Activation of myocardial constitutive nitric oxide synthase during coronary artery surgery

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Abstract

Objective: The role of nitric oxide (NO) in myocardial ischemia/reperfusion is controversial. While some studies have shown cardio-protective effects of NO, others suggested that increased myocardial NO release secondary to ischemia may contribute to reperfusion injury. However, the impact of cardioplegia-induced myocardial ischemia/reperfusion on the activity of the NO-producing enzyme constitutive NO-synthase (cNOS or NOS-III) has not been investigated. Methods: Twenty elective CABG patients were randomized to receive myocardial protection using either intermittent cold blood cardioplegia with ‘hot-shot’ (CBC; \( n = 10 \)) or continuous warm blood enriched with the ultra-fast-acting \( \beta \)-blocker esmolol (WBE; \( n = 10 \)). We collected transmural LV biopsies prior to cardiopulmonary bypass (CPB), at the end of the cross-clamp period, and at the end of CPB. Specimen were subjected to immunocytochemical staining against myocardial NOS-III and cGMP using polyclonal antibodies. NOS-III activity was determined using TV-densitometry (gray units) and cGMP content using a semiquantitative score. Global myocardial metabolism was assessed by arterio-coronary sinus lactate concentration difference (a-csDLAC). Results: For LV function determination we measured the fractional area of contraction (FAC) using TEE. Results: In CBC hearts a-csDLAC was significantly decreased following cross-clamp removal as compared to pre-CPB indicating global ischemia during cross-clamp. In contrast, a-csDLAC was unchanged in WBE hearts indicating absence of relevant ischemia in this group. In CBC hearts NOS-III activity did not change from pre-CPB (35.6 ± 11.1 U) to the end of the cross-clamp period (38.0 ± 8.1 U; \( P = 0.2 \)), but increased significantly to 48.5 ± 12.1 U at the end of CPB following initial warm blood reperfusion (\( P = 0.026 \)). In WBE hearts NOS-III activity remained unchanged throughout (29.2 ± 10.8, 35.1 ± 11.8, and 32.2 ± 14.7 U, respectively; \( P > 0.3 \)). At the end of CPB, nine CBC hearts, but only one WBE heart showed increased cGMP content (\( P = 0.002 \)). Compared to pre-CPB, FAC in the CBC group was 199 ± 25% following weaning off CPB (\( P = 0.26 \)), but was slightly decreased to 87 ± 22% at 4 h post-CPB (\( P = 0.03 \)). In the WBE group FAC remained unchanged compared to pre-CPB throughout (103 ± 21 and 96 ± 37%; respectively; \( P > 0.5 \)). Conclusions: Our data show that global myocardial ischemia and reperfusion induced by CBC is associated with myocardial NOS-III activation and increased cGMP content suggesting increased NO release. In contrast, avoidance of ischemia by use of WBE prevented NOS-III and cGMP increase. As LV function was decreased at 4 h post-CPB in the CBC group, these data suggest that increased NO release secondary to NOS-III activation may have contributed to ischemia-reperfusion injury as has been shown experimentally. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The role of nitric oxide (NO) in myocardial ischemia/reperfusion is controversial. Potential cardioprotective effects of NO release during reperfusion include inhibition

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mental studies investigating either increased NO release by NO precursor application or decreased NO release by NO synthase (NOS) inhibition have yielded conflicting data showing protective, deleterious, or no effects for both increased as well as decreased NO during reperfusion [3,11–15]. Thus, it has been speculated that the effects of NO during reperfusion could be dose dependent [3].

To gain more insight into the pathophysiology of NO in myocardial ischemia/reperfusion it appears important to elucidate the impact of myocardial ischemia/reperfusion on the activity of the NO-producing enzyme NOS. At least two NOS isoforms have been detected in human myocytes and coronary endothelium: the constitutively expressed NOS (cNOS), synonymously termed Ca2+-dependent endothelial NOS (eNOS) or NOS type III (NOS-III) and the inducible, Ca2+-independent NOS (iNOS) or NOS-II [3]. Inducible NOS is not expressed in normal myocardium but various stimuli including endotoxins, cytokines, and experimental infarction have been shown to increase myocardial iNOS [3]. However, iNOS activation requires 4–6 h [16]. In contrast, NOS-III activity can increase much faster. We and others have recently demonstrated that in both rat and rabbit heart detectable NOS-III activity is increased after only 5 min of global myocardial ischemia suggesting a conformational change of the enzyme [17,18]. This rapidly increased NOS-III activity resulted in increased NO release as indicated by increased myocardial cGMP content [17]. Thus, potential NOS-III activity and cGMP content changes could be of clinical relevance in myocardial ischemia induced by cardioplegia during routine cardiac surgery.

