Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 516 (2005) 60 – 70

www.elsevier.com/locate/ejphar

# Role of nitric oxide during coronary endothelial dysfunction after myocardial infarction

An Berges[a,b], Luc Van Nassauw[b], Jean-Pierre Timmermans[b], Christiaan Vrints[a,*]

[a]*Laboratory of Cardiology, University of Antwerp, Wilrijkstraat 10, Edegem 2650, Belgium*
[b]*Laboratory of Cell Biology and Histology, University of Antwerp, Belgium*

Received 18 March 2005; accepted 15 April 2005

**Abstract**

This study aimed to investigate whether permanent ischaemia influences subacute vasodilatation responses of non-infarcted rat coronary vasculature, and to characterise these coronary changes. Ischaemia led to a significant impairment of the endothelium-dependent vasodilator response, while coronary vasodilatory capacity remained unaltered. In normal coronary circulation, nitric oxide (NO) and prostanoids contributed to vasodilatation, while basal involvement of endothelium-derived hyperpolarising factor was limited. Vasodilatory impairment following myocardial infarction did not originate from alterations in the prostanoid pathway, and only a slightly increased influence of $K^+$ channels was observed. However, NO-mediated vasodilatation was significantly increased after ischaemia, as also confirmed by higher mRNA and protein levels of iNOS and eNOS. Additionally, the amount of superoxide was enhanced following infarction. We conclude that subacute postinfarction remodeling is accompanied by endothelial dysfunction in non-infarcted coronary arteries. Although the NO-mediated response is increased after ischaemia, its final action is restricted due to the presence of superoxide.
© 2005 Elsevier B.V. All rights reserved.

*Keywords:* Coronary circulation; Endothelial function; Myocardial infarction; Remodeling; Vasodilatation; Nitric oxide; Superoxide

## 1. Introduction

A large transmural myocardial infarction initiates a cascade of changes, including myocardial hypertrophy, to deal with reduced contractility and increased workload (Vannan and Taylor, 1992). In addition, clinical and experimental reports (Gibson et al., 1999; Karam et al., 1990; Uren et al., 1994) have described a reduced basal coronary flow and an impaired flow reserve in the infarcted and non-infarcted myocardium after myocardial infarction and heart failure. While research interest has focussed largely on coronary blood flow in the culprit artery after ischaemia, the flow in the non-infarcted arteries has received little attention and has been assumed to be normal. However, it has been reported that the reactivity of endothelium in remote vessels is impaired after an acute ischaemic insult (Gibson et al., 1999; Uren et al., 1994; Vérroneau et al., 1997).

The initial clinical event of myocardial infarction is a rupture of an atherosclerotic lesion in a coronary vessel. Atherosclerosis is an inflammatory disease of the vessel wall, strongly characterised by a diffuse endothelial dysfunction (Ross, 1999). This endothelial dysfunction is an early marker of atherosclerosis (Bonetti et al., 2003) and contributes to enhanced plaque vulnerability, triggers plaque rupture, and favours thrombus formation (Behrendt and Ganz, 2002). However, it is not clear whether the reduced endothelium-dependent vasodilatation response observed after myocardial infarction is a result of atherosclerosis and concomitant endothelial dysfunction in non-infarcted arteries, or a supplementary negative influence of ischaemia on coronary vessels.

The aim of this study was to elucidate whether permanent ischaemia itself can influence endothelium-dependent vasodilatation responses in non-infarcted coronary vessels, and to characterise these coronary changes

* Corresponding author. Tel.: +32 3 821 35 29; fax: +32 3 830 23 05.
*E-mail address:* christiaan.vrints@ua.ac.be (C. Vrints).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.04.028

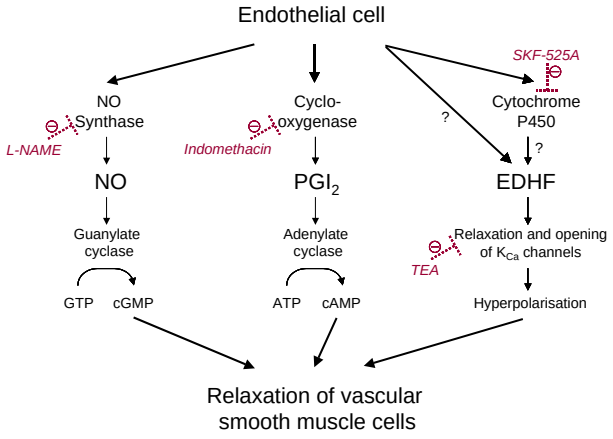


Fig. 1. Schematic representation of endothelial cells and vascular smooth muscle cells, illustrating the possible pathways of endothelium-dependent vasodilatation. In addition, the site of interaction of the antagonists used in this study is shown (modified from Félétou and Vanhoutte, 1999) (TEA: tetraethyl-ammonium sulphate).

during subacute postinfarction remodeling. To this end, we used an experimental model of myocardial infarction in healthy, normocholesterolemic rats. Metabolically active endothelium produces a number of factors maintaining an adequate vascular tone and resistance. The major endothelium-derived vasodilators include nitric oxide (NO), prostanoids, and endothelium-derived hyperpolarising factor (EDHF) (Fig. 1). First, we studied the contribution of these endothelium-derived mediators to an endothelium-dependent vasodilator, bradykinin, in the isolated perfused rat heart after sham operation. Second, we examined whether ischaemia leads to the impairment of coronary flow responses during subacute postinfarction remodeling. To define the nature of this endothelial dysfunction, the contributions of the endothelium-derived mediators have been estimated and compared after ischaemia. In addition, the presence of inducible NO synthase (iNOS), endothelial NO synthase (eNOS), and reactive oxygen species has been investigated.

## 2. Materials and methods

### 2.1. Myocardial infarction model

The coronary artery ligation model in the rat was used as a model for myocardial infarction (Selye et al., 1960). Adult male Wistar rats were randomised to the coronary artery ligation (MI) group or to the sham group. The MI group underwent permanent ligation of the left anterior descending coronary artery, resulting in a reproducible large anterior myocardial infarction. Sham-operated rats underwent the same operation without ligation of the coronary artery.

The animals were anaesthetised by intramuscular injection of 2.5 mg/kg body weight of diazepam (Valium; Roche, Basel, Switzerland), 1.25 mg/kg body weight of droperidol, and 0.025 mg/kg body weight of fentanyl (Thalamonal; Janssen Pharmaceutica, Beerse, Belgium). After intubation and ventilation (60 respirations/min, 1.0 l/min $O_2$), a left thoracotomy was performed via the fourth intercostal space. The pericardium was opened and the left anterior descending coronary artery was ligated proximally with a 6-0 polyester suture. The chest was closed in layers. After surgery, animals were treated with 0.1 mg of piritramide, i.m. (Dipidolor; Janssens Pharmaceutica), for analgesia and were put in an $O_2$-rich environment for several hours. One day after surgery, the rats were placed in a normal cage with free access to water and standard food. All experiments were approved by the local ethics committee of the University of Antwerp.

