Serotonin-Induced Coronary Contraction Increases After Blood Cardioplegia-Reperfusion
Role of COX-2 Expression

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Background—Coronary contraction has been implicated in causing suboptimal myocardial function after coronary bypass surgery. Addition of blood to cardioplegic solutions has been shown to improve endothelial function after cardioplegia. In this study, the effects of blood cardioplegia and brief reperfusion on vascular reactivity in patients with coronary artery disease and the expression (mRNA and protein) of enzymes involved in vasomotor regulation were examined.

Methods and Results—The atrial appendages of patients undergoing coronary artery surgery were harvested before cardiopulmonary bypass (control, n=8) and after bypass from a nonischemic tissue atrial segment exposed to cold, hyperkalemic blood cardioplegia (mean, 60 minutes) and a brief period (10 minutes) of reperfusion (CP-Rep, n=8). Responses of atrial arterioles were studied in vitro with video-microscopy. Reverse-transcriptase polymerase chain reaction and Western blotting were used to examine the expressions and protein content, respectively, of enzymes involved in vasomotor regulation. Serotonin caused a minimal dilation under baseline conditions but after CP-Rep elicited a potent contractile response that was inhibited in the presence of the selective inducible cyclooxygenase (COX-2) inhibitor NS398. Substance P caused an endothelium-dependent relaxation of atrial arterioles through release of nitric oxide, and ADP caused relaxation mediated through release of prostaglandins. After CP-Rep, relaxation to substance P was impaired, whereas endothelium-independent relaxation to nitroprusside and response to ADP were unchanged. Expression and protein level of COX-2 were significantly increased after CP-Rep. In contrast, expression of inducible (nitric oxide synthase-2) or constitutive endothelial (nitric oxide synthase-3) nitric oxide synthase, prostacyclin synthase, and constitutive cyclooxygenase (COX-1) were not altered after CP-Rep.

Conclusions—CP-Rep increases serotonin-induced contraction of human microvessels caused by the release of products of COX-2 and the impaired release of nitric oxide. These findings have implications regarding altered coronary microvascular regulation and the cause of coronary spasm after cardiac surgery. (Circulation. 1999;100[suppl II]:II-328–II-334.)

Key Words: coronary disease ■ heart diseases ■ endothelium ■ nitric oxide ■ surgery ■ microcirculation

Cardioplegia is widely and successfully used for the protection of the myocardium against ischemic injury during cardiac surgery. Cardiac dysfunction after cardiopulmonary bypass and ischemic arrest may be related to reduced myocardial perfusion secondary to altered vasomotor regulation and coronary artery spasm. Although the effects of cardioplegic solutions on endothelium-dependent relaxation, β-adrenergic regulation, and myogenic vasomotor regulation are well known, these experiments have been performed in normal animals without coronary artery disease. Patients undergoing CABG surgery suffer from atherosclerosis and in general have underlying endothelial dysfunction. Therefore, cardioplegia-induced endothelial dysfunction may be a moot point in these patients who already possess dysfunctional endothelium. No studies have examined the effects of cardioplegia and reperfusion (CP-Rep) on vascular reactivity in patients with underlying endothelial dysfunction caused by atherosclerotic vascular disease. The alteration in vasomotor pathways after cardioplegia and reperfusion in patients may be due to changes in expression or activities of either nitric oxide synthase (NOS) or cyclooxygenase (COX).

This study was designed to examine the effect of blood cardioplegia and brief reperfusion on vascular responses of human atrial microvessels to serotonin (5-HT) and other vasoactive substances and to correlate these responses to possible alterations in gene expressions and protein levels of constitutive endothelial NOS-3 and inducible NOS-2 in the human heart, as well as the constitutive cyclooxygenase (COX-1), inducible cyclooxygenase (COX-2), and prostacyclin synthase.