Therefore, the purpose of our clinical study was to investigate (1) if cardioplegia-induced global myocardial ischemia/reperfusion affects myocardial NOS-III activity and cGMP content, and (2) if ischemia avoidance using a non-ischemic myocardial protection technique prevents NOS-III and cGMP changes in patients subjected to coronary artery surgery.

2. Material and methods

2.1. Patients

Following approval by the University of Cologne Human Ethics Committee, written, informed consent was obtained from each patient during the preoperative interview. Twenty patients scheduled for elective coronary artery surgery were randomized into either the ‘warm blood + esmolol group’ (WBE; n = 10) or the ‘cold blood cardioplegia group’ (CBC; n = 10). In WBE patients the myocardium was protected during aortic cross-clamping using continuous antegrade coronary perfusion with warm cardiopulmonary bypass (CPB) blood enriched with the ultra short-acting β-blocker esmolol (Brevibloc®) as previously described [19–21]. Briefly, aortic root pressure is maintained at 50–70 mmHg during cross-clamping, coronary blood flow is maintained at 150–350 ml/min, and esmolol is infused at 10–15 mg/min following an initial 100 mg bolus [19]. This technique has been shown to virtually avoid myocardial ischemia, thus minimizing subsequent reperfusion injury [19–21]. In CBC patients we used intermittent (every 20 min) cold (6–8°C) antegrade blood cardioplegia with ‘hot shot’ prior to cross-clamp removal according to Buckberg [22]. Patients admitted to the study were men and women with isolated coronary artery disease, and left ventricular (LV) ejection fraction >25%. Exclusion criteria included heart valve disease, myocardial infarction within 2 weeks prior, evidence of renal or hepatic failure, and history of severe asthma.

2.2. Monitoring

Following anesthesia induction and standard hemodynamic monitoring including pulmonary artery catheter, a 5 MHz transesophageal echocardiography (TEE) probe (Vingmed CFM 800®, Sonotron, Horten, Norway) was placed to provide a LV short-axis image at the midpapillary level. From the TEE recordings we derived the fractional area of contraction (FAC) as a measure of LV ejection fraction [19]. Following sternotomy and pericardiotomy a 5F catheter was introduced via a right atrial purse string into the coronary sinus for coronary sinus blood sampling.

2.3. Clinical protocol

Prior to cannulation for CPB, we recorded baseline measurements of all hemodynamic parameters and a one minute TEE reading. Five milliliters of arterial as well as coronary sinus blood were drawn simultaneously for blood gas analysis and determination of arterio-coronary sinus lactate concentration difference (a-csDlac) [19,20]. We then collected a transmural biopsy from a fat-free area of the LV anterior wall using a 14G biopsy needle (Gallini®, Modena, Italy). Simultaneous arterial and coronary sinus blood collection was repeated after each distal coronary anastomosis completion. Following the last distal anastomosis a second LV biopsy was taken prior to aortic cross-clamp removal. At 2 and 5 min after cross-clamp removal arterial and coronary sinus blood samples were simultaneously drawn. A third LV biopsy was collected just prior to weaning from CPB. At 10–15 min after separation from CPB we repeated measurements of all hemodynamic parameters, TEE, and blood sampling. Final hemodynamic and TEE measurements were performed at 4 h post-CPB.

2.4. LV biopsies

The LV biopsies were placed in 4% paraformaldehyde for 4 h and then rinsed in 0.1 M phosphate-buffered saline (PBS) for 24 h followed by storage for 12 h in PBS solution with 18% sucrose for cryoprotection and frozen at −80°C. As 4% paraformaldehyde has been shown to result in opti-
2.5. Immunocytochemistry

Prior to immunohistochemical examination 20 µm slices from the biopsies were placed in a bathing solution of 3% H₂O₂ and 60% methanol PBS for 30 min, then permeabilized with 0.2% Triton-X 100 in 0.1 M PBS. Thereafter, specimens were treated with 5% normal goat serum (NGS) and 5% bovine serum (BSA) solution in PBS. Prior to each step the sections were rinsed three times in PBS buffer. Incubation with primary polyclonal rabbit anti-NOS-III antibody (Biomol, Hamburg, Germany) at a dilution of 1:1500 was performed in a PBS-based solution of 0.8% BSA and 20 mM Na₂S₂O₃ for 12 h at 4°C. For cGMP detection we used a polyclonal rabbit anti-cGMP antibody (Quartett, Hamburg, Germany) at a dilution of 1:600. After rinsing with PBS the sections were incubated with the corresponding secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. A streptavidin–horseradish peroxidase complex was then applied as a detection system (1:100 dilution) for 1 h. Finally, staining was developed for 3–5 min with 3,3-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris–HCl buffer and 0.1% H₂O₂.

