Impaired Endothelium-Dependent Regulation of Ventricular Relaxation in Pressure-Overload Cardiac Hypertrophy

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Background—Endothelium-derived nitric oxide (NO) selectively enhances myocardial relaxation and may benefit diastolic function. Left ventricular hypertrophy (LVH) is characterized by abnormal myocardial relaxation and endothelial dysfunction. We investigated endothelium-dependent regulation of LV relaxation in moderate pressure-overload LVH induced by aortic banding in guinea pigs.

Methods and Results—Isolated ejecting hearts of banded or sham-operated animals (shams) were studied. The specific agonists for endothelial release of NO, bradykinin (10 nmol/L), and substance P (100 nmol/L) both induced earlier onset of LV relaxation in shams (time to LV dP/dt\textsubscript{min} [tdP/dt\textsubscript{min}], −13.4±3.0 and −10.4±2.5 ms, respectively) without altering peak LV pressure or LV dP/dt\textsubscript{max}. Neither agent altered tdP/dt\textsubscript{min} in banded animals. The ACE inhibitor captopril (1 μmol/L) also selectively reduced tdP/dt\textsubscript{min} in shams via a bradykinin/NO-dependent mechanism but had no effect in banded animals. An exogenous NO donor, sodium nitroprusside (0.1 μmol/L), selectively reduced tdP/dt\textsubscript{min} to a similar extent in both shams and banded animals. Endothelial-type NO synthase (eNOS) protein expression in whole LV homogenate was unaltered in banded animals.

Conclusions—Endothelium-dependent enhancement of LV relaxation is impaired in moderate pressure-overload LVH, despite a preserved response to exogenous NO. This is not accounted for by altered eNOS expression. These abnormalities may contribute to diastolic dysfunction in LVH. (Circulation. 2000;101:1854-1860.)

Key Words: nitric oxide ▪ diastole ▪ hypertrophy ▪ endothelium

It is now well established that agents released by coronary endothelial cells exert direct effects on cardiac myocyte function, analogous to endothelial regulation of vascular smooth muscle tone.1 The paracrine release of nitric oxide (NO) from coronary microvascular endothelial cells exerts specific effects on normal myocardial function, in particular (1) selective enhancement of myocardial relaxation and diastolic left ventricular (LV) function1 and (2) reduction in myocardial O\textsubscript{2} consumption.2 Cardiac myocytes themselves also express endothelial-type NO synthase (eNOS).3 Indeed, several other effects of NO on myocardial function have been described, eg, positive and negative inotropic effects, inhibition of ß-adrenergic responsiveness, modulation of sarcosomal Ca\textsuperscript{2+} influx and sarcoplasmic reticular Ca\textsuperscript{2+} release, and changes in heart rate.1,3

The modulatory effects of NO on basal myocardial relaxation (ie, in the absence of stimulation by other agonists) have been confirmed in several species and preparations. In isolated ferret papillary muscles, stimulation of endothelial NO release with substance P induced earlier onset of isometric twitch relaxation without any reduction in systolic function as assessed by the maximal rate of force development.4 Similar effects were noted with the NO donor sodium nitroprusside (SNP) and with 8-bromo-cGMP (a lipid-soluble analogue of cGMP, a downstream messenger of NO).4 Identical effects were described in cat and human papillary muscles.5,6 In isolated rat ventricular myocytes, both 8-bromo-cGMP and SNP induced earlier isotonic twitch relaxation and an increase in diastolic cell length, which were not accompanied by changes in cytosolic Ca\textsuperscript{2+} transients and were therefore attributed to a reduction in myofilament responsiveness to Ca\textsuperscript{2+}.7,8 In isolated ejecting guinea pig hearts, either SNP, substance P, or bradykinin induced NO-dependent enhancement of LV relaxation without altering the maximal rate of LV pressure rise (LV dP/dt\textsubscript{max}).9,10 In human subjects with normal LV function undergoing diagnostic cardiac catheterization, bicoronal infusion of SNP or substance P induced earlier onset of LV relaxation without changes in LV dP/dt\textsubscript{max}.11,12 The beneficial effects of endothelium-derived NO on LV relaxation in isolated ejecting guinea pig hearts or on O\textsubscript{2} consumption in canine and human cardiac preparations were also reproduced by short-term administration of ACE inhibitors, through a mechanism that involved bradykinin receptors and NO.13,14 ACE inhibitors not only inhibit conversion of angiotensin I to angiotensin II but also reduce degradation of bradykinin, which may itself be released by...
endothelial cells. Indeed, this pathway may contribute to the beneficial cardiovascular effects of ACE inhibitors. 15