### 2.2. Isolation of rat hearts and pharmacological experiments in a Langendorff set-up

One week after surgery, the animals were killed with an intraperitoneal overdose of sodium pentobarbital (Nembutal; Sanofi-Synthelabo, Brussels, Belgium). Heparin (500 U, i.v.; Leo Pharma, Ballerup, Denmark) was given to prevent blood coagulation and intracoronary clot formation. The heart was rapidly excised and transferred to a modified Krebs–Henseleit buffer (118.5 mM NaCl, 1.25 mM $CaCl_2$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$, and 10 mM glucose). The aorta was cannulated to perfuse the heart with the modified Krebs–Henseleit buffer (pH 7.4, 37 °C), equilibrated with 95% $O_2$ and 5% $CO_2$. Perfusion pressure was kept constant at 75 mm Hg. Coronary flow was measured with an ultrasonic flowmeter (T106; Transonic Systems Inc., Maastricht, The Netherlands) located above the aortic cannula. Isovolumetric left ventricular pressure was measured with a pressure transducer (EMKA, Paris, France), which was connected to a fluid-filled balloon at the tip. The balloon was placed into the cavity of the left ventricle via the left atrium. Balloon volume was adjusted to a left ventricular end-diastolic pressure of about 10 mm Hg. Hearts were paced at approximately 300 beats/min. All physiological parameters were recorded simultaneously by IOX1565 software (EMKA).

Hearts were allowed to equilibrate for 30 min until constant coronary flow and left ventricular pressure values were attained. Vasoactive substances were administered by bolus injection (50 μl) with stepwise increasing dosage, with the injection site located just above the heart. Bradykinin ($10^{-9}$–$10^{-5}$ M) (Calbiochem, Darmstadt, Germany) was used as an endothelium-dependent vasodilator, while adenosine ($10^{-8}$–$10^{-4}$ M) (Adenocor, Sanofi-Synthelabo) served to visualise the endothelium-independent vasodilatory response. Antagonists were added to the perfusate to investigate the separate and combined effects of NO, prostanoids, and EDHF on coronary flow response (Fig. 1).

First, the basal bradykinin and adenosine dose–response curves were assessed in all sham and infarcted hearts. Second, the involvement of NO, prostanoids, and EDHF metabolites in vasodilatation responses to bradykinin in sham and infarcted hearts was analysed. Inactivation of the NO and cyclo-oxygenase (COX) pathways was achieved by adding $N^G$-monomethyl-L-arginine ester (L-NAME; 100 μM) (Sigma, St. Louis, MO) and indomethacin (Indom; 10 μM) (Sigma), respectively, to the perfusate. To evaluate the impact of iNOS-derived NO on the coronary flow response, 1400W (10 μM) (Alexis, Zandhoven, Belgium) was applied. Although the exact nature of EDHF has not been identified yet, most reports (Campbell et al., 1996; Fisslthaler et al., 1999; Gauthier et al., 2002) suggest that EDHFs are short-living metabolites of arachidonic acid, viz. epoxyeicosatrienoic

acids, which are produced through the cytochrome *P*450 (CYP450) monooxygenase pathway. These EDHF metabolites diffuse to the vascular smooth muscle cells where they induce an increase in $K^+$ conductance through activation of $Ca^{2+}$-dependent $K^+$ ($K_{Ca}$) channels (Busse et al., 2002; Félétou and Vanhoutte, 1999). The CYP450 inhibitor used in our experiments was proadifen (SKF-525A; 10 μM) (Sigma), a substance that irreversibly blocks the production of epoxyeicosatrienoic acids. In addition, we used a non-specific $K_{Ca}$ channel inhibitor, tetraethyl-ammonium sulphate (TEA, 1 mM) (Sigma), to block the action of EDHF. We investigated the basal involvement of $K^+$ channels in coronary vasodilatation and after co-application with L-NAME and indomethacin.

### 2.3. Immunohistochemical detection of iNOS, eNOS, COX-2, and NAD(P)H oxidase

Additional hearts were used for the immunohistochemical visualisation of iNOS, eNOS, COX-2, and subunits of NAD(P)H oxidase, p22phox, and p47phox. One week after surgery, hearts were dissected, fixed for 3 h in a 4% phoshate-buffered (pH 7.4) paraformaldehyde solution, and processed for embedding in paraffin. Five-micron-thick sections were pretreated for 30 min in 3% $H_2O_2$ in methanol and for 1 h in 0.01 M PBS (pH 7.4) with 5% normal donkey serum, 5% bovine serum albumin, and 0.01% $NaN_3$. Subsequently, sections were incubated overnight with a rabbit polyclonal anti-iNOS (diluted 1/20; BD Transduction Laboratories, Franklin Lakes, NY), rabbit polyclonal anti-eNOS (diluted 1/400; BD Transduction Laboratories), rabbit polyclonal anti-COX-2 (diluted 1/400; Chemicon, Temicula, CA), goat polyclonal anti-p47phox (diluted 1/100; Santa Cruz Biotechnology, Santa Cruz, CA), or goat polyclonal anti-p22phox (diluted 1/20; Santa Cruz Biotechnology) in 0.01 M PBS with 0.1% bovine serum albumin-c and 0.01% $NaN_3$. After rinsing with PBS, sections were subjected to anti-rabbit EnVision peroxidase (DAKO, Glostrup, Denmark) for 1 h (Sabattini et al., 1998) or biotinylated donkey–anti-goat (diluted 1/100; Amersham Biosciences, Roosendaal, The Netherlands) for 1 h and subsequently extravidin-conjugated horseradish peroxidase (diluted 1/1500; Sigma) for an additional hour. Next, 3,3′-diaminobenzidine solution (DAB; Pierce Biotechnology, Rockford, IL) was applied for 5 min. Finally, sections were counterstained with methyl green and mounted in Entellan (Merck, Darmstadt, Germany). Sections were quantified on an Olympus BX50 microscope (Olympus, Tokyo, Japan) using CAST grid analysis (Olympus, Albertslund, Denmark). With the computer-assisted stereological toolbox, a point grid and counting field were superimposed on the image obtained with an objective magnification of 20×. Subsequently, the proportion of immunoreactivity of each enzyme in relation to total ventricular surface was calculated.

### 2.4. RNA extraction and real-time PCR

Total RNA was isolated from the LV myocardium using a commercially available kit (Qiagen, Venlo, The Netherlands). Cells were lysed by guanidine thiocyanate and homogenised by QIAshredder homogenizer (Qiagen). Lysates were treated with proteinase K and DNase according to the manufacturer's instructions. Total RNA was quantified spectrophotometrically at $\lambda = 260$ nm. The purity of samples was assessed by measuring OD 260:280 nm. Reverse transcriptase was performed with 1 μg of total RNA in a reaction volume of 20 μl containing 4 μl of 25 mM $MgCl_2$, 2 μl of reaction buffer, 2 μl of deoxyribonucleoside triphosphates, 0.5 μl of RNasis ribonuclease inhibitor, 0.7 μl of reverse transcriptase, and 0.5 μl of random primers (Promega, Madison, WI). cDNA templates were stored at −20 °C.

Real-time PCR for eNOS and iNOS was performed on a LightCycler 1.5 (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (Farhangkhoee et al., 2003; Moritz et al., 2003). This technique allows amplification and kinetic detection in a single microcapillary tube (Roche Molecular Biochemicals) using a commercially available reaction mix containing FastStart Taq DNA polymerase, SYBR Green I, 10 mM $MgCl_2$, and deoxyribonucleoside triphosphates (Roche Molecular Biochemicals). For a final reaction volume of 20 μl, the following reagents were added: 11.6 μl of $H_2O$, 2 μl of reaction mix, 2.4 μl of 25 mM $MgCl_2$, 1 μl of each forward and reverse 10 μM primers, and 2 μl of cDNA template. The following primers were used: *eNOS*: 5′-GCA AGA CCG ATT ACA CGA CA-3′ (sense) and 5′-GTC CTC AGG AGG TCT TGC AC-3′ (antisense), *iNOS*: 5′-ATG GAA CAG TAT AAG GCA AAC ACC-3′ (sense) and 5′-GTT TCT GGT CGA TGT CAT GAG CAA AGG-3′ (antisense), and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*: 5′-TCC ATG ACA ACT TTG GCA TC-3′ (sense) and 5′-CAT GTC AGA TCC ACC ACG GA-3′ (antisense). Each sample was subjected to 50 cycles of denaturation (95 °C for 0 s), annealing (57 °C for 5 s), elongation (72 °C for 10 s), and single acquisition (85 °C for 1 s). To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and 2% agarose gel electrophoresis followed by ethidium bromide staining. Data were analysed with the RelQuant software (Roche Molecular Biochemicals). The levels of mRNA were quantified using the standard curve method, constructed with serial dilutions of control mRNA or PCR amplicons. Data were normalised to the housekeeping gene *GAPDH*.