2.6. NOS-III TV-densitometry

All LV biopsy slices were incubated and stored under identical conditions. For intensity analysis of NOS-III immunostaining in cardiomyocytes we measured the gray values of 50 cardiomyocytes from ten randomly selected areas. The intensity of immunostaining was reported as the mean of measured cardiomyocyte gray value minus background gray value. The background gray value was measured at a cell free area of the slice. For staining intensity detection a Zeiss Axio phot microscope coupled to a 3-chip CCD-camera was used and the analysis was performed using the Optimas 6.01 image analysis program installed on a Pentium PC.

2.7. cGMP semi-quantitative analysis

For semi-quantitative analysis of myocardial cGMP content we used a score to differentiate between no change and clearly increased cGMP content. All specimens were judged by two independent investigators in a double blinded fashion. Data were only accepted if both investigators agreed upon the score.

2.8. Statistical analysis

All data presented in the text are mean ± standard deviation (SD). Data presented in the figures are mean ± standard error of the mean (SEM). Biometric data were analyzed using two-tailed Student’s t-test for independent samples. We examined our data for changes over time (within group changes) and differences between WBE vs. CBC using two-way ANOVA for repeated measures (Statistica®, SoftStat® Inc., Tulsa, OK). Post hoc comparisons were performed using two-tailed Student’s t-test for dependent and independent samples with Bonferroni correction for multiple comparisons, where appropriate. The cGMP data were analyzed using two-tailed Fisher Exact Test. A value of P < 0.05 was considered significant.

3. Results

Age (WBE: 66 ± 7 years; CBC: 64 ± 6 years; P = 0.6) and body weight (WBE: 77 ± 10 kg; CBC: 78 ± 9 kg; P = 0.7) were similar for both groups. CBC patients received 2.9 ± 0.4 distal anastomoses during 48 ± 6 min aortic cross-clamp time (WBE: 3.0 ± 0.9 anastomoses during 49 ± 12 min; P = 0.6). WBE patients received a total esmolol dose of 867 ± 237 mg during continuous coronary perfusion with 247 ± 112 ml/min warm CPB blood at an aortic root pressure of 56 ± 13 mmHg.

All hemodynamic parameters were similar between both groups except cardiac index at 4 h post-CPB (CBC: 2.6 ± 0.6 vs. WBE: 3.0 ± 0.3 l/min per m²; P = 0.03). In the CBC group FAC was 56 ± 11% prior to CPB and 59 ± 11% following weaning off CPB (P = 0.2), but was slightly decreased to 47 ± 13% at 4 h post-CPB (P = 0.03). In the WBE group FAC remained unchanged throughout (59 ± 17, 60 ± 16, and 54 ± 16%, respectively; P > 0.2; ANOVA: P = 0.36 for changes between groups and P = 0.0013 for changes within groups). Looking at changes compared to pre-CPB values, FAC in the CBC group was 109 ± 25% (95% confidence interval: 96–122%) after weaning from CPB (P = 0.26), but was slightly decreased to 87 ± 22% (76–99%) at 4 h post-CPB (P = 0.03). In the WBE group we did not detect changes in FAC. FAC remained unchanged compared to pre-CPB throughput (103 ± 21% [93–113%] and 96 ± 37% [78–114%], respectively; P > 0.5).