An early feature of pressure-overload LV hypertrophy (LVH) is diastolic dysfunction, characterized by delayed or incomplete ventricular relaxation and/or increased diastolic stiffness, 16 often occurring in the absence of systolic dysfunction. 17 These abnormalities may lead to elevated filling pressures, pulmonary congestion, and dyspnea. Diastolic dysfunction in LVH is in part attributable to interstitial fibrosis and increased passive chamber stiffness but also involves intrinsic abnormalities of cardiac myocyte structure and function. 16,18 The influence of endothelium-derived NO on LV relaxation in LVH has not been studied previously. However, it is known that impaired NO-dependent coronary vasodilatation may be a feature of LVH, both experimentally 19–21 and in patients. 22,23 In the present study, we investigated the effects of endothelium-derived NO and of acute ACE inhibition on LV relaxation in an experimental model of compensated pressure-overload LVH in the guinea pig. We report that endothelium-dependent regulation of LV relaxation is markedly impaired in LVH, despite a preserved response to exogenous NO.

Methods

Induction of 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). Suprarenal abdominal aortic banding was performed in juvenile male guinea pigs (200 to 250 g; Charles River UK Ltd). Briefly, animals were anesthetized with a 4% halothane/96% oxygen mixture. After laparotomy, the subdiaphragmatic abdominal aorta was mobilized by blunt dissection, and aortic constriction was achieved by placement of a Weck Hemoclip (SkyMed Ltd) with application forces set to close the clip with an internal diameter of 0.5 mm. The peritoneum and oblique muscles were closed with a continuous suture (3/0 Prolene; Ethicon) and the skin with surgical staples. At the same surgical session, sham-operated animals underwent an identical procedure apart from aortic clip placement. Intramuscular buprenorphine was used for postoperative analgesia. Overall perioperative mortality was <5%. Animals were housed in identical, constant conditions and were fed FDI guinea pig diet (Special Diet Services) with water ad libitum. All experiments were performed 3 weeks after surgery at a stage when there was significant compensated LVH (see Results).

Isolated Ejecting Heart Studies

Isolated ejecting hearts were studied as previously described. 9,10 with minor modifications. Animals were euthanized by an overdose of sodium pentobarbitone (60 mg/kg IP). Excised hearts were rapidly transferred to ice-cold Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7, MgSO4·7H2O 1.2, NaHCO3 24, KH2PO4 1.1, glucose 10, and CaCl2·2H2O 1.25) gassed with 95% oxygen/5% CO2, and containing 1 μmol/L indomethacin to inhibit prostanoid effects. After aortic cannulation, hearts were initially perfused in Langendorff mode with Krebs-Henseleit buffer (37°C) at 80 cm H2O (58.8 mm Hg) pressure. The left atrium was cannulated via the largest pulmonary vein, and other pulmonary veins were tied off. A 2F micromanometer-tipped catheter (Millar Instruments) was inserted into the LV apex to record high-fidelity pressure, with care taken to avoid buffer leakage. 3 The heart was then switched to the ejecting, recirculating mode (total recirculating volume 150 mL). Aortic resistance was adjusted with a circumferential constrictor in the aortic outflow line to produce a mean coronary perfusion pressure of 50 mm Hg at a preload of 5 cm H2O and was then maintained at this level. Compliance was provided by a syringe containing 4 mL of air. Left atrial filling pressure (preload) was varied between 5 and 20 cm H2O (3.7 and 14.7 mm Hg) to generate Starling curves. Measurements of pressure and flow were made immediately (<40 seconds) after each change of preload. Aortic and coronary flows were measured by timed collections of aortic and pulmonary arterial effluent, respectively, and cardiac output was calculated as the sum of these measurements. The heart was paced at 10% above intrinsic rate via a right atrial electrode.