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The atrial appendages were harvested from patients undergoing CABG. Aspirin was discontinued ≥24 hours before surgery, but other medications were continued up to the time of surgery. Double cannulation of 3–0 polypropylene sutures was placed in the atrial appendage. After administration of heparin, a single 2-stage atrial cannula was placed and secured with the superior suture. The lower suture was not secured so that this portion of atrium would be exposed to cardiopulmonary bypass and be reperfused after release of the aortic cross clamp. A piece of right atrial appendage was harvested above the superior cannulation suture but before initiation of cardiopulmonary bypass (control group). Another piece of atrium was harvested after cross clamp removal and termination of cardiopulmonary bypass before protamine administration. An initial 800 to 1000 mL of cold-blood (0°C to 4°C) hyperkalemic (25 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, low-KCl (12 mmol/L K+) cardioplegic solution. The cardioplegic solution consisted of a 4:1 mixture of oxygenated blood with a hyperkalemic crystalloid solution, resulting in 25 or 12 mmol/L K+. The compositions of the high- and low-potassium crystalloid solutions were 25 and 12 mmol/L KCl, respectively, together with 5 g/L mannitol, 20 mL/L citrate phosphate dextrose solution, 4 mmol/L trihydroxy methylamino methane in 5% dextrose, and 0.2235% saline solution. A cold, moist gauze pad was placed on the heart for topical cooling. Patients were systemically cooled to 30°C to 32°C. Rewarming was initiated before the mammary distal anastomosis was begun. Mean cardioplegia time was 58±7 minutes, and the mean time from cross clamp removal until second tissue harvest was 10±3 minutes. Tissue in the control and CP-Rep groups was immediately frozen in liquid nitrogen after harvest for preparation for molecular biology studies or was placed in cold (5°C to 10°C) Krebs’ buffer solution for 15 to 30 minutes between times with Krebs’ buffer solution and allowed to equilibrate in drug-free Krebs’ buffer solution for 15 to 30 minutes between interventions.

Expression of NOS-3 and NOS-2 mRNA

For NOS-3 and NOS-2 mRNA studies, the semiquantitative reverse-transcriptase (RT) polymerase chain reaction (PCR) was performed because the signal intensities for NOS-3 and NOS-2 were not sufficient for quantitative analysis by Northern hybridization. Primers were designed on the basis of the published NOS-3 and NOS-2 sequences. The primers of the sense 5’-CAGTTTCCAC-ATGTCGCTGGAAATTG-3’ corresponding to bases 1050 through 1076 and the antisense 5’-TAAAAAGGTCTTCTTCTGTTGATGC-CC’ corresponding to bases 1511 through 1535 were used to amplify a 486-bp fragment of NOS-3. For NOS-2, the primer of sense 5’-GCTCTGCCTGGAAAG-3’ corresponding to bases 1425 through 1441 and the antisense 5’-TCCATGCAGACACCCGTTT-3’ corresponding to bases 1908 through 1924 were used to amplify a 500-bp fragment of NOS-2.

An equal amount of total RNA was used for RT-PCR. For quantification, GAPDH was amplified from the same amount of RNA to correct for variation of different samples. The PCR products were loaded in 1% agarose gel and then scanned and quantified with Image-Quant software (Molecular Dynamics).

Expression of COX-1, COX-2, and Prostacyclin Synthase mRNA

Primers were designed on the basis of the published COX-1 and COX-2, and prostacyclin synthase sequences. For COX-1, the primer of the sense 5’-TCTTTGCAACACACTCC-3’ corresponding to bases 601 through 620 and the antisense 5’-GTACTCATTGAAGGGCTGCA-3’ corresponding to bases 1050 through 1400 were used to amplify a 419-bp fragment of COX-1. For COX-2, the primer of the sense 5’-TAAACTGCGCTTTTICAAGG-3’ corresponding to bases 781 through 800 and the antisense 5’-GTGATACCTTCTGATCCTGCGG-3’ corresponding to bases 1381 through 1400 were used to amplify a 619-bp fragment of COX-2. For prostacyclin synthase, the primer of the sense 5’-AGTGTCAAAAATGTCGCTGGT-3’ corresponding to bases 721 through 740 and the antisense 5’-TCTTTTCTCTGAGCTCCATGG-3’ corresponding to bases 1261 through 1280 were used to amplify a 559-bp fragment of prostacyclin synthase.