In CBC hearts a-csDlac was significantly decreased during cross-clamp as well as at 2 and 5 min following cross-clamp release indicating anaerobic myocardial metabolism (Fig. 1). In contrast, a-csDlac in WBE hearts did not change over the time course of the operation (Fig. 1). ANOVA revealed P < 0.0001 for changes between groups and P < 0.0001 for changes within groups. Fig. 2 shows typical examples of myocardial NOS-III immunostaining in a CBC heart pre-CPB (A), at the end of the aortic cross-clamp period (B), and at the end of CPB (C) as well as the corresponding images of a WBE heart (D, E, F, respectively). Note the clearly enhanced NOS-III activity following ischemia and reperfusion in the CBC heart (C) as compared to the WBE heart (F) which was not subjected to ischemia, and thus, reperfusion. NOS-III activity quantified by TV densitometry is depicted in Fig. 3. In CBC hearts...
NOS-III activity was not affected at the end of the cross-clamp period, however, was significantly increased at the end of CPB following initial warm blood reperfusion ($P < 0.026$ end CPB vs. end AXC; $P = 0.017$ vs. WBE group). WBE hearts did not show changes in NOS-III activity ($P > 0.3$; ANOVA: $P = 0.47$ for changes between groups and $P = 0.012$ for changes within groups). Compared to pre-CPB, nine CBC hearts showed increased cGMP content at the end of CPB suggesting increased NO release, whereas only one WBE heart demonstrated increased cGMP content ($P = 0.002$ WBE vs. CBC group) (Fig. 4).

4. Discussion

Our data show that global myocardial ischemia and reperfusion induced by intermittent CBC is associated with
myocardial NOS-III activation and increased cGMP content indicating increased NO release. As LV function was slightly decreased at 4 h post-CPB in the CBC group, these data suggest that increased NO release secondary to myocardial NOS-III activation may contribute to reperfusion injury following global myocardial ischemia. In contrast, minimization of ischemia by use of WBE prevented both NOS-III and c-GMP increase and was associated with unchanged cardiac performance. It has to be emphasized, however, that the proposed association between NOS-III activation and reperfusion injury is based on the assumption that reperfusion injury was present since we did not measure it directly in our study.

Ischemia-reperfusion injury involves both cardiac myocytes and coronary endothelial cells and appears to be a major factor contributing to perioperative myocardial damage [3]. Even though the pathophysiology of ischemia-reperfusion injury is not yet fully understood, recent studies suggest that NO plays an important role in ischemia-reperfusion injury [3]. However, the various studies have yielded conflicting data showing both protective as well as deleterious effects of NO during reperfusion. Cardioprotective effects of NO include inhibition of neutrophil and platelet accumulation [1,2], inhibition of the release of cytotoxic mediators from neutrophils [2], direct cytoprotective effects on both endothelial cells and cardiomyocytes [2,3], amelioration of the 'no-reflow-phenomenon' [4] as well as potential infarct size reduction [5]. On the contrary, increased NO release during reperfusion has been suggested to contribute to reperfusion injury due to peroxynitrite-mediated lipid peroxidation [6], DNA synthesis inhibition [7], mitochondrial function inhibition [8], ribonucleotide reductase inhibition [9], decreased myocardial glucose utilization [24] as well as direct cardiac function depression [10]. To further elucidate the pathophysiology of NO in myocardial ischemia-reperfusion we intended to determine the impact of global myocardial ischemia on the NO-producing enzyme NOS-III during routine coronary artery surgery. Hearts subjected to intermittent CBC, and thus, repeated global ischemia (Fig. 1), showed significantly increased NOS-III activity (Figs. 2 and 3) and increased cGMP content (Fig. 4) at the end of CPB. In contrast, avoidance of global myocardial ischemia by use of continuous warm blood and esmolol perfusion in WBE hearts did neither result in NOS-III activation (Figs. 2 and 3) nor increased cGMP content (Fig. 4). These data suggest that myocardial ischemia acts as the stimulus for NOS-III activation resulting in increased NO release as indicated by cGMP increase during the early phase of reperfusion. This rapid NOS-III activation (Fig. 3; time between 'end AXC' and 'end CPB': 32 ± 12 min for WBE; 30 ± 4 min for CBC; P = 0.6) suggests a conformational change of the enzyme NOS-III from an inactive, not detectable state to an active form that can be detected by immunocytochemistry as supported by our recent experimental work [17].