Pressure data were sampled at 1 kHz via a MacLab module (ADI Instruments) and recorded on a personal computer. Four consecutive pressure traces were averaged for each measurement of peak LV pressure (LVPmax), LV dP/dtmax, and aortic pressure. Cardiac work was calculated as mean aortic pressure multiplied by cardiac output. Values of coronary flow, cardiac output, 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, tdP/dtmax.

Protocol

After stabilization, baseline measurements were obtained sequentially at preloads of 5, 10, 15, and 20 cm H2O. The following interventions were then studied, each in a separate group of banded and sham hearts (n=6 per group): (1) time controls, ie, no agents added; (2) substance P 100 nmol/L; (3) bradykinin 10 nmol/L; (4) SNP 0.1 μmol/L; and (5) captopril 1 μmol/L. In addition, we also studied (6) bradykinin 100 nmol/L (n=6 banded); (7) captopril 10 μmol/L (n=6 banded); (8) captopril 1 μmol/L in the presence of the NO scavenger hemoglobin 1 μmol/L (n=4 sham); (9) captopril 1 μmol/L in the presence of the bradykinin B2 receptor antagonist Hoe140 100 nmol/L (n=5 sham); and (10) the NOS inhibitor L-NMMA 10 μmol/L. After a stable response to interventions was achieved (~8 minutes for most agents but 4 minutes for bradykinin), a further set of measurements was recorded at preloads of 5, 10, 15, and 20 cm H2O. The doses of agents used were chosen on the basis of their effects previously characterized in this preparation. 9,10,13 In experiments with hemoglobin and Hoe140, these were added before the first set of measurements was taken. 13 Thus, for each group of hearts, we measured an identical set of parameters across a range of preloads both before and after a single intervention.

eNOS Expression

Hearts from banded or sham-operated animals were excised, immediately frozen in liquid nitrogen, and stored at −70°C. Particulate protein was extracted according to Hasenfuss et al. 19 Protein samples (100 μg in loading buffer: 250 mmol/L Tris HCl [pH 6.8], 4% SDS, 10% glycerol, 0.006% bromphenol blue, 2% mercaptoethanol) were denatured (100°C, 5 minutes) and run on 8% polyacrylamide gels. After overnight transfer onto Hybond-ECL nitrocellulose (Amersham), membranes were blocked for 90 minutes in 5% nonfat milk solution (20 mmol/L Tris HCl [pH 7.5], 150 mmol/L NaCl, 5% nonfat milk powder). The primary antibody was a mouse anti-bovine eNOS monoclonal antibody (Zymed Laboratories Inc) used at 1:500 dilution in 5% nonfat milk solution for 12 hours at 4°C. The secondary antibody was a 1:10 000 dilution of peroxidase-conjugated anti-mouse IgG (Transduction Laboratories). Blots were incubated with ECL detection reagent (Amersham) for 1 minute and developed on X-OMAT film (Kodak Inc) for 5 to 10 minutes. All blots were performed in triplicate and analyzed with a BioRad GS3000 densitometer and BioRad Molecular Analyst software.

Drugs and Chemicals

Captopril, bradykinin, SNP, L-NMMA, and indomethacin were obtained from Sigma, substance P was from Peninsula Chemicals, and Hoe140 was a gift from Hoechst. Hemoglobin was freshly prepared from human blood. 4 All agents were made up in distilled water except for indomethacin, which was dissolved in dimethylsulfoxide. The final concentration of dimethylsulfoxide (0.01%) had no effect on heart function.
Statistics
Comparison of measured parameters between banded and sham animals across the range of preloads was made by 2-factor ANOVA for repeated measures, followed by a post hoc Tukey’s test. This was performed for both (1) absolute values of baseline function and (2) the change in measured parameters induced by each intervention. A value of $P<0.05$ was considered significant.