### 2.5. Superoxide anion generation

Lucigenin-enhanced chemiluminescence was measured as an estimate of superoxide anion production. It does not directly react with superoxide, but must first be reduced to lucigenin radical, which then reacts with superoxide to give the chemiluminescent product (Li et al., 1998). Segments of hearts were placed in Krebs–Henseleit solution for 30 min and in Krebs–HEPES for another 30 min. Thereafter, the tissue was placed in a dark-adapted scintillation vial with 2 ml of Krebs–HEPES buffer (99 mM NaCl, 4.6 mM KCl, 1.0 mM $MgSO_4$, 0.4 mM $KH_2PO_4$, 0.15 mM $Na_2HPO_4$, and 20 mM HEPES) containing 5 μM lucigenin. Counts were acquired for 10 min at 1-min intervals in a Tri-carb 2300 TR liquid scintillation counter (Packard Instrument Company, Meriden, CT). Values for the first 2 min were discarded, and background and blanks (i.e., vials which were opened without adding tissue) were subtracted. The average value (counts per minute, cpm) was expressed per gram of tissue (wet weight).

### 2.6. Spectrophotometric determination of serum nitrite concentration

Nitrite ($NO_2^-$) and nitrate ($NO_3^-$) are the primary oxidation products of NO; therefore, a concentration of $NO_2^-$ plus $NO_3^-$ in serum was used as an indicator of total NO synthesis. Serum nitrate/nitrite level was measured after enzymatic conversion of $NO_3^-$ to $NO_2^-$ by nitrate reductase, using a commercial method (R&D Systems, Minneapolis, MN). Briefly, serum samples were diluted

and ultrafiltered through a 10,000 MW cut-off filter to eliminate proteins. After centrifugation (13,000×*g* for 40 min), each sample was incubated with nitrate reductase and NADH for a 30-min incubation at 37 °C. Subsequently, Griess reagents I and II were added and the mixture was incubated at room temperature for 10 min. Optical density at 550 nm ($OD_{550}$) was measured and total nitrite concentration for each sample was calculated by comparison with the $OD_{550}$ of a standard nitrite calibration curve.

### 2.7. Morphometric analyses

The size of myocardial infarction was determined in all hearts as the percentage of infarcted area vs. the total left ventricular circumference, using conventional haematoxylin–eosin staining. Hearts with an infarcted area <25% of the total left ventricle were discarded.

Transverse cut cells were randomly selected in haematoxylin–eosin sections of sham and infarcted hearts. The form factor (=4π area/perimeter$^2$) of these cells was calculated and only cells with factors above 0.6 were accepted as true cross sections and were used for further analyses (sham: 5 hearts, 185 cells; MI: 6 hearts, 155 cells). The cross-sectional area and minor axis length of these cells were measured using SCION software (SCION Corporation, Frederick, MD).

### 2.8. Statistics

Vasodilator responses were calculated as the absolute amplitude of the increase in coronary flow for each concentration. Statistical analysis was performed on the total data set by two-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test on each concentration. $ED_{50}$ and maximal dilatation values were obtained from nonlinear regression of the dose–response curve (GraphPad Prism, San Diego, CA). The relative change in area under the curve was calculated to allow comparisons between and within the sham and MI groups. Values were analysed using two-tailed non-paired *t*-test. Results were expressed as mean±standard error of the mean, with $P<0.05$ considered statistically significant.

## 3. Results

### 3.1. Basic parameters

Morphometric analysis of all infarcted hearts revealed that the mean size of infarction was 41.1±0.2% of the total left ventricular wall. Heart weights, cross-sectional area, and minor axis length of infarcted hearts were significantly higher than those of sham hearts (Table 1), suggesting postinfarction hypertrophy. In addition, left ventricular function was impaired after infarction. There was a significant reduction in maximal left ventricular pressure, contractility ($dp_{max}/dt$), and developed pressure following infarction. No signs of atherosclerosic lesions were observed in the sham or MI group.

Table 1
Basic parameters

| | Sham group | MI group |
|---|---|---|
| Body weight (g) | 320.9±3.1 | 326.8±4.2 |
| Heart weight (g) | 1.07±0.02 | 1.31±0.03[a] |
| Maximal pressure (mm Hg) | 100.5±2.3 | 62.5±2.3[a] |
| $dp_{max}/dt$ (mm Hg/s) | 2626±81 | 1526±79[a] |
| Developed pressure (mm Hg) | 86.1±2.7 | 49.8±2.6[a] |
| Cross-sectional area (μm$^2$) | 172.0±4.7 | 205.5±5.3[a] |
| Minor axis length (μm) | 11.7±0.2 | 12.5±0.2[a] |

Basic parameters of the sham and MI groups.
[a] $P<0.05$ vs. sham group.

Table 2
Influence of antagonists on the basal coronary flow

| | Sham group | | MI group | |
|---|---|---|---|---|
| | % Change | *n* | % Change | *n* |
| L-NAME | −26.3±3.3 | 8 | −40.8±3.9[a] | 11 |
| Indomethacine | 10.1±4.4 | 8 | 5.1±5.8 | 8 |
| L-NAME/Indom | −18.7±6.4 | 8 | −28.0±2.3 | 8 |
| 1400W | −8.8±5.2 | 6 | −0.4±4.7 | 8 |
| TEA | −23.8±10.1 | 8 | −14.3±2.6 | 8 |
| SKF-525A | −18.5±6.1 | 9 | −28.3±3.9 | 7 |
| L-NAME/Indom/TEA | −16.1±3.5 | 7 | −32.2±3.1[a] | 8 |

The relative change of antagonist-influenced coronary flow vs. basal coronary flow is described. The last column represents the number of rats used in that experiment.
[a] $P<0.05$ vs. sham group (Indom: indomethacine; TEA: tetraethylammonium sulphate).

### 3.2. Coronary flow and vasodilatory flow response

After the equilibration period, basal coronary flow was unchanged with total mean values of 10.7±0.3 ml/min in the sham group and 10.7±0.4 ml/min in the MI group (Table 2). Constant perfusion with L-NAME led to a significant decrease in basal coronary flow in both groups. However, this decrease was much more pronounced following infarction (Table 2). The iNOS antagonist 1400W had no effect on basal blood flow (Table 2). In addition, although indomethacin induced a small increase in basal coronary flow, combined perfusion with L-NAME and indomethacin significantly reduced basal coronary flow (Table 2). This decrease was only significant in the MI group, while there was a trend towards significance in the sham group. The $K^+$ channel inhibitor TEA reduced basal coronary flow in both groups, while SKF-525A induced a transient increase in coronary flow followed by a decreased flow (Table 2). Co-infusion of L-NAME, indomethacin, and TEA significantly reduced the basal flow in both groups. This reduction was significantly more pronounced in the MI group (Table 2).