In CBC hearts LV function as measured by fractional area of contraction was unchanged after separation from CPB but was slightly decreased at 4 h post-CPB indicating reperfusion injury, whereas cardiac performance remained unchanged in the WBE group despite similar inotropic medication (CBC: 3.9 ± 1.0 µg/min per kg dopamine vs. WBE: 4.0 ± 1.3 µg/min per kg; P = 0.8). As we did not collect corresponding LV biopsies at 4 h post-CPB, we can only speculate if increased NO release contributed to this decreased cardiac performance in the CBC group [10]. In addition, our data do not allow us to determine if potential iNOS expression which has been shown to require 4±6 h following an activating stimulus might have added to the observed changes [16]. However, other factors that have been shown to contribute to post cardioplegia cardiac dysfunction including myocardial stunning and myocardial edema formation [3,19–22] would be expected to depress cardiac function immediately post-CPB [19,22]. Thus, it appears unlikely that myocardial stunning or edema were responsible for the slight cardiac dysfunction we observed at 4 h post-CPB in the CBC group. To further understand the role of NO in myocardial ischemia/reperfusion injury, future studies are required to determine the time course of iNOS and NOS-III inactivation and NO release following restoration of myocardial perfusion as well as their effects on cardiac performance.

Several issues need to be discussed regarding interpretation of our data. First, measurement of a-csD_{LAC} in the absence of myocardial blood flow determination does not allow to establish the presence of anaerobic myocardial metabolism. However, as use of intermittent CBC necessarily involves myocardial ischemia, we believe it is reasonable to conclude that anaerobic myocardial metabolism was present during CBC. Second, the relationship between the presence of myocardial ischemia and the observed NOS-III activation may be an epiphenomenon for the following reasons: (1) the data from the present study do not allow to establish a causal relationship, because it was not possible to inhibit NOS-III in the CBC group and (2) there was no ischemia in the WBE group. However, in our recent experi-
mental study we have shown that in rat hearts NOS-III activation was strictly limited to an area of myocardium subjected to 5 min of regional ischemia followed by 2 min of reperfusion, whereas NOS-III activation was absent in non-ischemic regions of the same hearts [17]. Thus, it is likely that ischemia may be one stimulus for NOS-III activation as suggested by our and others’ work [17,18].

In conclusion, our data suggest that NOS-III activation and NO release induced by global myocardial ischemia may act to counterbalance the potentially deleterious effects of myocardial ischemia. In acute ischemia two basic mechanisms will attenuate myocardial damage: restoration of blood supply and reduction of metabolic demand. Increased NO release has been shown to support both mechanisms because of its potent vasodilatory [3,4] as well as negative inotropic and chronotropic [10,25] effects. However, overproduction of NO secondary to NOS-III activation may contribute to reperfusion injury as has been shown previously [3,6,12], and thus, it appears that very high NO concentrations during reperfusion may be as deleterious as absence of NO. This is supported by several experimental studies demonstrating that inhibition of NOS-III ameliorates ischemia reperfusion injury [13–15]. However, the potentially dose-dependent cardioprotective effects of NOS-III inhibition in the clinical setting remain to be established.

References


[6] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–1624.


Appendix A. Conference discussion

Mr M. Deja (Leicester, UK): I have two questions. First, there is some experimental evidence that nitric oxide can be cardioprotective actually. At least this was quite well proved showing the impact on the endothelial cells in the coronary endothelia. So can you comment on this, because it’s quite opposite to what you presented.
Dr Mehlhorn: Let me first answer this part, because it’s already two questions.

First, regarding the question if there is cardioprotective effects of nitric oxide. That’s what I showed in my first slide, actually, the role of nitric oxide during reperfusion is very controversial. Some studies have shown protective effects; but some studies have shown that if you block NOS-III activity, you have improved protection, decreased infarction size, for instance, after ischemia. So I don’t know what is the truth here. I think nobody does at this time.

The second part concerns if NOSIII activity is increased in the myocytes and endothelial cells, correct?

Mr Deja: I said that the study I know on the cardioprotective values of nitric oxide were that the endothelia in coronaries were well preserved even if the nitric oxide production was high or was induced during the ischemia time. I didn’t know of any studies regarding protection of the myocytes, but I knew the studies, experimental studies, showing that these coronary endothelia were better protected from ischemia by the high amount of nitric oxide.

Dr Mehlhorn: What is the question?

Mr Deja: It was related to the first one. The second question I have is regarding the reperfusion period. In stunning, as far as I understand, you deal mainly, if you don’t have too severe stunning, with the diastolic dysfunction, which obviously, is improved by high NOS-III level. So the question is whether really in stunning, as we have it, in a clinical situation with a relatively small systolic dysfunction and high diastolic dysfunction, nitric oxide shall not be beneficial? Have you studied diastolic function in your experimental model?

Dr Mehlhorn: No, we have not studied diastolic left ventricular function.