As above, an equal amount of total RNA was used for semiquantitative RT-PCR. For quantification, GAPDH was amplified from the same amount of RNA to correct for variation of different samples. The PCR products were loaded in 1% agarose gel and then scanned and quantified with Image-Quant software (Molecular Dynamics).

Expression of NOS-2, NOS-3, COX-1, and COX-2 Proteins

Total proteins from atrial tissues were obtained by homogenizing in a lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS and centrifuging at 12 000g for 10 minutes at 4°C. Protein concentration of the supernatant was measured by spectrophotometry at 595 nm (DU640, Beckman) of an aliquot developed for 10 minutes in protein assay dye reagent (Bio-Rad). Total protein (40 μg/lane) was fractionated on 10% SDS-PAGE transferred to a polysineulide difluoride membrane (Immobilon-P, Millipore). Equal protein loading and transfer efficiency were visualized by Western blotting.
Ponceau red staining. The membrane was incubated with 5% nonfat dry milk powder and 0.05% Tween-20 in PBS for 12 hours at 4°C to block nonspecific absorption and then was immunoblotted with the monoclonal mouse anti-endothelial NOS antibody (Transduction Laboratories) at 1:2500 (vol/vol) dilution or the monoclonal mouse anti-inducible NOS antibody (Transduction Laboratories) at 1:500 dilution (vol/vol) for NOS-3 and NOS-2 Western, or with the polyclonal goat anti–COX-1 (Santa Cruz) at 1:1000 dilution (vol/vol) or with the polyclonal goat anti–COX-2 at 1:500 dilution (vol/vol) antibody (Santa Cruz). After washing with PBS, the membrane was incubated for 1 hour in 5% milk powder PBS containing 1:3000 diluted goat anti-mouse IgG conjugated to horseradish peroxidase (Vector Laboratories) or anti-goat IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Peroxidase activity was visualized with an enhanced chemiluminescence substrate system (Amersham). Densitometry of digitized images of immunoprobed membranes (ScanJet 4c, Hewlett Packard) was performed by use of Image-Quant software (Molecular Dynamics).

Drugs
Substance P and NS-398 were obtained from RBI. LNNA, U46619, ADP, SNP, indomethacin, and 5-HT were obtained from Sigma Chemical Co. ADP, 5-HT, LNNA, and SNP were dissolved in ultrapure distilled water. U46619 was dissolved in ethanol to make a 10-mmol/L stock solution. Indomethacin was dissolved in minimal ethanol to make a 20-mmol/L stock solution. All stock solutions were stored at −20°C. Dilutions were prepared daily.

Data Analysis
The relaxation responses were expressed as percent relaxation of the spontaneous and/or U46619-induced vascular contraction (mean±SEM) of the microvessels. Because all vascular responses could not be performed before and after cardioplegia, paired comparisons could not be performed. Comparisons of dose-response curves were performed by 2-way ANOVA with repeated measures or 1-way ANOVA, followed by Scheffé’s multiple range test when indicated. Student’s t test was used to compare changes in gene and protein expressions. P<0.05 was considered to indicate significance.

Results
Patient Population
We studied responses of atrial microvessels from 20 patients undergoing nonemergent CABG. The average age was 68±5 years. Fifteen patients were male; 8 continued to smoke until just before surgery; and 6 had non–insulin-dependent diabetes mellitus. Mean total cholesterol concentration was 206±24 mg/dL; mean LDL was 140±20 mg/dL; and mean HDL was 34±5 mg/dL. Triglycerides were 190±30 mg/dL. Mean ejection fraction was 49±5%.

