Impaired Endothelial Regulation of Ventricular Relaxation in Cardiac Hypertrophy
Role of Reactive Oxygen Species and NADPH Oxidase

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Background—Endothelium-derived nitric oxide (NO) selectively enhances myocardial relaxation. In experimental left ventricular hypertrophy (LVH), this endothelium-dependent LV relaxant response is impaired despite a preserved response to exogenous NO. We investigated the potential role of reactive oxygen species (ROS) in this defect.

Methods and Results—Short-term treatment with the antioxidants vitamin C (10 μmol/L) or deferoxamine (500 μmol/L) restored LV relaxant responses to the NO agonists bradykinin (10 nmol/L) and substance P (100 nmol/L) in isolated ejecting hearts of aortic-banded guinea pigs. Substance P decreased the time to onset of LV relaxation (tdP/dt\textsubscript{min}) by −6.8±1.7 ms in the presence of vitamin C and by −8.9±2.2 ms in the presence of deferoxamine compared with −0.8±2.2 ms in the absence of antioxidants (P<0.05 either antioxidant versus control). A similar restoration of relaxant response to substance P was observed in the presence of the superoxide dismutase mimetic, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (10 μmol/L), but not with tetrahydrobiopterin or L-arginine. Protein expression of the NADPH oxidase subunits gp91-\textsuperscript{phox} and p67-\textsuperscript{phox} and myocardial NADPH oxidase activity were significantly increased (P<0.05) in the banded group compared with shams.

Conclusions—An increase in ROS, most likely derived at least in part from NADPH oxidase, is responsible for the impaired endothelial regulation of LV relaxation in LVH. These are the first data to potentially link increased NADPH oxidase–derived ROS with a defect in cardiac contractile function in a pathological setting. (Circulation. 2001;104:2967-2974.)

Key Words: nitric oxide ● hypertrophy ● endothelium ● free radicals ● myocardial contraction

Nitric oxide (NO) exerts biologically important effects on cardiac myocyte function, including inotropic and chronotropic effects, modification of β-adrenergic responsiveness, and an influence on growth and survival. The paracrine release of NO from coronary microvascular endothelial cells selectively enhances myocardial relaxation and diastolic left ventricular (LV) function and reduces myocardial O\textsubscript{2} consumption. The effects of NO on myocardial relaxation have been demonstrated in several species and preparations, including the normal human heart in vivo. Endothelium-mediated, NO-dependent enhancement of LV diastolic function may facilitate cardiac filling and subendocardial coronary perfusion and may accordingly be beneficial in such settings as tachycardia or increased volume loading, when intracardiac NO release is augmented.

Whereas the contribution of endothelial dysfunction to abnormalities of vascular regulation is well recognized, its impact on myocardial function has received little attention. Recently, we reported that endothelial regulation of LV relaxation was markedly impaired in experimental pressure-overload LV hypertrophy (LVH) in the guinea pig, despite a preserved response to exogenous NO. In that study, the NO-dependent LV relaxant effects of substance P and bradykinin were significantly blunted in hypertrophied hearts. Importantly, this defect was not attributable to alterations in coronary flow but rather reflected a direct impact of cardiac endothelial dysfunction on LV contractile function. The mechanisms underlying this abnormality are unknown. The two main mechanisms that lead to reduction in NO bioactivity are either a decrease in its production and/or an increase in inactivation by reactive oxygen species (ROS), such as superoxide (O\textsubscript{2}⁻). In our previous study, no significant change in cardiac endothelial NO synthase (eNOS) expression was found. The aims of the present study were to (1) examine the potential role of increased ROS in the impaired LV relaxant response in experimental LVH and (2) define the source(s) of any such increase in ROS.
Pressure-Overload LVH
All procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (Her Majesty’s Stationery Office, London, UK). Juvenile male guinea pigs (200 to 250 g; Charles River, Margate, UK) underwent suprarenal abdominal aortic banding or sham banding. Experiments were performed 3 weeks after surgery at a stage of significant compensated LVH in banded animals.

