It is well established that the endothelium regulates coronary vascular tone via the controlled release of vasoactive agents.\(^1\) NO appears to be the major endothelium-derived relaxing factor (EDRF) and is synthesized continuously under basal conditions by endothelial NO synthase (eNOS). Its production may be increased by agonists such as bradykinin and acetylcholine or by physiological stimuli such as shear stress.\(^1\) Changes in the release and bioactivity of NO occur in many cardiovascular diseases and are thought to contribute to the associated vascular dysfunction.\(^1\)

There is much controversy as to whether coronary eNOS expression and the biological activity of NO are increased or decreased in left ventricular (LV) hypertrophy (LVH). Both impaired and enhanced NO-dependent coronary artery vasodilation has been found in LVH and heart failure, experimentally and clinically.\(^2\)\(^-\)\(^7\) Likewise, other groups have reported increased,\(^8\)\(^,\)\(^9\) decreased,\(^3\)\(^,\)\(^10\) or unaltered\(^11\)\(^,\)\(^12\) coronary eNOS expression and activity in spontaneously hypertensive rats and in compensated LVH. LVH is initially adaptive, but it is associated with a progressive decline in LV function and ultimate cardiac failure. It is characterized by structural and functional changes that depend on the stage of LVH and result at least in part from changes in gene expression.\(^13\) It is possible that the conflicting data on cardiac eNOS expression and NO bioactivity may be due to varying stages and severity of LVH in different models. Previous reports have studied purely molecular or biochemical aspects of coronary eNOS regulation and have not assessed whether changes in eNOS expression and functional activity depend on the stage of LVH.

The aim of the present study was to investigate changes in coronary NO bioactivity and eNOS expression during the progression of LVH. We addressed this issue in a guinea pig model of pressure-overload LVH in which functional stages of LVH were precisely defined.

Divergent Biological Actions of Coronary Endothelial Nitric Oxide During Progression of Cardiac Hypertrophy

David J. Grieve, Philip A. MacCarthy, Nicholas P. Gall, Alison C. Cave, Ajay M. Shah

Abstract—Coronary endothelial NO synthase expression and NO bioactivity were investigated at sequential stages during the progression of left ventricular hypertrophy. Male guinea pigs underwent abdominal aortic banding or sham operation. Left ventricular contractile function was quantified in isolated ejecting hearts. Coronary endothelial and vasodilator function were assessed in isolated isovolumic hearts in response to boluses of bradykinin (0.001 to 10 \(\mu\)mol/L), substance P (0.01 to 100 \(\mu\)mol/L), diethylamine NONOate (DEA-NO) (0.1 to 1000 \(\mu\)mol/L), \(N^G\)-monomethyl-L-arginine monoacetate (L-NMMA) (10 \(\mu\)mol/L), and adenosine (10 \(\mu\)mol/L). At a stage of compensated left ventricular hypertrophy (3 weeks), left ventricular endothelial NO synthase protein expression was unaltered (Western blot and immunocytochemistry). Vasoconstriction in response to L-NMMA was increased in banded animals compared with sham-operated animals (13.8\(\pm\)2.1\% versus 6.2\(\pm\)1.3\%, \(n=10; P<0.05\)), but agonist- and DEA-NO–induced vasodilation was similar in the 2 groups. At a stage of decompensated left ventricular hypertrophy (8 to 10 weeks), left ventricular endothelial NO synthase protein expression was significantly lower in banded animals (on Western analysis: banded animals, 7.8\(\pm\)0.4 densitometric units; sham-operated animals, 12.2\(\pm\)1.7 densitometric units; \(n=5; P<0.05\)). At this time point, vasoconstriction in response to L-NMMA was similar in the 2 groups, but vasodilation in response to bradykinin (30.9\(\pm\)2.4\% versus 39.7\(\pm\)2.2\%, \(n=10; P<0.05\)), DEA-NO (26.2\(\pm\)1.8\% versus 34.6\(\pm\)1.8\%, \(n=10; P<0.05\)), and adenosine (24.3\(\pm\)2.0\% versus 35.7\(\pm\)2.0\%, \(n=10; P<0.01\)) was attenuated in banded animals. These findings indicate that there is an increase in the basal activity of NO (without a significant change in endothelial NO synthase expression) in early compensated left ventricular hypertrophy, followed by a decrease in both endothelial NO synthase expression and NO bioactivity during the transition to myocardial failure. (Hypertension. 2001;38:267-273.)