Vessel Characteristics
Atrial microvessel ID ranged from 71 to 180 μm, averaging 106±4 μm in the control group and 123±13 μm in the CP-Rep group. Precontraction after spontaneous constriction and/or after application of the thromboxane A2 analog was 59±3% in the control group and 64±6% in the CP-Rep group. Similar amounts of U46619 were required to produce adequate precontraction in both groups.

In Vitro Response to 5-HT
5-HT, a platelet-derived substance that produces both receptor-mediated endothelium-dependent relaxation and direct vascular smooth muscle contraction, had a minimal net effect on control microvessels. In contrast, 5-HT elicited a significant contraction after CP-Rep. This contraction was significantly reduced in the presence of the COX inhibitor indomethacin and was completely inhibited in the presence of the selective COX-2 inhibitor NS-398 (Figure 1A). This implies that a significant portion of the enhanced contractile response observed after cardioplegia is due to the release of prostanoid contracting substances. Indomethacin prevented the contractile response to 5-HT and induced a significant relaxation of control microvessels. This suggests that 5-HT causes the release of contractile prostaglandins even under nonischemic conditions in atherosclerotic human microvessels. LNNA produced a significant contraction response to 5-HT in control microvessels, suggesting that part of the response to 5-HT is due to the stimulated release of nitric oxide (Figure 1B).
In Vitro Response to ADP and Substance P

The responses of microvessels to ADP before and after cardioplegia were similar. The responses of microvessels to ADP were not altered in the presence of LNNA but were significantly reduced in the presence of indomethacin (Figure 2A). Thus, in contrast with most other species, ADP does not elicit the release of endothelium-derived nitric oxide in human atrial microvessels. This response was not affected by CP-Rep. The response of microvessels to substance P was markedly reduced in the presence of LNNA. The endothelium-dependent relaxation response to substance P was significantly reduced after CP-Rep compared with the response of microvessels from the control group (Figure 2B).

In Vitro Response to SNP

The relaxations of atrial microvessels to SNP, which operates through an endothelium-independent cGMP-mediated pathway, were similar in both groups, suggesting no alteration of the ability of the smooth muscle to relax through the cGMP pathway after CP-Rep (Figure 3).

Gene Expression of NOS-3, NOS-2, COX-1, COX-2, and Prostacyclin Synthase

To examine whether the endothelium dysfunction observed after cardioplegia is due to an altered expression of 1 of the isoforms of NOS or to enzymes responsible for synthesis of prostaglandins, the expressions of NOS-3, NOS-2, COX-1, COX-2, and prostacyclin synthase were analyzed by semi-quantitative RT-PCR. Gene expressions of NOS-3 and NOS-2, as well as the expressions of COX-1 and prostacyclin synthase, were not altered after cardioplegia. In contrast, COX-2 expression was significantly increased (2.5-fold, \( P < 0.05 \)) after CP-Rep (Figure 4).

Protein Expression of NOS-3, NOS-2, COX-1, and COX-2

NOS-3 was slightly but significantly reduced after CP-Rep, possibly because of a posttranscriptional mechanism. NOS-2 was not affected by CP-Rep (Figure 5). COX-1 protein level was significantly reduced and COX-2 was significantly increased after CP-Rep (Figure 6).

