to 37°C was continuously circulated through the organ bath. The microvessels were pressurized to 40 mmHg in a non-flow state filled with KHB solution. With an inverted microscope (Olympus Optical) connected to a video camera, the vessel image was projected onto a television monitor. An electronic dimension analyzer (Living System Instrumentation, Burlington, VT) was used to measure the internal diameter, and measurement was recorded.

After 60 minutes of equilibrating period, pre- and post-CP/CPB microvessels received ET-1 (10⁻¹²-10⁻⁷ M) for examining the dose-dependency of ET-1-induced vasoconstriction. The vessels were pretreated with ET-A receptor antagonist BQ123 (10⁻⁷ M) or ET-B antagonist BQ788 (10⁻⁷ M) (Sigma, St. Louis, MO) 5 minutes before ET-1 (10⁻¹²-10⁻⁷ M) administration. Some vessels were pre-incubated with PKC-α inhibitor safingol (2.5 × 25 µM, Avanti Polar Lipids, Alabaster, AL) 20 minutes before ET-1 (10⁻¹²-10⁻⁷ M) perfusion. Baseline diameter was defined as the diameter measured after cannulation and equilibration in the KHB solution. Internal diameters measured after treatment with ET-1 were normalized to the baseline diameter. The microvessels were washed with a KHB solution and allowed to equilibrate 15-30 min between interventions.

Immunoblot

Human discarded atria tissue (n = 6) collected from pre-CP/CPB and post-CP/CPB was analyzed for levels of total ET-A and ET-B receptor proteins by immunoblotting. Total protein (40 µg) was fractionated on an 8-16 % SDS-PAGE, 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, CA). The membranes were then incubated for 1 hour with horseradish peroxidase-conjugated secondary anti-Ig washed 3x in Tris saline buffer (TBS), and processed for chemiluminescent detection (Pierce, Rockford, IL) on X-ray film (Kodak, Rochester, NY). Band intensity was measured by densitometric analysis of autoradiograph films using NIH Image J 1.33.

Immunofluorescence photomicroscopy

Atrial tissue sections from five 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 h. After PBS wash, overnight incubation with anti-ET-A and ET-B (each used 1:200) (Santa Cruz Biotechnology) were performed at 4°C. Anti-mouse, α-smooth muscle actin (1:1000) (Sigma, 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.

Chemicals

ET-1, BQ123, and BQ788 were obtained from Sigma. Safingol was purchased from Avanti Polar Lipids (Alabaster, AL). 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 -20°C . All dilutions were prepared fresh daily.

Data Analysis

Data are presented as the mean and standard error of the mean (SEM). Repeated-measures ANOVA and Student's *t*-test were used to compare variables among or between vessels. The treatment effects were statistically examined by paired or independent two-tailed Student's *t*-test. Statistical significance was taken at a probability value of <0.05 .

Results

Patient Characteristics

Tissue samples from 21 patients were studied. Twenty-one patients (mean age 68 ± 6 years) underwent coronary artery bypass graft (CABG) with duration of cardioplegic arrest of 60 ± 4 min and CPB time of 73.0 ± 16 min. Fifteen patients were male and six were female. Eighteen out of the 21 patients carried a preoperative diagnosis of hypertension. All patients with preoperative hypertension were on medication (β -blockers, aspirin, calcium channel blockers, or angiotensin-converting enzyme inhibitors), and received perioperative β -blockade. Diabetes mellitus (type I or type II) was present in 5 of 21 patients. All patients received perioperative aspirin.

Coronary Microvascular Responses

The post-CP/CPB contractile responses of coronary arterioles to ET-1 was significantly decreased compared with the pre-CP/CPB response ($P < 0.05$, Fig. 1A). Pretreatment of pre-CP/CPB coronary arterioles with the ET-A-receptor antagonist BQ123 significantly inhibited ET-1-induced vasoconstriction ($P < 0.05$, Fig. 1 B). Pre-incubation of post-CP/CPB coronary arterioles with BQ123 also resulted in an inhibition of vasoconstriction ($P < 0.05$, Fig. 2A). In contrast, exposure of pre- or post-CP/CPB arterioles to the ET-B receptor antagonist BQ788 failed to affect ET-1-induced vasoconstriction (Fig. 1C, Fig. 2B). Pretreatment with the PKC- α inhibitor safingol ($2.5 \times 10^{-5}\text{M}$) of pre- and post-CP/CPB arterioles significantly reversed the ET-1 response from contraction into relaxation, respectively (Fig. 3A, B).

Effect of CPB on levels of ETA and ETB polypeptides

Pre-CP/CPB and post-CP/CPB expression of the atrial ET-A and ET-B polypeptides were similar as detected by immunoblot (Fig. 4).

Effect of CP/CPB on microvessel distribution of ETA and ETB polypeptides

Immunofluorescent staining of coronary microvessels displayed a strong signal for ET-A (Fig. 5A) localized to the microvascular smooth muscles and a relatively weak signal for ET-B (Fig. 5B). Negative controls documented low level of background fluorescence (red) and strong signal of α -actin stained on smooth muscle (green, Fig. 5A, B).