Bolus injections of bradykinin or adenosine induced a sigmoidal dose-dependent increase in coronary flow in both groups. Coronary flow increases to bradykinin were significantly reduced in the MI group, as compared to the sham group (% change sham vs. MI: −25.5±5.7%), while the flow response after administration of adenosine remained unaltered in both groups (Fig. 2). In both the sham and MI groups, the bradykinin-induced vasodilatory response after continuous perfusion with L-NAME was significantly reduced (Fig. 3). This decrease, however, was much more pronounced after infarction (Table 3). The iNOS antagonist 1400W had no effect on vasoresponse in both groups (Table 3). In contrast, coronary vasodilatation to bradykinin was significantly reduced by COX inhibition (Fig. 4). This reduction was apparently not changed after ischaemia (Table 3). Co-infusion of L-NAME and indomethacin elicited a significant decrease in flow response after administration of bradykinin in both groups (Fig. 5). However, this decrease did

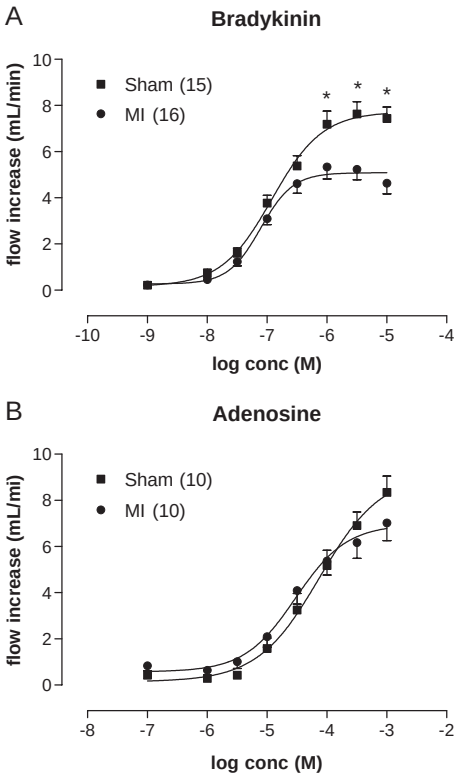


Fig. 2. Basal dose–response curves of bradykinin ($10^{-9}$–$10^{-5}$ M) (A) and adenosine ($10^{-8}$–$10^{-4}$ M) (B) in the sham group (■) and after myocardial infarction (MI, ⋆). Note the significantly impaired endothelium-dependent vasoresponse after infarction, while the vasodilatory capacity after adenosine remained unaltered. The number of rats used for these experiments was shown between parentheses. *$P<0.05$ vs. sham.

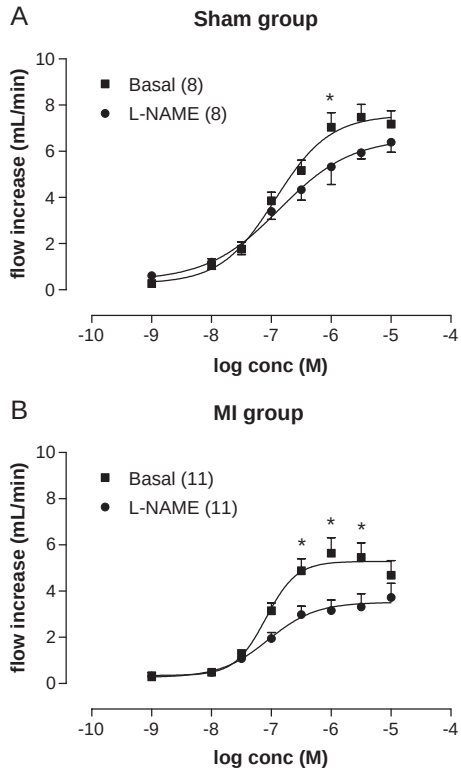


Fig. 3. Dose–response curves of bradykinin during Krebs–Henseleit (■) and L-NAME (⋆) perfusion, in the sham (A) and MI groups (B). Continuous addition of L-NAME led to a significant decrease in vaso-response in both groups. However, this decrease was much more pronounced after myocardial infarction. The number of rats used for these experiments was shown between parentheses. *$P<0.05$ vs. basal.

not reflect the sum of the separate decreases after perfusion with either L-NAME or indomethacin (Table 3). The EDHF antagonists alone did not significantly influence the endothelium-dependent coronary vasoreactivity in both groups, although there was a slight decrease in vasodilatation response in the MI group (data not shown). After additionally blocking the NO and the prostanoid pathways, TEA induced a large and significant decrease in vasoresponse in both groups (Table 3, Fig. 6). Compared to the response after co-infusion of L-NAME and indomethacin in the absence of TEA, the influence of antagonists on vasodilatation response was increased. However, this was only a trend, which did not reach statistical significance.

### 3.3. Immunohistochemical visualisation and quantification

iNOS immunoreactivity was sparse in the sham group, but the number of iNOS-immunopositive cells was significantly increased after myocardial infarction (sham: 0.28±0.05, $n=4$; MI: 1.27±0.25, $n=4$, $P<0.05$). iNOS synthesis was found in endothelial cells, cardiomyocytes, macrophages, and other connective tissue cells (Fig. 7A). In addition, strong immunostaining for eNOS was observed in endothelial cells and some cardiomyocytes of sham and infarcted hearts (Fig. 7B). eNOS immunoreactivity was more pronounced following ischaemia (sham: 1.6±0.3, $n=4$; MI: 3.4±0.6, $n=5$, $P<0.05$). In contrast, no difference was seen between COX-2 immunoreactivity in both groups (sham: 1.8±0.5, $n=3$; MI: 2.8±0.3, $n=5$). Mainly vascular smooth muscle cells and inflammatory cells from the ischaemic area stained positive for COX-2 antibodies (data not shown). In addition, p22phox and p47phox, two subunits of NAD(P)H oxidase, were particularly found in cardiomyocytes of the peri-infarcted area and, to a lesser extent, in endothelial cells. No or sparse immunoreactivity was seen after sham operation, while the presence of both NAD(P)H oxidase subunits was clearly increased after infarction (p22phox: sham: 0.4±0.2, $n=4$; MI: 2.2±1.1, $n=4$; p47phox: sham: 0.5±0.2, $n=4$; MI: 1.9±0.3, $n=4$, $P<0.05$).

Table 3
Changes in area under the curve

| | Sham group | | MI group | |
|---|---|---|---|---|
| | % Change | $n$ | % Change | $n$ |
| L-NAME | −14.1±3.9 | 8 | −34.8±5.4[a] | 11 |
| Indom | −16.3±2.8 | 8 | −23.2±7.4 | 8 |
| L-NAME/Indom | −25.7±7.9 | 8 | −17.1±4.7 | 9 |
| 1400W | −1.6±4.7 | 6 | 0.3±6.4 | 8 |
| TEA | 8.8±13.3 | 8 | −10.8±1.8 | 8 |
| SKF-525A | 1.6±9.2 | 9 | −6.4±6.1 | 7 |
| L-NAME/Indom/TEA | −33.2±8.3 | 7 | −30.1±5.7 | 8 |

The first column represents the relative difference in area under the curve between basal and antagonist-influenced curves for each antagonist. The last column represents the number of rats used in that experiment.

[a] $P<0.05$ vs. sham group (Indom: indomethacine; TEA: tetraethyl-ammonium sulphate).