Isolated Heart Studies
Isolated hearts were studied in ejecting mode under controlled loading and constant paced rate. The perfusate was (mmol/L) NaCl 118, KCl 4.7, MgSO4·7H2O 1.2, NaHCO3 24.5, KH2PO4 1.1, glucose 10, CaCl2·2H2O 1.25 (37°C), gassed with 95% oxygen/5% CO2, and containing 1 μmol/L indomethacin to inhibit prostanoxid effects. Left atrial filling pressure was varied between 5 and 20 cm H2O to generate Starling curves. LV pressure was recorded with a 2F Millar catheter, coronary flow and cardiac work were normalized for LV weight. The duration of systolic contraction was assessed by the time interval from onset of LV pressure development to the time of LV dP/dtmax, ie, dP/dtmax.

We studied the following interventions, each in a separate group of hypertrophied hearts (n=6, except for group 9, where n=3): (1) substance P 100 nmol/L, (2) bradykinin 10 nmol/L, (3) substance P 100 nmol/L, the presence of vitamin C 10 μmol/L, (4) bradykinin 10 nmol/L in the presence of vitamin C 10 μmol/L, (5) substance P 100 nmol/L in the presence of deferoxamine 500 μmol/L, (6) bradykinin 10 nmol/L in the presence of deferoxamine 500 μmol/L, (7) substance P 100 nmol/L in the presence of the cell-permeable superoxide dismutase (SOD) mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP, 10 μmol/L, Calbiochem), (8) substance P 100 nmol/L in the presence of tetrahydrobiopterin (BH4) 10 μmol/L, and (9) substance P 100 nmol/L in the presence of l-arginine 100 μmol/L. We also studied the effects of short-term vitamin C pretreatment on responses to substance P and bradykinin in control (nonhypertrophied) hearts (n=3 each). Vitamin C, deferoxamine, MnTMPyP, BH4, or l-arginine was added to hearts ≥10 minutes before subsequent addition of substance P or bradykinin. Pilot studies (n=4 per group) showed that none of these agents exerted significant effects on baseline cardiac function (data not shown). Doses and protocols for these agents were based on previous studies. Stable responses to substance P and bradykinin were achieved after ~8 minutes and ~4 minutes, respectively.

ROS Generation
Myocardial ROS production was assessed by lucigenin (10 μmol/L)-enhanced chemiluminescence. A low concentration of lucigenin was used to minimize artifactual O2 production due to redox cycling. Excised hearts (3 banded, 3 shams) were washed in ice-cold PBS. The LV free wall was cut into 10-mg blocks, which were gassed with 95% O2/5% CO2 in a Krebs buffer solution at 37°C for 30 minutes. Tissue blocks (2 per well) were transferred to a microplate luminometer (Anthos “Lucy 1,” Labtech). O2 production was measured in the presence of (1) no additional agents, (2) the calcium ionophore A23187 10 μmol/L, and (3) NADPH 200 μmol/L. In some experiments, samples were preincubated with diphenyleeniodionium 100 μmol/L, SOD 100 U/mL, the NOS inhibitor Nω-nitro-l-arginine methyl ester (L-NAME) 100 μmol/L, or rotenone 50 μmol/L. Chemiluminescence readings were expressed as arbitrary light units per minute over a period of 20 minutes.

Immunoblotting
LV tissue from banded or sham-operated animals (n=3 each) was immunoblotted for the NADPH oxidase subunits gp91-phox and p47-phox with anti-human neutrophil polyclonal antibodies provided by Dr F. Wiant (University College, London, UK). The monoclonal anti-cardiac troponin I antibody was a gift from Dr I. Trayer (University of Birmingham, UK). Protein bands were detected by enzyme-linked chemiluminescence (Amersham, UK) and visualized by autoradiography.

Oxidative Stress and Antioxidant Levels
Four banded and 4 sham hearts were homogenized in ice-cold PO4/EDTA/KCl buffer containing 0.004% BHT at 100 mg/mL. Malondialdehyde (MDA) content was assessed by detection of thiobarbituric acid (MDA adduct. For determination of total glutathione (reduced [GSH] + oxidized [GSSG]), tissue samples (4 bands, 4 shams) were homogenized in cold 5% metaphosphoric acid, and the homogenate was diluted 1:100 with phosphate buffer. Total glutathione was assayed by a recycling assay in the presence of 5,5′-dithio-bis(2-nitrobenzoic acid); NADPH, and glutathione reductase at 412 nm. GSSG was assayed by first derivatizing GSH by direct homogenization in the presence of 12.5 N-ethylmaleimide. Standard curves for GSH and GSSG of 0 to 6.6 and 0 to 3.3 μmol/L, respectively, were used. Vitamin C and uric acid contents were determined by reverse-phase high-performance liquid chromatography with electrochemical detection using the homogenates from the glutathione preparations. Total ascorbate was estimated by full reduction of dehydroascorbate to ascorbate with d-L homocysteine.