Key Words: hypertrophy ■ pressure overload ■ nitric oxide ■ arteries ■ endothelium ■ nitric oxide synthase
Morphometric Data From Sham-Operated and Banded Animals at 3 to 10 Weeks After Aortic Banding

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Operated</th>
<th>Banded</th>
<th>Sham Operated</th>
<th>Banded</th>
<th>Sham Operated</th>
<th>Banded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>418±6</td>
<td>405±14</td>
<td>597±17</td>
<td>575±12</td>
<td>658±19</td>
<td>666±23</td>
</tr>
<tr>
<td>RA/body, mg/g</td>
<td>0.16±0.01</td>
<td>0.19±0.02</td>
<td>0.14±0.01</td>
<td>0.17±0.02</td>
<td>0.13±0.004</td>
<td>0.20±0.02*</td>
</tr>
<tr>
<td>LA/body, mg/g</td>
<td>0.23±0.01</td>
<td>0.27±0.03</td>
<td>0.18±0.01</td>
<td>0.25±0.02*</td>
<td>0.22±0.01</td>
<td>0.39±0.05*</td>
</tr>
<tr>
<td>RV/body, mg/g</td>
<td>0.62±0.02</td>
<td>0.63±0.03</td>
<td>0.53±0.03</td>
<td>0.61±0.05</td>
<td>0.44±0.03</td>
<td>0.59±0.04*</td>
</tr>
<tr>
<td>LV/body, mg/g</td>
<td>2.03±0.06</td>
<td>2.50±0.13*</td>
<td>1.87±0.05</td>
<td>2.50±0.08*</td>
<td>1.93±0.03</td>
<td>2.63±0.15*</td>
</tr>
<tr>
<td>Lung/body, mg/g</td>
<td>6.62±0.24</td>
<td>7.17±0.33</td>
<td>5.51±0.26</td>
<td>5.73±0.19</td>
<td>4.35±0.17</td>
<td>8.90±1.4*</td>
</tr>
</tbody>
</table>

RA indicates right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.
Values are mean±SEM for ≥6 animals.
*P<0.01 vs appropriate sham-operated control animal.

Methods

Male Dunkin-Hartley guinea pigs (Harlan UK Ltd, Bicester, UK) were used in this study. 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).

Experimental Cardiac Hypertrophy

Suprarenal abdominal aortic banding was performed as previously described.14 In a series of 100 operations, the mortality rate was 3.8% in banded animals and 2.1% in sham-operated animals. Animals were studied for physiological and molecular analyses 3 to 10 weeks after surgery.

Isolated Heart Studies

Animals were anesthetized (60 mg/kg sodium pentobarbitone IP) and heparinized (1000 IU/kg IP). Hearts were dissected into ice-cold buffer consisting of (in mmol/L) NaCl 118, KCl 3.8, KH2PO4 1.18, NaHCO3 25, MgSO4 1.19, CaCl2 1.25, and glucose 10, gassed with 95% O2/5% CO2, and containing indomethacin (10 μmol/L). Ejecting heart studies were undertaken as previously described.14 Hearts were paced at 10% above the intrinsic rate via a right atrial electrode, and measurements of pressure and flow were made immediately after each change in preload.14 For Langendorff studies, hearts were perfused at 37°C and the flow rate was adjusted to a coronary perfusion pressure of 60 mm Hg. LV end-diastolic pressure was adjusted to 10 mm Hg with a fluid-filled balloon. Hearts were paced at 10% above the intrinsic rate. Coronary perfusion pressure was measured via a transducer (Bell & Howell). After 15 minutes of stabilization, coronary vasodilatation was studied on the basis of responses to boluses (50 μL over 3 seconds) of bradykinin (0.001 to 10 μmol/L), substance P (0.01 to 100 μmol/L), diethylamine NONOate (DEA-NO) (0.1 to 1000 μmol/L), and adenosine (10 mmol/L). Basal NO release was assessed on the basis of contraction in response to the NOS inhibitor L-NAME (1 mmol/L), which had no effect on cardiac function at its final concentration of 0.1%.