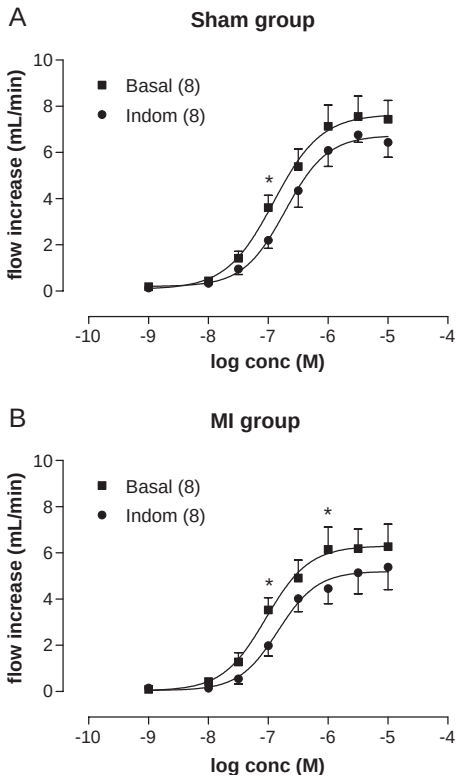


Fig. 4. Dose–response curves of bradykinin during Krebs–Henseleit (■) and indomethacin (⋆) perfusion, in the sham (A) and MI groups (B). Continuous addition of indomethacin led to a significant but similar decrease in vasoresponse in both groups. The number of rats used for these experiments was shown between parentheses. *$P<0.05$ vs. sham (Indom: indomethacin).

### 3.4. Determination of mRNA levels of eNOS and iNOS

Relative quantification of the mRNA levels of eNOS or iNOS in relation to the housekeeping gene *GAPDH* was increased after myocardial infarction (Fig. 8). The mRNA ratio of iNOS vs. GAPDH was significantly enhanced from 0.9±0.2 in the sham group to 2.4±0.2 following infarction, while the ratio of eNOS vs. GAPDH increased from 9.0±0.8 in the sham group to 14.7±1.6 in the MI group.

### 3.5. Determination of serum nitrites and superoxide production

Serum levels of nitrites, which are the sole stable metabolites of NO, are shown in Fig. 9. There was a significant increase in serum nitrite concentration after myocardial infarction (sham: 29.0±1.8, $n=6$; MI: 40.1±1.5, $n=8$, $P<0.05$), representing a higher NO production following ischaemia. In addition, a trend towards significantly higher superoxide concentration was observed after myocardial infarction (sham: 58,403±12,126 cpm/g, $n=6$; MI: 93,444±8990 cpm/g, $n=20$) (Fig. 10).

## 4. Discussion

The present study demonstrates that permanent ischaemia impairs endothelium-dependent subacute vasodilatation responses in the coronary vasculature of the remote myocardium, while endothelium-independent vasodilatation remains unaltered. In the coronary circulation of the sham-operated rat heart, NO and prostanoids were found to contribute to the vasodilatory response to an endothelium-dependent stimulus. The vasodilatory impairment following myocardial infarction did not originate from alterations in the prostanoid-mediated pathway and only a slightly increased influence of $K_{Ca}$ channels was observed. However, ischaemia significantly increased the impact of the NO-mediated coronary vasoresponse during the subacute remodeling phase.

Basal coronary flow was not changed 1 week after experimental induction of myocardial infarction. These findings are in accordance with a previous study (Nelissen-Vrancken et al., 1996), which described that basal coronary flow was acutely reduced after ischaemia, but normalised within 1 week postinfarction. The decrease in $O_2$ supply immediately following ligation of the coronary artery activates a remodeling process, compensating for the loss of ventricular contractile tissue and thereby maintaining left ventricular function (Vannan and Taylor, 1992). This process initiates architectural changes in the remote myocardium, such as eccentric and concentric hypertrophy (Swynghedauw, 1999; Vannan and

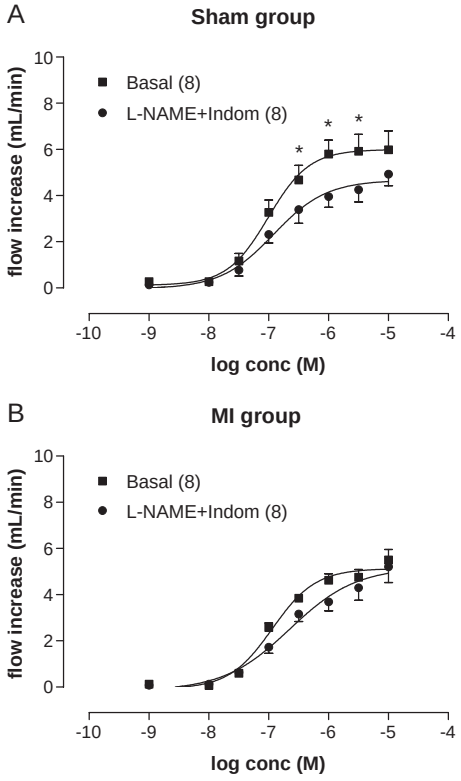


Fig. 5. Dose–response curves of bradykinin during Krebs–Henseleit (■) and L-NAME and indomethacin (⋆) perfusion, in the sham (A) and MI groups (B). A significant decrease in vasoresponse was observed in both groups. This decrease did not reflect the sum of the separate decreases of L-NAME and indomethacin perfusion. The number of rats used for these experiments was shown between parentheses. *$P<0.05$ vs. basal (Indom: indomethacin).

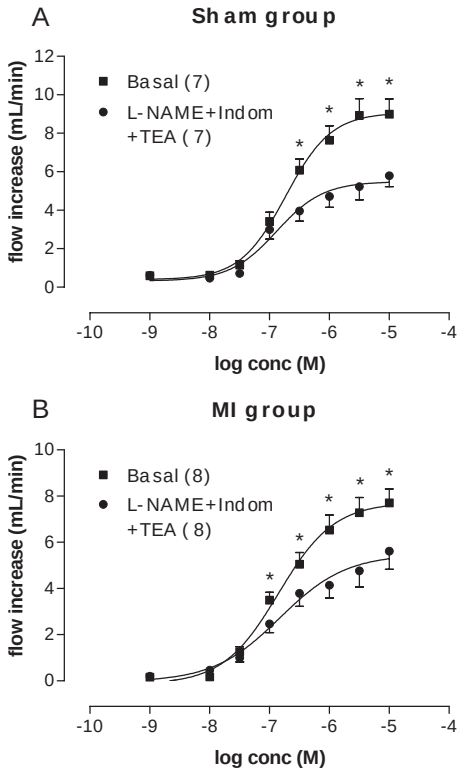


Fig. 6. Dose–response curves of bradykinin during Krebs–Henseleit perfusion (■) and after combined perfusion with L-NAME, indomethacin, and TEA (⋆), in the sham (A) and MI groups (B). There was a significant and large effect in both groups. However, no difference was seen between both groups. The number of rats used for these experiments was shown between parentheses. *$P<0.05$ vs. basal (Indom: indomethacin; TEA: tetraethyl-ammonium sulphate).

Taylor, 1992). After infarction, the mean heart weight, cross-sectional area, and minor axis of transverse myocardial sections were indeed significantly increased in our study, suggesting myocardial hypertrophy. Consequently, although part of the coronary circuit was interrupted due to ligation of the left anterior descending coronary artery, no reduction in basal coronary flow was observed in the subacute phase following ischaemia because of higher metabolic demands.

Our experiments revealed that L-NAME had a significant impact on coronary flow, suggesting that NO plays an important role in the maintenance of coronary flow. Other studies (Bauersachs et al., 1994; Graves et al., 2000) have confirmed that NO is a major contributor of vascular tone. The influence of NO on coronary tone was significantly higher following myocardial infarction, possibly due to higher baseline concentrations of NO after ischaemia. In addition, we and others (Clark and Fuchs, 1997) observed that $K_{Ca}$ channels and/or CYP450 metabolites participate in the modulation of basal tone of coronary arteries, while some investigators found no tonic release of EDHF in coronary circulation (Nishikawa et al., 1999). Consequently, the role of EDHF metabolites in regulating coronary tone remains elusive. The basal release of COX products was reported to be too limited to significantly influence the tone of coronary arteries (Bauersachs et al., 1994).