Discussion

The major finding of this study is that blood cardioplegia and brief reperfusion cause an enhanced contractile response to 5-HT of human atrial microvessels that is completely prevented by COX-2 inhibition. This enhanced microvascular response to 5-HT is due to an increased production and release of contractile prostaglandin substances, likely due to increased expression of the inducible COX isoform. As opposed to the inducible isoform of NOS, COX-2 seems to be regulated by agonist stimulation, such as to 5-HT. Prostaglandins are formed by the action of isoforms of COX in a 2-step conversion of arachidonic acid. First, the enzyme converts
arachidonic acid to a cyclic endoperoxide (PGG₂) by the action of COX. This is followed by cleavage of the peroxide to yield an endoperoxide (PGH₂). These unstable intermediate products of arachidonic acid metabolism are rapidly converted to the prostaglandins (prostaglandins E₂, F₂, I₂, and thromboxane A₂) by specific isomerase enzymes. ¹² Prostacyclin synthase transforms PGH₂ into what is the most characteristic prostanoid formed by resting endothelial cells, prostacyclin. Prostacyclin is a potent platelet antiaggregatory agent and has antiadhesive and smooth muscle relaxing properties. PGH₂ also undergoes spontaneous or enzymatic transformation to prostaglandins F₂α, E₂, and D₂. Two isoforms of COX exist. The constitutive isoform (COX-1) is present in such tissues as the heart, gut, and kidney, in which prostaglandin production plays a cytoprotective role in maintaining normal physiological process.¹³ Both COX-1 and COX-2 use the same endogenous substrate, arachidonic acid, and form the same product by the same catalytic mechanism; their major difference lies in their pathological functions. In inflammatory processes, the inducible isoform of cyclooxygenase (COX-2) is expressed in many cells, including fibroblasts and macrophages, and accounts for the release of large quantities of proinflammatory prostaglandins at the site of inflammation.¹³ Which prostaglandin products (constrictor versus vasodilator) predominate depends mostly on the relative activity of the 2 isoforms of COX but also on the secondary pathways that yield the different prostanoids. When COX activity is overexpressed, prostacyclin synthase activity could be the limiting step in the biosynthesis of prostaglandin I₂; other prostanoids may be synthesized instead. In addition, the oxidative state and other conditions in the tissue could influence which prostaglandins are synthesized.

Indomethacin may not have completely prevented the enhanced microvascular contraction to 5-HT after cardioplegia because indomethacin is roughly 60 times more potent at inhibiting COX-1 than COX-2 in intact cells.¹⁴ The inducing factor leading to increased expression of COX-2 was not examined in the present study, but it is most likely related to myocardial ischemia or hypoxia or the exposure of vessels to inflammatory cytokines during cardiopulmonary bypass and cardioplegic arrest. Endothelial injury, platelet accumulation and aggregation, and release of serotonin and other cell-derived mediators could promote COX-2 expression. It has been shown that COX-2 can be induced by the platelet product serotonin in rat mesangial cells.¹⁵

Figure 4. NOS-3, NOS-2, COX-1, COX-2, prostacyclin synthase (Prost.S), and GAPDH gene expression in human atrial tissue from patients undergoing CABG before (control, n=4) and after (n=4) CP-Rep. Atrial tissue (1 μg) was subjected to RT-PCR, and reaction mixture was amplified by PCR and then analyzed by agarose gel expression. Positions and sizes of the DNA markers are shown at right.

Figure 5. Western blot analysis of NOS-3 and NOS-2 in human atrial tissue from patients undergoing CABG before (control, n=4) and after (n=4) CP-Rep. Protein was fractionated by 10% SDS-PAGE. Results are expressed as percent (mean±SEM, n=4) of control. *P<0.05 vs control (Student t test).

Figure 6. Western blot analysis of COX-1 and COX-2 in human atrial tissue from patients undergoing CABG before (control, n=4) and after (n=4) CP-Rep. Protein was fractionated by 10% SDS-PAGE. Results are expressed as percent (mean±SEM, n=4) of control. *P<0.05 vs control.
Cardiac operations incorporating cardiopulmonary bypass cause a systemic inflammatory response, which can lead to organ injury and postoperative morbidity. Causative factors include surgical trauma, contact of blood with the extracorporeal circuit, and lung reperfusion injury on discontinuing bypass. The perioperative response to such procedures includes activation of the complement, coagulation, fibrinolytic, and kallikrein cascades; activation of neutrophils with degranulation and protease enzyme release; oxygen radical production; and synthesis of various cytokines from mononuclear cells (including tumor necrosis factor, interleukin-1, interleukin-1β, tumor necrosis factor-α, and other cytokines) are important components of inflammation and the immune response. Adherence to foreign surfaces activates monocyte production of interleukin-1.