Statistical Analysis

A 2-way repeated measures ANOVA, a 1-way ANOVA with Bonferroni post hoc testing, an unpaired Student’s t test, or a Mann-Whitney U test was used as appropriate. P<0.05 was considered significant.

Results

Functional Stages of Cardiac Hypertrophy

Morphometric data are shown in the Table, and parameters of isolated ejecting heart contractile function are shown in Figure 1. By 3 weeks, the LV-to–body weight ratio was significantly greater in the banded group, confirming LVH. By 6 weeks, left atrium–to–body weight ratio was also greater in the banded group. By 8 to 10 weeks, the right atrium–to–body weight, right ventricle–to–body weight, and lung-to-body weight ratios were significantly increased in the banded group, indicating pulmonary congestion and heart failure. At 3 weeks, both LV dp/dtmax and cardiac work were similar in the 2 groups, consistent with compensated LVH. Bby 8 to 10 weeks, however, cardiac work was decreased and the Frank-Starling response was significantly blunted, indicating decompensated LVH. At this stage, SERCA 2a protein levels were reduced in the banded group versus the sham-operated group (10.4±0.6 versus 13.4±0.2 densitometric
units, n = 5; P < 0.01). At an intermediate point (6 weeks), LV dP/dt max and cardiac work were similar in the 2 groups.

**Coronary Vascular Responses in LVH**

No significant differences in basal coronary flow were observed between the banded group and the sham-operated group at any point (3 weeks, 16.7 ± 0.5 versus 16.5 ± 0.9 mL/min; 6 weeks, 18.3 ± 1.4 versus 19.4 ± 1.8 mL/min; 8 to 10 weeks, 19.2 ± 0.9 versus 18.6 ± 0.8 mL/min; n = 6 to 10). Coronary flow normalized by LV weight was slightly but significantly less in banded hearts at all time points (3 weeks, 11.9 ± 0.6 versus 13.9 ± 0.8 mL · min⁻¹ · g⁻¹; 6 weeks, 9.6 ± 0.7 versus 12.6 ± 0.6 mL · min⁻¹ · g⁻¹; 8 to 10 weeks, 8.1 ± 0.2 versus 10.2 ± 0.5 mL · min⁻¹ · g⁻¹; n = 6 to 10; all, P < 0.05). At 3 weeks, vasodilation in response to bradykinin, substance P, or DEA-NO was unaltered between the banded group and the sham-operated group (Figure 2). However, by 8 to 10 weeks, relaxations in response to bradykinin (Figure 2B), DEA-NO (Figure 2F), and adenosine (Figure 3A) were significantly reduced in the banded group, although those in response to substance P were unchanged. At an intermediate point of 6 weeks, vasodilator responses were similar between groups (data not shown). At 3 weeks, constriction in response to L-NMMA was significantly greater in the banded group than in the sham-operated group (Figure 3B), but at later points, the response was similar in the 2 groups.

eNOS Protein Expression During Progression of LVH

At 3 weeks, there was no significant difference in LV eNOS protein expression between groups (Western analysis, 10.7 ± 0.5 versus 9.3 ± 0.7 arbitrary densitometric units for banded versus sham operated; n = 5). However, by 8 to 10 weeks, eNOS expression was significantly decreased in the banded group versus the sham-operated group (7.8 ± 0.4 versus 12.2 ± 1.7 arbitrary densitometric units, n = 5; P < 0.05; Figure 4A). Immunocytochemical images are shown in Figures 4B through 4F. The negative control showed no staining (Figure 4B). Immunolabeling for eNOS showed clear localization to capillaries, endothelium, and endocardium, which is consistent with previous reports.17 After 3 weeks, the distribution/intensity of eNOS staining was similar between groups (Figures 4C and 4D; 18 738 ± 5233 versus 21 962 ± 3850 arbitrary densitometric units for banded versus sham operated; n = 5; P > 0.05). By 8 to 10 weeks, although the distribution of eNOS was similar, the intensity of eNOS immunostaining was less in failing myocardium (Figures 4E and 4F; 5895 ± 672 versus 21 953 ± 2149 arbitrary densitometric units for banded versus sham operated; n = 5; P < 0.05).