Apart from affecting coronary flow, NO also proved to be an important mediator of vasorelaxation responses in the coronary circulation of sham-operated rat hearts. Indomethacin administration, on the other hand, suggested that the endothelium-dependent vasoresponse was partly mediated by COX products, such as prostacyclin ($PGI_2$). Moreover, no basal involvement of $K_{Ca}$ channels was observed in the vasodilatation of rat coronary arteries. Additional blocking of NO and prostanoid pathways revealed that $K_{Ca}$ channels might be functional in rat heart but only after deprivation of NO and $PGI_2$. It has been shown that NO and EDHF fulfill complementary roles. Under physiological conditions, EDHF-mediated dilatations are attenuated by NO (Bauersachs et al., 1996; Oltman et al., 2001). This inhibitory effect results from interference with the synthesis and/or release of EDHF rather than from its mechanism of action (Bauersachs et al., 1996). Therefore, when NO synthesis is impaired, EDHF formation is no longer restricted and may maintain endothelial vasodilator response. In addition, prostanoids compete with EDHF for arachidonic acid, thereby exerting an inhibitory action on the release of EDHF (Yajima et al., 1999). Our results and these data suggest that endothelium-dependent hyperpolarisation backs up or enhances the relaxing action of NO, but that it is not an important primary mediator of endothelium-dependent relaxation. In addition, our findings are in agreement with earlier studies (Edwards et al., 1998; Yamanaka et al., 1998), showing that CYP450 metabolites, such as epoxyeicosatrienoic acids, are probably not involved in the hyperpolarisation-mediated coronary vasoresponse. Moreover, a large portion of vasodilatation is resistant to L-NAME, indomethacin, and TEA treatment, suggesting the involvement of additional pathways, vasodilators, and/or $K^+$ channels in coronary vasodilatation of rats. Additionally, the close proximity of beating cardiomyocytes might contribute to vasoreactivity when other mechanisms fail.

Occlusion of part of the coronary vasculature diminishes coronary flow substantially (Nelissen-Vrancken et al., 1996). In the acute and subacute phases after myocardial infarction, blood supply to the ischaemic area can be improved by the recruitment and dilatation of pre-existing vessels, while neovascularisation occurs in the chronic phase (Nelissen-Vrancken et al., 1996). As a consequence, coronary flow reserve could be reduced early after infarction due to basal dilatation of the remaining coronary arteries. However, only bradykinin-induced vasodilatation was significantly reduced 1 week after myocardial infarction, while the endothelium-independent vasoresponse remained unaltered, indicating no change in vasodilatory capacity of the vascular smooth muscle cells. Therefore, the reduction in coronary vasodilatation during subacute postinfarction remodeling is caused by an endothelial dysfunction in the remote myocardium. These findings are in accordance with the results of other investigators (Hasegawa et al., 2000;

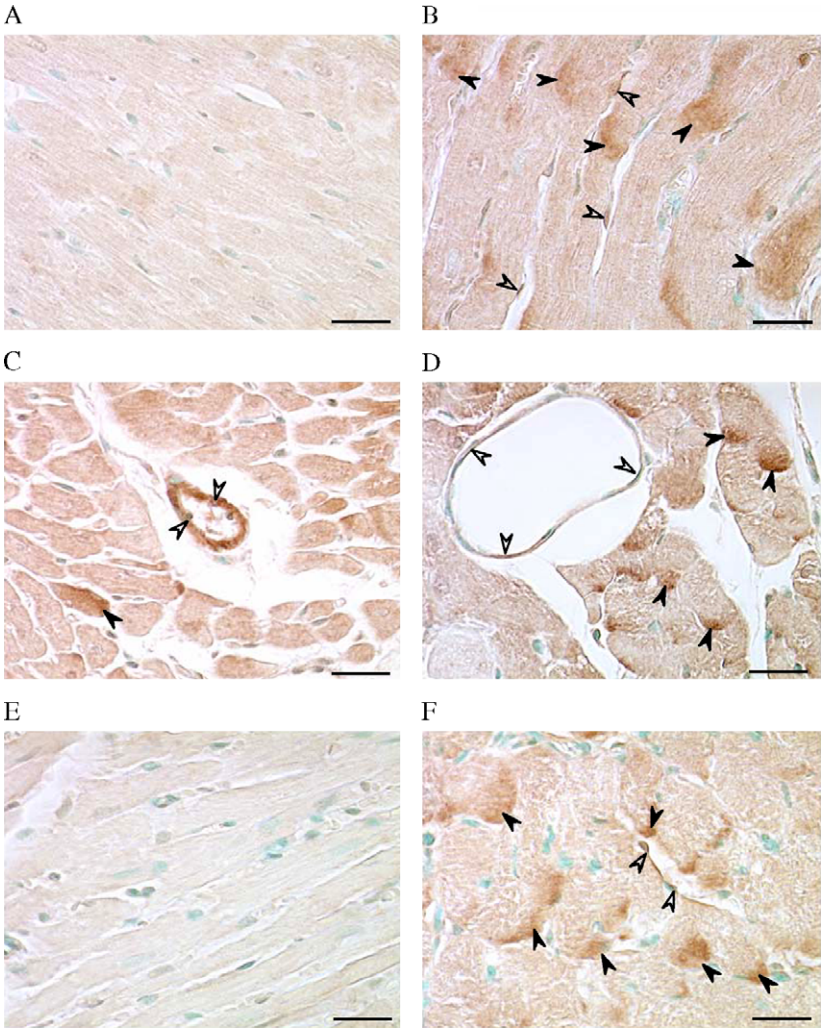


Fig. 7. (A and B) Representative images of iNOS immunoreactivity in sham (A) and infarcted hearts (B). Shams showed only weak iNOS immunoreactivity, while there was a clear upregulation of iNOS-positive cells after ischaemia. (C and D) Localisation of eNOS immunoreactivity in the sham group (C) and after infarction (D). Note the strong immunostaining in endothelial cells in both groups. The number of eNOS-positive cells was increased after ischaemia. (E and F) Expression of p22phox immunoreactivity in sham (E) and infarcted hearts (F). No or sparse immunoreactivity was seen after sham operation, while the presence of NAD(P)H oxidase was clearly increased after infarction. Immunostaining for p22phox was particularly found in cardiomyocytes and, to a lesser extent, in endothelial cells. Open arrowheads point at endothelial cells, while closed arrowheads indicate immunostaining in cardiomyocytes (scale bar 50 μm).

Kubo et al., 1991). In addition, ischaemia itself is able to induce endothelial dysfunction in non-infarcted, non-atherosclerotic arteries, without the presence of additional inflammatory processes.

The influence of COX-2 was of similar magnitude in sham and infarcted hearts, suggesting that this coronary dysfunction does not originate from an altered activation of the prostanoid pathway. Conversely, there was a slight increase in involvement of $K_{Ca}$ channels under basal conditions and particularly after deprivation of NO and $PGI_2$. Combined blockade of NOS and COX pathways led to a small activation of EDHF in the sham group, while the effect was much more pronounced following infarction, even largely neutralising the effect of L-NAME and indomethacin.