Increased production of interleukin-1 has been transiently found after cardiopulmonary bypass, maximal at 24 hours coinciding with a peak in body temperature. By contrast, plasma interleukin-1 was not detected in 2 other reports, perhaps reflecting a more important role as a paracrine mediator. Interleukin-1β has been shown to induce expression of COX-2 mRNA as early as 15 minutes after exposure.

In atrial tissue was significantly expressed even before cardiopulmonary bypass. Other studies have demonstrated NOS-2 mRNA and protein expression in human arteries with transplant coronary artery disease or in myocardium of patients with heart failure caused by either dilated cardiomyopathy or ischemic heart disease. No induction of NOS-2 after CP-Rep could be observed in our study, probably because the time that the hearts were subjected to cardioplegia was not long enough for observation of such induction. Synthesis of nitric oxide by NOS-2 is typically delayed 4 to 6 hours in response to inflammatory stimuli such as cytokines. This hypothesis remains to be determined because it has been shown that nitric oxide amplifies interleukin-1–induced COX-2 expression. Interleukin-1 also induces NOS-2 expression in various cell types and may contribute to the inflammatory state observed after cardiac surgery. Interestingly, the NOS-3 protein level was decreased after CP-Rep, possibly because of alterations in mRNA or protein half-life. This may contribute to the endothelial dysfunction observed after cardiac surgery.

In conclusion, patients with atherosclerotic coronary arteries and baseline endothelial dysfunction may develop coronary vasoconstriction in response to 5-HT after CP-Rep. The release of 5-HT associated with platelet activation and the increased expression of COX-2 after CP-Rep may potentially contribute to myocardial ischemia after cardiac surgery.

Clinical Implications
Coronary artery contraction or spasm has been estimated to occur in ≈2.5% of patients after cardiac surgery. In addition, ECG changes detected by Holter monitoring occur in 1% to 3% of patients in the postoperative period. Furthermore, a reduction in coronary performance has been described in young patients undergoing repair of transposition of the great vessels, a condition not associated with coronary obstruction of altered endothelial function. It is possible that altered myocardial perfusion, in addition to myocardial edema and inflammation, is 1 cause of this decrease in myocardial function. 5-HT is a platelet-derived vasoactive agent that is released on platelet activation during myocardial ischemia or cardiopulmonary bypass or in response to other stimuli. 5-HT is a prototypical agent to examine vascular responsiveness because it both affects vascular smooth muscle and causes relaxation through the release of endothelium-dependent substances. It has been shown that serotonin is released into the coronary circulation during angioplasty and that this vasoactive substance may contribute to the occurrence of vasoconstriction distal to the dilated site. Arterial 5-HT concentrations of $0.5 \times 10^{-8}$ mol/L have been reported in patients without coronary artery disease, and in a canine model of coronary thrombosis, coronary sinus levels $>2 \times 10^{-4}$ mol/L were found. It is likely that local levels of 5-HT are increased even more in the coronary microcirculation and other regions in which platelet activation occurs during myocardial ischemia.

A previous study in pigs demonstrated that cold-blood cardioplegia preserves endothelium-dependent relaxation better than a purely crystalloid cardioplegic solution. In this previous study, indomethacin inhibited the increased contractile response to 5-HT, as was the case in the present study. However, in the present study, microvessels were examined from human subjects with underlying endothelial dysfunction as a consequence of hypercholesterolemia and other risk factors. The short- and long-term patency of saphenous vein bypass grafts in the arterial circulation could be increased if aspirin (cyclooxygenase inhibitor) is administered before the postoperative period. It is possible that this is due not only to reduced platelet activity but also to improved perfusion of myocardium distal to the site of a vein to coronary anastomosis. Finally, because only atrial microvascular responses were examined, it remains to be determined whether responses of the ventricular microcirculation and other vascular territories are affected in the same manner.

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