Data and Statistics
All data are mean±SEM. Ejecting heart data are presented for a preload of 10 cm H2O. In these experiments, changes in measured parameters induced by each intervention across a range of preloads were compared between groups by 2-way repeated-measures ANOVA, followed by a post hoc Tukey’s test. Chemiluminescence data were compared by independent 2-tailed Student’s t tests.

Results
Baseline parameters of cardiac function in each group of hypertrophied hearts were similar (not shown).

Effects of Vitamin C Pretreatment
In the absence of vitamin C, substance P had no significant effect on any parameter in hypertrophied hearts. In the presence of vitamin C, however, substance P significantly reduced dP/dtmin (ie, induced an earlier onset of LV relaxation) in hypertrophied hearts without altering cardiac work, LV dP/dtmax, or coronary flow (Figure 1). Substance P had no effect on LVR, LV minimum diastolic pressure, or LV end-diastolic pressure (not shown). These selective effects of substance P on LV relaxation were similar to its previously described effects in nonhypertrophied control hearts.

Figure 2 shows analogous data for hypertrophied hearts treated with bradykinin. Bradykinin induced a significant reduction in dP/dtmax in the presence of vitamin C but not in its absence. Bradykinin increased coronary flow in both groups, but the magnitude of increase did not differ between the groups. There were no significant changes in cardiac work, LV dP/dtmax, or other indices after bradykinin in either group. The effects of bradykinin in hypertrophied hearts in the presence of vitamin C were similar to its previously described effects in nonhypertrophied control hearts.

In nonhypertrophied control hearts, vitamin C pretreatment had no effect on LV relaxant responses to substance P or bradykinin (data not shown).

Effects of Deferoxamine Pretreatment
Responses to substance P and bradykinin were also studied in hearts pretreated with deferoxamine. In deferoxamine-pretreated hearts, both substance P and bradykinin-induced...
reductions in tdP/dtₘᵢₙ were restored (Figures 3 and 4, respectively). Changes in other parameters (ie, cardiac work, LV dP/dtₘᵢₓ, coronary flow) were not affected by deferoxamine pretreatment, indicating a selective change in tdP/dtₘᵢₙ.

**Effects of MnTMPyP**

In an additional group of hypertrophied hearts, we studied responses to substance P after pretreatment with the cell-permeable SOD mimetic, MnTMPyP. MnTMPyP pretreat-
ment restored the LV relaxant response to substance P to the same extent as with vitamin C or deferoxamine, while having no significant effect on other cardiac functional parameters (Figure 5).

**Pretreatment With BH₄ or L-Arginine**
The above results with vitamin C, deferoxamine, and MnTMPyP suggested that ROS production was increased in hypertrophied hearts. To assess whether a dysfunctional NOS deficient in BH₄ or L-arginine was involved, 5 experiments were undertaken in BH₄- or L-arginine-pretreated hearts. Substance P had no significant effect on tdP/dtₘ₉ either in BH₄-pretreated hearts (maximum change in tdP/dtₘ₉/₉/H₁₁₀₀₂/₉/H₁₁₀₀₆ 2.7/₉/H₁₁₀₀₂/₉/H₁₁₀₀₆ 1.7 ms; P=NS) or L-arginine-pretreated hearts (maximum change in tdP/dtₘ₉/₉/H₁₁₀₀₂/₉/H₁₁₀₀₆ 1.6/₉/H₁₁₀₀₂/₉/H₁₁₀₀₆ 4.3 ms; P=NS).

**Myocardial ROS Production**
Basal O₂⁻ production in the absence of added agents was minimal and did not differ between the banded and sham groups (Figure 6A). In the presence of NADPH (200 μmol/L), there was significantly greater O₂⁻ production in hypertrophied than nonhypertrophied myocardium. Addition of A23187 had no effect on O₂⁻ production in either group (data not shown).