However, more importantly, the NO-mediated part of coronary vasodilatation was significantly increased after ischaemia, suggesting augmented production of eNOS and/or induction of iNOS. After ischaemia, an acute and rapid induction of iNOS, which remained elevated for a prolonged period of time, was demonstrated (Dudek et al., 1994; Wang et al., 1999; Wildhirt et al., 1995). Nevertheless, these high levels of iNOS are associated with impaired endothelium-dependent vasorelaxation (Kessler et al., 1997). Additionally, although different cardiovascular syndromes, such as ischaemia, are characterised by an increase in eNOS activity and NO release, endothelial dysfunction occurs (Bauersachs et al., 1999; Bloch et al., 2001). Indeed, increased mRNA and protein levels of iNOS and eNOS were observed after myocardial infarction. However, we did not detect a pharmacological impact of iNOS inhibition on vasoreactivity, indicating that the increased influence of L-NAME is related to eNOS formation and activation.

NO plays an important role in the modulation of vasodilatation, vascular tone, and myocardial function, while

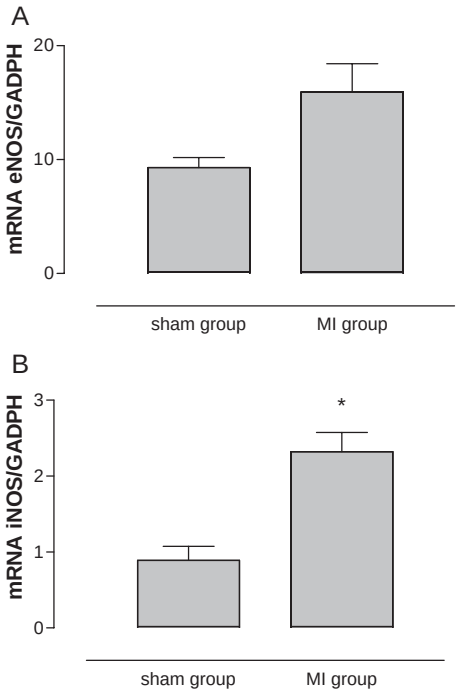


Fig. 8. Relative quantification of mRNA levels of eNOS (A) or iNOS (B) in relation to mRNA concentrations of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). *$P<0.05$ vs. sham.

increased pathophysiological concentrations of NO within the myocardium may have detrimental effects. However, the final action of NO is not only influenced by the amount of NO, but also strongly depends on the environmental state of the heart, more precisely, on the level of oxidative stress in the myocardium (Berges et al., 2003; Ferdinandy and Schulz, 2003). Under normal circumstances, the production of NO predominates, scavenging small amounts of superoxide radicals. In pathological states, large amounts of reactive oxygen species are produced in the vasculature (Bauersachs et al., 1999; Fukui et al., 2001). Superoxide is able to react with most or all NO, leading to the formation of reactive nitrogen intermediates, thereby decreasing the bioavailability of NO and consequently impairing endothelium-dependent vasodilatation (Arimura et al., 2001; Guzik et al., 2000; Kojda and Harrison, 1999). Indeed, the presence of NAD(P)H oxidase was found to be significantly increased

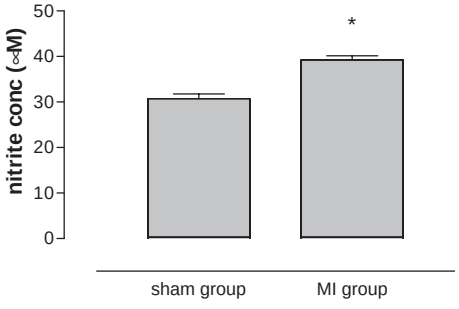


Fig. 9. Serum concentrations of nitrite in the sham and MI groups. Nitrite levels were significantly increased following infarction. *$P<0.05$ vs. sham.

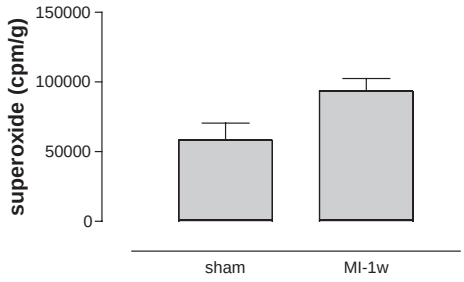


Fig. 10. Amount of reactive oxygen species in both groups, measured by lucigenin-enhanced chemiluminescence. There was a trend towards a higher amount of superoxide radicals after myocardial infarction.

after myocardial infarction and there was a trend towards significantly higher superoxide production. As a consequence, although the formation of NO is enhanced, the final bio-availability and vasodilatory action of NO will be limited due to increased scavenging of superoxide radicals.

In conclusion, we demonstrated that subacute postinfarction remodeling in rats impairs coronary endothelium-dependent vasodilatation in the remote myocardium, without affecting endothelium-independent vasodilatatory capacity. In the normal rat circulation, NO and prostanoids contributed to the coronary vasodilatation, while basal involvement of EDHF was restricted. Endothelial dysfunction following myocardial infarction was not mediated by alterations in the prostanoid-mediated response and was only slightly affected by increased involvement of $K_{Ca}$ channels. In contrast, NO concentration and NO-mediated coronary vasodilatation response were significantly increased after infarction. This apparent duality between increased NO levels and NO-mediated response, on the one hand, and decreased coronary vasodilatation, on the other hand, could be explained by increased production of superoxide radicals. Increased scavenging of superoxide radicals by NO molecules limits the bio-availability and final action of NO.

## Acknowledgements

The authors wish to thank the technical staff of the Laboratory of Cell Biology and Histology, the Laboratory of Pharmacology, and especially Dr. Dominique Ursi of the Laboratory of Microbiology. We also thank Mieke Hoppenbrouwers and Prof. Dr. Wouter Creten for the assistance with the statistical analyses. This work is supported by FWO grant G.0422.02.

## References

Arimura, K., Egashira, K., Nakamura, R., Ide, T., Tsutsui, H., Shimokawa, H., Takeshita, A., 2001. Increased inactivation of nitric oxide is involved in coronary endothelial dysfunction in heart failure. Am. J. Physiol. 280, H68–H75.

Bauersachs, J., Hecker, M., Busse, R., 1994. Display of the characteristics of endothelium-derived hyperpolarisation factor by a cytochrome *P*450-derived arachidonic acid metabolite in the coronary microcirculation. Br. J. Pharmacol. 113, 1548–1553.

Bauersachs, J., Popp, R., Hecker, M., Sauer, E., Fleming, I., Busse, R., 1996. Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. Circulation 94, 3341–3347.

Bauersachs, J., Bouloumié, A., Fraccarollo, D., Hu, K., Busse, R., Ertl, G., 1999. Endothelial dysfunction in chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression: role of enhanced vascular superoxide production. Circulation 100, 292–298.

Behrendt, D., Ganz, P., 2002. Endothelial function: from vascular biology to clinical applications. Am. J. Cardiol. 90, L40–L48.

Berges, A., Van Nassauw, L., Bosmans, J., Timmermans, J.-P., Vrints, C., 2003. Role of nitric oxide and oxidative stress in ischaemic myocardial injury and preconditioning. Acta Cardiol. 58, 119–132.

Bloch, W., Mehlhorn, U., Krahwinkel, A., Reiner, M., Dittrich, M., Schmidt, A., Addicks, K., 2001. Ischemia increases detectable endothelial nitric oxide synthase in rat and human myocardium. Nitric Oxide 5, 317–333.

Bonetti, P.O., Lerman, L.O., Lerman, A., 2003. Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler. Thromb. Vasc. Biol. 23, 168–175.

Busse, R., Edwards, G., Feletou, M., Fleming, I., Vanhoutte, P.M., Weston, A.H., 2002. EDHF: bringing the concepts together. Trends Pharmacol. Sci. 23, 374–380.

Campbell, W.B., Gebremedhin, D., Pratt, P.F., Harder, D.R., 1996. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circ. Res. 78, 415–423.