Discussion

There are several new findings in the present study: ET-1 induced a dose-dependent vasoconstriction of human coronary arterioles. The contractile response of atrial microvessels to ET-1 was significantly reduced after CP/CPB. The response to ET-1 was significantly inhibited in the presence of the ET-A-receptor antagonist BQ123, but unaffected with the pretreatment with ET-B-receptor antagonist BQ788. The presence of ET-A and ET-B polypeptides in human coronary microvasculature was documented by immunoblot and by immunofluorescence microscopy. Positive ET-A immunostaining was present predominantly in smooth muscle cells, whereas ET-B appeared less abundant. Finally, CP/CPB changed neither total polypeptide levels of ET-A or ET-B.

Our and several other studies in the past have shown that CP/CPB results in vasomotor dysfunction in animals and humans.¹⁻⁵ The present study demonstrated a reduced contractile response of human coronary microvessels to ET-1 after CP/CPB. The mechanism responsible for this dysfunction may be related in part to the ET-1-receptor responses to CP/CPB. The endogenous stress response to CP/CPB results in an enhanced release of vasoactive ET-1, which acts predominately on ET-A or some of ET-B receptors.⁷⁻¹³ The sustained increase in circulating levels of ET-1 in vivo, or prolonged exposure to ET-1 in vitro, may result in subsequent loss of ET-1 mediated vascular smooth muscle cell contraction.⁹⁻¹¹ In addition, the inflammatory response to CP/CPB can result in activation or release of oxygen free radicals, prostaglandins, nitric oxide, complement, and pro-inflammatory cytokines, all of which can contribute to vasomotor dysfunction through vasodilatation and increased vascular permeability.¹⁻⁵

Endothelin-1, a vasoactive biopeptide, is synthesized from the precursor “big endothelin”. The diverse physiologic and pathophysiologic effects of ET-1 are mediated via two subtypes of receptors, the ET-A and ET-B receptors.⁶ Various types of cells, such as endothelium, smooth muscle cells, and cardiac myocytes may attribute to the increased release of ET-1 after CP/CPB.⁶⁻¹³ The present work has demonstrated that ET-A receptor is the predominant subtype found within the human myocardium and coronary microvasculature, and ET-B receptor is less abundant. The vasoconstriction was inhibited in the presence of ET-A receptor antagonist, not ET-B antagonist, further suggesting that ET-A receptors are responsible for ET-1-induced vasoconstriction, not ET-B. These findings are consistent with those of previous studies.⁶⁻⁹ ET-A and ET-B receptor polypeptides were detected by immunoblot and immunofluorescence microscopy of extracts from human atria tissue and coronary microvessels. CP/CPB did not alter expression of ET-A and ET-B polypeptides, suggesting that it may modify the functional state of receptor protein activation or intracellular distribution rather than the steady state levels of protein.

PKC- α has been found to be the predominant conventional PKC present in human coronary microcirculation.^{1, 4, 5, 16} Alpha-adrenergic-induced vasoconstriction in the human coronary microvasculature was partially mediated by PKC- α , and CP/CPB resulted in reduced PKC- α activity of coronary microcirculation.⁵ The present study indicates that ET-1-induced vasoconstriction in the human coronary arterioles is also in part due to PKC- α activation. The upstream molecular mechanism contributing to this dysfunction may be due to diminished 1, 4, 5-triphosphate turnover, which results in decreased concentrations diacylglycerol and calcium, both of which are required for conventional PKC activation.⁵ 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.

In conclusion, CP/CPB decreases the contractile response of human coronary microvessels in responses to ET-1. ET-A receptors are predominantly present in human coronary microcirculation, but ET-B receptors appear to be less abundant. The contractile responses to ET-1 are via activation of ET-A receptors and PKC- α . This evidence may suggest that the diminished contractile response to ET-1 seen in coronary microvessels after CP/CPB may be secondary to stress CP/CPB induced intraoperative overstimulation of ET receptors. These results provide novel pathways of ET-1-induced contraction in the vasomotor dysfunction after cardiac surgery.

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Fig 1.

Coronary microvascular vasoconstriction in response to endothelin-1 (ET-1) (**A**) pre- vs post-cardioplegic arrest /cardiopulmonary bypass (CP/CPB), (**B**) pre-CP/CPB vs pre-CP/CPB + BQ123, (**C**) pre-CP/CPB vs pre-CP/CPB + BQ788, * $P < 0.05$.

Fig 2. Coronary microvascular vasoconstriction in response to endothelin-1 (ET-1) (**A**) post-cardioplegic arrest /cardiopulmonary bypass (post-CP/CPB) vs post-CP/CPB + BQ123, (**B**) post-CP/CPB vs post-CP/CPB + BQ788, * $P < 0.05$

Fig 3. Coronary microvascular vasoconstriction in response to Endothelin-1 (ET-1) (**A**) pre-cardioplegic arrest /cardiopulmonary bypass (pre-CP/CPB) vs pre-CP/CPB + safingol, (**B**) post-CP/CPB vs post-CP/CPB + safingol, * $P < 0.05$.

Fig 4.

Representative immunoblot of human atria tissue. Lanes 1-2 loaded with 40 μ g protein were developed for ET-A and ET-B receptor polypeptides, respectively, showing unaltered levels of ET-A and ET-B polypeptides after pre- vs post-cardioplegic arrest /cardiopulmonary bypass (CP/CPB).

Fig 5.

Immunolocalization of ET-A and ET-B receptors (ET-AR and ET-BR) polypeptides in human coronary microvessels. Vessels were co-stained for smooth muscle α -actin and either (A) ET-AR, or (B) ET-BR antibody. Matched negative controls for ET-AR or ET-BR are displayed below each row, indicating only α -actin signals in α -actin staining and merged images.