**Discussion**

Previous investigations into possible abnormalities of NO-dependent coronary vasodilation in cardiac hypertrophy have either involved purely molecular/biochemical aspects or vas-
cular physiology in isolation or have failed to relate findings to the functional stage(s) of LVH. Conflicting data have been reported, including increased, decreased, and unaltered eNOS expression or biochemical Ca\(^{2+}\)-dependent NOS activity in LVH. The present study was designed to address this issue by investigating both eNOS protein expression and coronary NO bioactivity at sequential stages during the progression of cardiac hypertrophy, from compensated LVH through the transition to cardiac failure. Using an experimental model of progressive pressure-overload LVH that was carefully characterized functionally, we found that the expression of eNOS and biological actions of NO did indeed vary significantly during the progression of hypertrophy. Therefore, it is likely that the contrasting findings of previous studies may have been a consequence of the variation in the stages of LVH that were studied.

The experimental guinea pig model of progressive pressure-overload LVH that we used resulted in an initial phase of well-compensated LVH, followed by transition to LV decompensation and failure. Detailed contractile characterization of isolated ejecting hearts under controlled loading and heart rate, using well-established afterload-independent indices of cardiac function (LV dP/dt\(_{max}\) and cardiac work), demonstrated compensated LVH at 3 weeks after surgery. By 8 to 10 weeks, LV function was significantly depressed in the banded group compared with the sham-operated animals, and the lung-to-body weight ratio was significantly increased, indicating pulmonary congestion. Consistent with the development of myocardial failure, SERCA 2a protein expression was significantly reduced in the banded animals at this stage, similar to previous reports. At an intermediate stage of 6 weeks, LV function remained compensated, although cardiac work tended to be decreased in the banded group versus the sham-operated group.

Using this model, we found that during compensated LVH (3 weeks after surgery), there was no significant change in LV eNOS protein expression, but basal NO activity of NO was increased as indicated by increased vasoconstriction in response to the NOS inhibitor L-NMMA in the banded group. However, agonist- and DEA-NO–induced vasodilation was unaltered at this stage. In contrast, with LV decompensation (by 8 to 10 weeks), eNOS protein was significantly reduced on both Western blot and immunocytochemistry analyses. At this stage, basal NO bioactivity was similar in the 2 groups (as assessed with vasoconstriction to L-NMMA), but the vasodilator responses to both bradykinin and DEA-NO were attenuated in the banded group.

Because the basal activity of NO and responses to agonist/DEA-NO differed markedly between the stages of compensated LVH and cardiac failure, we also studied an intermediate time point of 6 weeks, which represented a transition stage. At this time point, neither vasoconstriction in response to L-NMMA nor agonist/DEA-NO–induced vasodilation differed significantly between the banded group and the sham-operated group. This suggests that the increased basal activity of NO is synonymous with a stage of early compensated LVH, whereas the decreased responsiveness to bradykinin/DEA-NO is evident only in the coronary circulation during myocardial failure.

The increased basal activity of NO in early compensated LVH in the absence of a change in eNOS expression may be due to the increased shear stresses and mechanical forces caused by the initial pressure-overload and could be beneficial during the development of compensated LVH. Increased basal NO-dependent vasodilation would maintain basal coronary flow, thereby facilitating oxygen and substrate...
supply for the increased myocardial mass. NO may also have beneficial effects on myocardial contractile function (eg, positive inotropic effects, improved diastolic function, and improved muscle efficiency), which could be adaptive during early LVH. On the other hand, the reduction in basal NO activity at later stages of LVH and failure (to levels similar to those in the sham-operated group) could be related to the observed decrease in eNOS expression and/or a reduction in shear stresses and mechanical forces as cardiac function declines. This reduction may contribute to an impaired coronary flow–myocardial function relationship, as well as to the contractile dysfunction and energetic imbalance evident at this stage.