Results
Baseline Characteristics of LVH
At 3 weeks after surgery, LV/body weight ratios (mg/g) were $3.49\pm0.05$ in the banded group and $2.87\pm0.03$ in the sham-operated animals (shams) (n=53 banded, 50 sham; $P<0.001$), indicating significant LVH. Lung/body weight ratios (mg/g) were not significantly different (banded versus sham, $5.8\pm0.4$ versus $6.2\pm0.2$; $P=NS$). Figure 1 shows a comparison of absolute values for baseline cardiac work, LV $dP/dt_{max}$, $t_{dp/dt_{min}}$, and coronary flow across a range of preloads in all banded and sham isolated ejecting hearts studied. LV $dP/dt_{max}$ and cardiac work were slightly but significantly higher in the sham group, and there was a significant group-preload interaction for cardiac work ($P<0.01$). Coronary flow normalized by LV weight was lower in the LVH hearts at all preloads ($P<0.001$), but coronary flow normalized by cardiac work was not significantly different between the groups ($P=NS$). Baseline $tdP/dt_{min}$ was similar in both groups.

Effect of Substance P and Bradykinin on Isolated Hearts
Figure 2 shows a typical example of the LV response to substance P (100 nmol/L) in a sham heart and an LVH heart. There was an earlier onset of LV relaxation (ie, a reduction in $tdP/dt_{min}$) without significant change in LV pressure development in the sham heart but no effect in the LVH heart. Mean data for changes in measured parameters after substance P in the 2 groups are shown in Figure 3. Substance P significantly reduced $tdP/dt_{min}$ in shams but not in the banded group. There was no significant effect on cardiac work, LV $dP/dt_{max}$, or coronary flow in either group. When no agent was added to sham or banded hearts (ie, time controls; n=6 each), there were no significant changes in any of these parameters over a 15-minute period (data not shown).

Treatment of the sham group with bradykinin (10 nmol/L) caused a reduction in $tdP/dt_{min}$ similar to that observed with substance P, again without significant changes in LV $dP/dt_{max}$ or cardiac work (Figure 4). This response to bradykinin was markedly blunted in the LVH group. In contrast to substance
P, bradykinin induced a significant rise in coronary flow, but its magnitude was not significantly different between the groups. In LVH hearts treated with a 10-fold higher dose of bradykinin (100 nmol/L), the change in tdp/dtmin was no different from that seen with 10 nmol/L bradykinin (maximal reduction 7.4±2.3 compared with 5.1±1.6 ms; P=NS).

Effect of Short-Term ACE Inhibition
Treatment of sham group hearts with captopril (1 μmol/L) resulted in effects similar to (but larger than) those with substance P and bradykinin, ie, significant reductions in tdp/dtmin without changes in LV dP/dt max (Figure 5). This response was significantly blunted in the LVH group, and the changes in tdp/dtmin were also not significantly different from those in LVH time controls. No changes in cardiac work or coronary flow were observed in either group. As previously observed in normal guinea pig hearts, the LV relaxant effect of captopril in the sham group was abolished by hemoglobin or Hoe140 (maximal changes in tdp/dtmin, +1±1.2 and +1.5±1.4 ms, respectively; P=NS). A 10-fold higher dose of captopril (10 μmol/L) in the LVH hearts also had no significant LV relaxant effect (maximal change in tdp/dtmin, −2.6±1.5 ms; P=NS versus time control).