Clark, S.G., Fuchs, L.C., 1997. Role of nitric oxide and $Ca^{++}$-dependent $K^+$ channels in mediating heterogeneous microvascular responses to acetylcholine in different vascular beds. J. Pharmacol. Exp. Ther. 282, 1473–1479.

Dudek, R.R., Wildhirt, S., Conforto, A., Pinto, V., Suzuki, H., Winder, S., Bing, R.J., 1994. Inducible nitric oxide synthase activity in myocardium after myocardial infarction in rabbit. Biochem. Biophys. Res. Commun. 205, 3–1671.

Edwards, G., Dora, K.A., Gardener, M.J., Garland, C.J., Weston, A.H., 1998. $K^+$ is an endothelium-derived hyperpolarizing factor in rat arteries. Nature 396, 269–272.

Farhangkhoee, H., Khan, Z.A., Mukherjee, S., Cukiernik, M., Barbin, Y.P., Karmazyn, M., Chakrabarti, S., 2003. Heme oxygenase in diabetes-induced oxidative stress in the heart. J. Mol. Cell. Cardiol. 35, 1439–1448.

Félétou, M., Vanhoutte, P.M., 1999. The alternative: EDHF. J. Mol. Cell. Cardiol. 31, 15–22.

Ferdinandy, P., Schulz, R., 2003. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia–reperfusion injury and preconditioning. Br. J. Pharmacol. 138, 532–543.

Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D.R., Fleming, I., Busse, R., 1999. Cytochrome *P*450 2C is an EDHF synthase in coronary arteries. Nature 401, 493–497.

Fukui, T., Yoshiyama, M., Hanatani, A., Omura, T., Yoshikawa, J., Abe, Y., 2001. Expression of p22-phox and gp91-phox, essential components of NADPH oxidase, increases after myocardial infarction. Biochem. Biophys. Res. Commun. 281, 1200–1206.

Gauthier, K.M., Deeter, C., Krishna, U.M., Reddy, Y.K., Bondlela, M., Falck, J.R., Campbell, W.B., 2002. 14,15-Epoxyeicosa-5(*Z*)-enoic acid: a selective epoxyeicosatrienoic acid antagonist that inhibits endothelium-dependent hyperpolarization and relaxation in coronary arteries. Circ. Res. 90, 1028–1036.

Gibson, C.M., Ryan, K.A., Murphy, A.A., Mesley, R., Marble, S.J., Giugliano, R.P., Cannon, C.P., Antman, E.M., Braunwald, E., 1999. Impaired coronary blood flow in nonculprit arteries in the setting of acute myocardial infarction. J. Am. Coll. Cardiol. 34, 974–982.

Graves, J.E., Greenwood, I.A., Large, W.A., 2000. Tonic regulation of vascular tone by nitric oxide and chloride ions in rat isolated small coronary arteries. Am. J. Physiol. 279, H2604–H2611.

Guzik, T.J., West, N.E.J., Black, E., McDonald, D., Ratnatunga, C., Pillai, R., Channon, K.M., 2000. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. Circ. Res. 86, e85–e90.

Hasegawa, T., Miura, T., Tsuchida, A., Miki, T., Nakano, A., Kuno, A., Shimamoto, K., 2000. Endothelium-dependent coronary response is impaired in the myocardium at the early phase of post-infarct remodeling. Jpn. Heart 41, 743–755.

Karam, R., Healy, B.P., Wicker, P., 1990. Coronary reserve is depressed in postmyocardial infarction reactive cardiac hypertrophy. Circulation 81, 238–246.

Kessler, P., Bauersachs, J., Busse, R., Schini-Kerth, V.B., 1997. Inhibition of inducible nitric oxide synthase restores endothelium-dependent relaxations in proinflammatory mediator-induced blood vessels. Arterioscler. Thromb. Vasc. Biol. 17, 1746–1755.

Kojda, G., Harrison, D., 1999. Interaction between NO and reactive oxygen species: pathological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc. Res. 43, 562–571.

Kubo, S.H., Rector, T.S., Bank, A.J., Williams, R.E., Heifetz, S.M., 1991. Endothelium-dependent vasodilation is attenuated in patients with heart failure. Circulation 84, 1589–1596.

Li, Y., Zhu, H., Kuppusamy, P., Roubaud, V., Zweier, J.L., Trush, M.A., 1998. Validation of lucigenin (bis-*N*-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. J. Biol. Chem. 273, 2015–2023.

Moritz, F., Monteil, C., Isabelle, M., Bauer, F., Renet, S., Mulder, P., Richard, V., Thuillez, C., 2003. Role of reactive oxygen species in cocaine-induced cardiac dysfunction. Cardiovasc. Res. 59, 834–843.

Nelissen-Vrancken, H.J.M., Debets, J.J.M., Snoeckx, L.H.E.H., Daemen, M.J.A.P., Smits, J.F.M., 1996. Time-related normalization of maximal coronary flow in isolated perfused hearts of rats with myocardial infarction. Circulation 93, 349–355.

Nishikawa, Y., Stepp, D.W., Chilian, W.M., 1999. In vivo location and mechanism of EDHF-mediated vasodilation in canine coronary microcirculation. Am. J. Physiol. 277, H1252–H1259.

Oltman, C.L., Kane, N.L., Fudge, J.L., Weintraub, N.L., Dellsperger, K.C., 2001. Endothelium-derived hyperpolarizing factor in coronary microcirculation: responses to arachidonic acid. Am. J. Physiol. 281, H1553–H1560.

Ross, R., 1999. Atherosclerosis: an inflammatory disease. N. Engl. J. Med. 340, 115–126.

Sabattini, E., Bisgaard, K., Ascani, S., Poggi, S., Piccioli, M., Ceccarelli, C., Pieri, F., Fraternali-Orcioni, G., Pileri, S.A., 1998. The EnVision$^{++}$ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. J. Clin. Pathol. 51, 506–511.

Selye, H., Bajusz, E., Grasso, S., Mendell, P., 1960. Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 11, 398–407.

Swynghedauw, B., 1999. Molecular mechanisms of myocardial remodeling. Physiol. Rev. 79, 215–262.

Uren, N.G., Crake, T., Lefroy, D.C., de Silva, R., Davies, G.J., Maseri, A., 1994. Reduced coronary vasodilator function in infarcted and normal myocardium after myocardial infarction. N. Engl. J. Med. 331, 222–227.

Vannan, M.A., Taylor, D.J., 1992. Ventricular remodelling after myocardial infarction. Br. Heart J. 68, 257–259.

Vérroneau, M., Tanguay, M., Fontaine, E., Jasmin, G., Dumont, L., 1997. Reactivity to endothelium-dependent and -independent vasoactive substances is maintained in coronary resistance vessels of failing hamster heart. Cardiovasc. Res. 33, 623–630.

Wang, D., Yang, X.P., Liu, Y.H., Carretero, O.A., LaPointe, M.C., 1999. Reduction of myocardial infarct size by inhibition of inducible nitric oxide synthase. Am. J. Hypertens. 12, 174–182.

Wildhirt, S.M., Dudek, R.R., Suzuki, H., Bing, R.J., 1995. Involvement of inducible nitric oxide synthase in the inflammatory process of myocardial infarction. Int. J. Cardiol. 50, 253–261.

Yajima, K., Nishiyama, M., Yamamoto, Y., Suzuki, H., 1999. Inhibition of endothelium-dependent hyperpolarization by endothelial prostanoids in guinea-pig coronary artery. Br. J. Pharmacol. 126, 1–10.

Yamanaka, A., Ishikawa, T., Goto, K., 1998. Characterization of endothelium-dependent relaxation independent of NO and prostaglandins in guinea pig coronary artery. J. Pharmacol. Exp. Ther. 285, 480–489.