To investigate the sources of NADPH-dependent myocardial O₂⁻ production, experiments were repeated in the presence of inhibitors of potential ROS-generating enzymes. NADPH-dependent O₂⁻ production was virtually abolished by a flavoprotein inhibitor, diphenyleneiodonium, but was unaltered by a NOS inhibitor (L-NAME) or an inhibitor of the mitochondrial electron transport chain, rotenone (Figure 6B). SOD significantly inhibited chemiluminescence, confirming O₂⁻ as the measured ROS.

**NADPH Oxidase Expression**
The finding of significant NADPH-dependent O₂⁻ production, inhibitable by diphenyleneiodonium but not L-NAME, suggested that a phagocyte-type NADPH oxidase¹⁴ may be responsible. Consistent with this, expression of the gp91-phox subunit of the oxidase was significantly increased in hypertrophied myocardium (Figure 7). Likewise, expression of the regulatory p67-phox subunit was also increased (Figure 7). Equal loading of gels was confirmed by immunoblotting for cardiac troponin I.

**Antioxidant Status and Markers of Oxidative Stress**
Ascorbate concentrations were 245±59 vs. 318±40 nmol/g wet wt and GSH concentrations were 1.18±0.18 vs. 1.41±0.10 μmol/g wet wt in sham and banded animals, respectively (P=NS). Likewise, concentrations of their oxidation products were similar: dehydroascorbate 45±5 vs. 42±12 nmol/g wet wt and GSSG 84±4 vs. 88±4 nmol/g wet wt in sham and banded animals, respectively (P=NS). Therefore, the redox balance of both antioxidants was unaltered. The MDA content of sham and banded hearts was 19±2 vs. 18±2 nmol/g wet wt, respectively (P=NS), indicating no evidence of undue oxidative stress.

**Discussion**
The major new findings of this study are that (1) an increase in ROS production is responsible for impaired endothelial regulation of LV relaxation in LVH, (2) a major source of increased ROS is likely to be a phagocyte-type NADPH oxidase, and (3) the physiological abnormality is reversible by short-term treatment with antioxidants or a SOD mimetic. To the best of our knowledge, these are the first data to potentially link increased NADPH oxidase–derived ROS with a defect in cardiac contractile function in a pathological setting.
Effect of Antioxidants and a SOD Mimetic

Our previous study in this experimental model demonstrated a direct impact of cardiac endothelial dysfunction on LV contractile function in pressure-overload LVH, independent of coronary flow. Potential underlying mechanisms include impaired NO production and/or its increased inactivation by ROS. The previous study showed that eNOS protein abundance was unaltered, although eNOS activity can change independent of expression level. In the present study, LV relaxant responses to substance P and bradykinin were
restored in hypertrophied hearts after short-term treatment with vitamin C or deferoxamine. Responses to substance P and bradykinin were unaffected by vitamin C pretreatment in control hearts. Vitamin C and deferoxamine are highly effective water-soluble antioxidants, which in the short term improved vascular endothelial dysfunction. Impot-
antly, in the present study, the LV relaxant response to substance P was also restored in the presence of the cell-
permeable SOD mimetic MnTMPyP, suggesting that there was an increase in ROS within the hypertrophied hearts. These results therefore suggest that increased ROS production with a consequent decrease in NO bioavailability is the major defect underlying the cardiac endothelial dysfunction. The experiments with MnTMPyP suggest that the ROS responsible for these effects is likely to be O$_2^-$; definitive identification of the precise oxidant species, however, would require a technique such as electron paramagnetic resonance spectroscopy. Some previous studies have reported increased myocardial oxidative stress in advanced decompensated LVH, but this was not linked to abnormalities of cardiac endothelial function, nor were ROS sources defined.

Sources of ROS

Two major ROS sources considered to be important in the genesis of vascular endothelial dysfunction are (1) dysfunctional NOS enzymes deficient in BH$_4$ or l-arginine and (2) phagocyte-type NADPH oxidases. Addition of exogenous BH$_4$ or l-arginine usually inhibits O$_2^-$ production by dysfunctional NOSs, restores NO production, and corrects endothelial dysfunction. In the present study, neither BH$_4$ nor l-arginine corrected the cardiac endothelial dysfunction, suggesting that dysfunctional NOS was unlikely to be an important source of O$_2^-$. Consistent with this, ex vivo myocardial ROS production was unaffected by L-NAME or the Ca$^{2+}$ ionophore A23187 (which stimulates eNOS).