Effect of SNP
SNP (0.1 μmol/L) reduced tdp/dtmin in the sham group without significantly altering LV dP/dt max or cardiac work and with a nonsignificant rise in coronary flow (Figure 6). A similar LV relaxant effect was also observed with SNP (0.1 μmol/L) in the banded group (Figure 6).
Effect of L-NMMA
To assess the role of basal (tonic) NO production, we tested the effects of \( \text{N}^\text{G} \)-monomethyl-L-arginine (L-NMMA). However, these studies were difficult, because an L-NMMA dose \( >50 \mu\text{mol/L} \) induced marked coronary vasoconstriction and depression of systolic LV function, resulting in instability of the preparation. The highest dose that was possible to study was \( 10 \mu\text{mol/L} \), which reduced coronary flow by a maximum of \( -4.4 \pm 1.5 \text{ mL} \cdot \text{min}^{-1} \cdot \text{g}^{-1} (-20.8\%) \) in the sham group and \( -2.5 \pm 0.7 \text{ mL} \cdot \text{min}^{-1} \cdot \text{g}^{-1} (-16.2\%) \) in the banded animals. L-NMMA (10 \( \mu\text{mol/L} \)) tended to increase \( \text{tdP/dt}_{\text{min}} \) to a greater extent in shams (+3.7 \pm 1.7 ms) than in banded animals (+2.1 \pm 1.1 ms), but this did not achieve statistical significance. There were no significant changes in cardiac work or \( \text{dP/dt}_{\text{max}} \) with this dose of L-NMMA (data not shown).

Expression of eNOS
Figure 7 shows a representative Western blot of LV eNOS protein expression in banded and sham-operated animals. There was no significant difference between the groups (LVH, 10.7 \pm 0.5 compared with shams, 9.3 \pm 0.7 densitometric units; \( n=5 \) animals per group, with blots performed in triplicate; \( P=\text{NS} \)).

Discussion
The main finding of this study was that cardiac endothelial dysfunction in moderate pressure-overload LVH was associated with impaired regulation of LV relaxation. We believe that this is the first demonstration of a direct impact of cardiac endothelial dysfunction on LV contractile function in LVH, independent of changes in coronary flow. Two different agonists for release of NO from endothelium both had little or no effect on LV relaxation in hypertrophied hearts. Similarly, an ACE inhibitor (captopril) known to have bradykinin/NO-dependent LV relaxant effects in this preparation\(^{13} \) had a markedly blunted effect in hypertrophied hearts. This difference in response between the sham and banded groups was not attributable to differential effects of these agents on coronary flow, because (1) substance P and captopril had no significant effects on coronary flow in either group, and (2) bradykinin induced a similar rise in coronary flow in both groups. The latter is consistent with the suggestion from some studies that there is a significant NO-independent component to bradykinin-mediated coronary vasodilatation,\(^{10} \) although this idea is somewhat controversial. The LV relaxant response to an exogenous NO donor, SNP, however, was preserved in hypertrophied hearts. This finding indicated that hypertrophied myocardium remained responsive to exogenous NO and that the impaired response to endothelium-dependent agonists or to captopril was due to endothelial dysfunction. It is unlikely that the impaired action of captopril was due to higher local ACE activity in the hypertrophied heart,\(^{18} \) because a 10-fold greater dose (10 \( \mu\text{mol/L} \)) of...
captopril, which effectively inhibits cardiac ACE,25 had effects similar to those of 1 μmol/L captopril. Whether basal as well as stimulated release of NO was impaired in the LVH hearts could not be ascertained with certainty because of the difficulties of undertaking studies with the NOS inhibitor L-NMMA in the ejecting heart preparation.

Impairment of endothelium-dependent regulation of LV relaxation in LVH is analogous to the impaired endothelium-dependent vascular regulation reported in many cardiovascular disorders.19–23 Potential underlying mechanisms include reduced eNOS expression, eNOS substrate (L-arginine) or cofactor (tetrahydrobiopterin) deficiency, impaired agonist-induced NO release, and/or increased NO inactivation.26 Expression of eNOS was reportedly decreased in the coronary endothelium of spontaneously hypertensive rats (SHR).19,27 Other authors reported increased eNOS expression in SHR hearts, but this was accompanied by dysfunctional enzyme activity, resulting in generation of reactive oxygen species and increased inactivation of NO.28 Increases in Ca2+-dependent NOS activity in coronary endothelium or in whole LV homogenate of SHR have also been reported,28,29 but the relationship between NOS activity assays and functional effects of NO is complex; activity assays measure the maximal potential for NO formation but do not account for changes in substrate/cofactor concentration or other variables that may influence NO production in vivo. In the present study, we found no significant differences in LV eNOS expression, suggesting that the abnormal endothelial responses were attributable either to reduced enzyme activity independent of the amount expressed or to increased NO inactivation.