In the present study, vasodilation in response to bradykinin was decreased in decompensated LVH. Many previous studies have reported an impairment of agonist-induced, endothelium-dependent coronary vasodilation in LVH, but the precise underlying mechanisms and, in particular, the role of altered eNOS expression remain unclear. We found that eNOS expression was significantly decreased concomitant with the impairment of responses to bradykinin, suggesting that reduced NO production may be at least partially involved. However, the overall underlying mechanism is likely to be more complex. In addition to bradykinin-induced vasodilation, DEA-NO–induced responses were impaired during decompensated LVH. Possible reasons for this include (1) increased inactivation of NO by reactive oxygen species or (2) reduced vascular smooth muscle response to NO. An increase in reactive oxygen species in cardiac hypertrophy has been previously reported; potential sources include a dysfunctional NOS deficient in tetrahydrobiopterin or L-arginine. Structural abnormalities of the coronary vasculature are also well recognized in LVH, being attributable to changes such as a reduction in resistance vessel density, decreased vascular luminal radius, and upregulation of extracellular matrix. Indeed, it has been suggested that increased coronary artery wall thickness in LVH may limit vasodilator responses to both NO-dependent and independent stimuli. Coronary flow reserve is reported to be reduced in LVH, thus rendering the heart more vulnerable to ischemia. Consistent with this, here we observed a

Figure 4. LV eNOS expression in banded and sham-operated animals. A, Representative Western blot at 8 to 10 weeks. C indicates bovine aortic endothelial cell protein as a positive control. B to E, LV sections of the banded and sham-operated groups immunostained for eNOS. B, Negative control incubated with secondary antibody only. C, Banded, 3 weeks. D, Sham operated, 3 weeks. E, Banded, 8 to 10 weeks. F, Sham operated, 8 to 10 weeks. Magnification, ×40.
significant decrease in adenosine-induced relaxation after 8 weeks of banding.

An additional factor to consider is the role of other EDRFs, such as prostacyclin and endothelium-derived hyperpolarizing factor (EDHF). In the present study, the inclusion of indomethacin excluded a role for prostacyclin. However, EDHF may be involved in the responses to bradykinin. In the guinea pig coronary vasculature, vasodilation in response to substance P was shown to be mediated almost exclusively by NO, whereas vasodilation in response to bradykinin appeared to also involve other mechanisms. 31,32 Bradykinin induces hyperpolarization of guinea pig coronary endothelial cells, 33 so it is probable that EDHF contributed to the relaxation responses to bradykinin observed in the present study. The extent of this contribution is difficult to quantify but has been estimated to be <50% by using both inhibitors of NOS 34 and hemoglobin, which inactivates NO. 31 The fact that bradykinin-induced coronary vascular relaxation is not exclusively NO mediated may explain the greater magnitude of these responses in the present study, compared with those seen with substance P. This may also explain the finding that after 8 to 10 weeks, responses to bradykinin were significantly attenuated in the banded group versus the sham-operated group, whereas those to substance P only tended to be reduced.

In conclusion, we demonstrated in a well-characterized model of pressure-overload cardiac hypertrophy with transition to heart failure that eNOS expression and coronary NO bioactivity change in a complex manner dependent on the functional stage of LVH. We found that early compensated LVH was associated with an increase in the basal activity of coronary NO in the absence of changes in eNOS expression and with no change in agonist-induced vasodilation. Decompensated LVH, however, was associated with a decrease in eNOS expression, a decline in basal NO bioactivity to the same level as that of the control group, and a reduction in agonist- and NO donor–induced vasodilation. These data emphasize the importance of carefully relating molecular and physiological alterations in the coronary vasculature to the corresponding cardiac functional status in conditions such as LVH.

Acknowledgments
This work was supported by the Medical Research Council and the British Heart Foundation.

References
Divergent Biological Actions of Coronary Endothelial Nitric Oxide During Progression of Cardiac Hypertrophy

David J. Grieve, Philip A. MacCarthy, Nicholas P. Gall, Alison C. Cave and Ajay M. Shah

Hypertension. 2001;38:267-273
doi: 10.1161/01.HYP.38.2.267

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/38/2/267

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/