Phagocyte-type NADPH oxidases are reportedly expressed in several nonphagocytic cells, including the endothelium, vascular smooth muscle, fibroblasts, and cardiac myocytes. In these cells, NADPH oxidases generate low amounts of ROS, which may be involved in redox signaling, in contrast to the high-output O$_2^-$ generation by neutrophil NADPH oxidase. NADPH oxidase-derived ROSs are implicated in vascular pathophysiology, for example angiotensin II–induced smooth muscle hypertrophy and hypertension, and the endothelial dysfunction associated with hypercholesterolemia. The results of the present study suggest that NADPH oxidase–derived ROS are likely to be a major source of ROS, contributing to the
cardiac endothelial dysfunction observed in hypertrophied myocardium. In support of this possibility, (1) the isolated heart experiments with MnTMPyP suggested that O₂⁻ production was increased in situ, (2) there was no evidence for dysfunctional NOS activity, (3) myocardial NADPH oxidase activity was significantly increased in hypertrophied myocardium compared with the sham group, and (4) protein expression of the oxidase subunits gp91-phox and p67-phox was increased. Increased expression of these subunits may account for at least part of the increase in oxidase activity. On the basis of previous studies in phagocytes and nonphagocytes, stimuli that could increase NADPH oxidase expression and/or activity include angiotensin II, tumor necrosis factor-α, and increased mechanical forces. These factors are all likely to be relevant in LVH.

The phagocyte NADPH oxidase comprises a heterodimeric b-cytochrome formed from p22-phox and gp91-phox, which is the site of enzymatic activity, and several regulatory subunits (p47-phox, p40-phox, p40-phox, and rac1).29 The subunit composition of nonphagocytic NADPH oxidases varies significantly among different tissues.14 Whereas gp91-phox is expressed in endothelium23,24 and fibroblasts,26 in vascular smooth muscle it is replaced by a homologue called Nox-1 (or Mox-1).30,31 The anti-cytochrome formed from p22-phox was raised against a synthetic peptide corresponding to the 30 C-terminal amino acids of gp91-phox.32 The homology between gp91-phox (Nox-2) and Nox-1 in this region is 50%,31 and we cannot exclude the possibility that both isoforms are detected by this antibody. The cell type(s) in which myocardial NADPH oxidase up-regulation occurred was not specifically addressed in the present study. Preliminary immunohistochemistry studies, however, suggest that the oxidase is expressed in both endothelial cells and cardiac myocytes, with no significant phagocyte infiltration being evident.

**Potential Effects of ROS**

The main physiological end point studied in this report was cardiac endothelial function and its impact on LV contraction. Apart from decreasing NO bioavailability, ROS production in tissues can be accompanied by lipid peroxidation and oxidative modification of proteins, which can have several deleterious effects. We found no evidence of significant oxidative stress in myocardium as a whole, however, which may be a result of a relatively low level of ROS production restricted to specific cellular or subcellular compartments. Because low-level ROS production can modulate redox-sensitive signaling pathways, such as activation of protein kinases and gene transcription factors,23 it will be of interest in future studies to investigate the contribution of ROS to other aspects of LVH, such as gene expression and chronic remodeling.

**Conclusions**

This study demonstrates that antioxidants and a SOD mimetic improve LV relaxation in pressure-overload LVH in the short term, via their effect to improve cardiac endothelial dysfunction. This endothelial dysfunction is largely attributable to increased ROS production, which probably results in a decrease in NO bioavailability. Although these results cannot be directly extrapolated to clinical LVH, they suggest the potential for antioxidant therapy to improve abnormal LV relaxation by correcting cardiac endothelial dysfunction. We also show that a major source of increased ROS in compensated LVH is a phagocyte-type NADPH oxidase. In addition to the short-term effects on LV relaxation, increased ROS production in LVH could affect other aspects of cardiac structure and function.

**Acknowledgments**

This work was supported by British Heart Foundation (BHF) program grant RG/98008 and by the UK Medical Research Council (MRC). Dr MacCarthy was an MRC Clinical Training Fellow. Dr Shah holds the BHF Chair of Cardiology in King’s College, London.

**References**


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Circulation. 2001;104:2967-2974
doi: 10.1161/hc4901.100382

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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