An intriguing finding in the present study was the dichotomy between endothelium-dependent effects on coronary flow and those on LV relaxation, as also observed in previous studies.9,10,13 It might be anticipated that NO-mediated enhancement of LV relaxation would be paralleled by NO-mediated rises in coronary flow. The lack of such parallel effects was most obvious with captopril, which had the largest effects on LV relaxation yet no effect on coronary flow, as reported previously.13 The simplest explanation for this dichotomy could be that the effects of NO on LV relaxation reflect its release at the capillary level (where the spatial separation between endothelial cells and cardiac myocytes is lowest), whereas the effects on coronary flow reflect release mainly at the arteriolar level. Then, the effects of ACE inhibition might reflect an action predominantly at the post-arteriolar (ie, capillary) level.14 With respect to the blunting of endothelium-dependent LV relaxant effects independent of changes in endothelium-dependent coronary vasodilatation, this could again reflect either a differential release of NO or its differential inactivation at different sites in the vascular bed.

We studied cardiac endothelial function quite early during LVH progression (ie, 3 weeks after banding). At this stage, there was a 22% increase in LV/body weight ratio compared with shams, a minor reduction in isolated heart contractile function, and no evidence of heart failure. It is feasible that both endothelium-dependent effects and myocardial responses to exogenous NO may alter with increasing severity of LVH. Indeed, a recent study reported an impairment of cardiac myocyte response to SNP in rats with severe pressure-overload LVH, although no investigation of endothelium-dependent effects on myocardial function was undertaken.8 In view of the early occurrence of cardiac endothelial dysfunction in the present study, an interesting question is whether reduced endothelial NO “activity” influences aspects of LV function other than diastolic function in LVH. NO is reported to have antihypertrophic activity,30,31 raising the possibility that impaired NO action may promote LVH progression. Another possibility is an influence on myocardial metabolism and O2 consumption. Studies by Hintze et al have shown that endothelium-derived NO reduces myocardial O2 consumption and influences substrate utilization, the net effect being an increase in cardiac efficiency.2,14,32 These investigators recently also reported that in canine pacing-induced heart failure, development of decompensated heart failure was accompanied by a reduction in total cardiac NO production, a switch in myocardial substrate utilization from free fatty acids to glucose, a decrease in cardiac efficiency, and diastolic dysfunction.32 Whether the same applies in pressure-overload LVH merits investigation.

In the present study, short-term treatment of isolated hearts with an ACE inhibitor failed to alter LV relaxation in hypertrophied hearts. However, long-term administration of ACE inhibitors is beneficial with respect to LVH regression and improvement of endothelial function, both experimentally and in patients.15,33 It is reported that chronic ACE inhibitor therapy increases eNOS expression and NO production.27 It is therefore conceivable that the beneficial effects of long-term ACE inhibition on diastolic function in LVH and heart failure may, at least in part, involve improvement in endothelial function.

In conclusion, we have shown that the NO-mediated LV relaxant effects of substance P, bradykinin, and captopril are significantly blunted in pressure-overload LVH in the guinea pig but that the hypertrophied myocardium remains responsive to an exogenous NO donor. Thus, coronary endothelial dysfunction leads to impaired endothelium-dependent regulation of LV relaxation. Coronary endothelial dysfunction is a prominent feature of several pathological conditions, including ischemic heart disease, hypertension, LVH, diabetes mellitus, dilated cardiomyopathy, and transplant vasculopathy.26 In all these disorders, endothelial dysfunction can contribute to impaired vascular regulation. The findings of the present study raise the possibility that coronary endothelial dysfunction in these conditions may also have a direct impact on LV contractile function. Furthermore, therapeutic interventions that correct coronary endothelial dysfunction could lead to improvements in LV diastolic function.

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