Endomyocardial Nitric Oxide Synthase and Left Ventricular Preload Reserve in Dilated Cardiomyopathy

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Background—Patients with heart failure have modified myocardial expression of nitric oxide synthase (NOS), as is evident from induction of calcium-insensitive NOS isoforms. The functional significance of this modified NOS gene expression for left ventricular (LV) contractile performance was investigated in patients with dilated nonischemic cardiomyopathy.

Methods and Results—In patients with dilated, nonischemic cardiomyopathy, invasive measures of LV contractile performance were derived from LV microtip pressure recordings and angiograms and correlated with intensity of gene expression of inducible (NOS2) and constitutive (NOS3) NOS isoforms in simultaneously procured LV endomyocardial biopsies (n=20). LV endomyocardial expression of NOS2 was linearly correlated with LV stroke volume (P=0.001; r=0.66), LV ejection fraction (P=0.007; r=0.58), and LV stroke work (P=0.003; r=0.62). In patients with elevated LV end-diastolic pressure (>16 mm Hg), a closer correlation was observed between endomyocardial expression of NOS2 and LV stroke volume (P=0.001; r=0.74), LV ejection fraction (P=0.0007; r=0.77), and LV stroke work (r=0.82; P=0.0002). LV endomyocardial expression of NOS3 was linearly correlated with LV stroke volume (P=0.01; r=0.53) and LV stroke work (P=0.01; r=0.52). To establish the role of nitric oxide (NO) as a mediator of the observed correlations, substance P (which causes endothelial release of NO) was infused intracoronarily (n=12). In patients with elevated LV end-diastolic pressure, an intracoronary infusion of substance P increased LV stroke volume from 72±13 to 91±16 mL (P=0.06) and LV stroke work from 67±11 to 90±15 g · m (P=0.03) and shifted the LV end-diastolic pressure–volume relation to the right.

Conclusions—In patients with dilated cardiomyopathy, an increase in endomyocardial NOS2 or NOS3 gene expression augments LV stroke volume and LV stroke work because of a NO-mediated rightward shift of the diastolic LV pressure-volume relation and a concomitant increase in LV preload reserve. (Circulation. 1999;99:3009-3016.)

Key Words: nitric oxide • cardiomyopathy • ventricles • diastole

In patients with heart failure, the functional significance for left ventricular (LV) performance of modified myocardial expression of nitric oxide synthase (NOS) remains unclear.1 Expression of cytokine-inducible, calcium-insensitive NOS isoform (NOS2) was first reported in the ventricular myocardium of patients with dilated cardiomyopathy after myocarditis2 and, subsequently, in the myocardium of failing hearts, regardless of the underlying cause.3 In the latter study, NOS2 gene expression was more frequent in NYHA class II patients than in class IV patients, but in a subsequent study,4 high NOS2 gene expression was associated not with functional class but with low LV ejection fraction. In allograft recipients, a Doppler echocardiographic study5 showed that NOS2 gene expression correlated with LV dysfunction, but an invasive assessment of LV performance6 failed to demonstrate any effects of high myocardial NOS2 expression on LV ejection fraction or on the peak rate of rise of LV pressure (LV dP/dtmax). In the normal human heart and in allograft recipients, intracoronary infusions of NO donors or of substance P, which releases nitric oxide (NO) from the coronary endothelium, also failed to alter LV ejection fraction or LV dP/dtmax; instead, they hastened LV relaxation and increased LV diastolic distensibility.7,8 These effects of NO on diastolic LV function were recently corroborated by experimental observations. In isolated guinea pig hearts, administration of a NOS inhibitor reduced LV diastolic distensibility,9 and in the beating rabbit heart, a porphyrinic microsensor in the LV wall revealed a prominent diastolic peak of myocardial NO concentration.10

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In end-stage explanted human cardiomyopathic hearts, expression and activity of endothelial constitutive NOS isoform (NOS3) was reduced, but expression and activity of
NOS2 were enhanced. In trabeculae isolated from these hearts, NOS2 activity hastened isometric tension decline and blunted the β-agonist–induced increase in twitch tension. A blunted myocardial contractile response to β-agonists because of NOS activity was also observed in adult rat cardiomyocytes,6 in dogs with pacing-induced heart failure,12 in patients with LV dysfunction,13,14 and in the human allograft.6,13

To investigate the in vivo functional significance of modified myocardial NOS expression for LV contractile performance in patients with nonischemic, dilated cardiomyopathy, invasive measures of LV function were derived from LV angiograms and high-fidelity tip-micromanometer LV pressure recordings; they were then correlated with the intensity of myocardial NOS2 and NOS3 gene expression in simultaneously procured LV endomyocardial biopsies. To confirm the role of NO as a mediator of the observed correlation between LV stroke volume or LV stroke work and myocardial NOS2 or NOS3 activity, repeat LV angiograms and LV pressure recordings were obtained during intracorony substance P infusion, which causes receptor-mediated release of NO from the coronary endothelium.8,15

Methods

Patients

A total of 32 patients with nonischemic, dilated cardiomyopathy underwent diagnostic cardiac catheterization and coronary angiography. The group consisted of 9 women and 23 men (mean age, 51 years; range, 23 to 75 years). For ethical reasons, heart failure therapy was maintained; it consisted of angiotensin-converting enzyme (ACE) inhibitors (n = 31), diuretics (n = 31), digitalis (n = 14), and β-blockers (n = 2; Table; patients 11 and 31). LV angiography revealed a LV end-diastolic volume index of 148±37 mL/m² and a LV ejection fraction of 29±10%. A LV end-diastolic volume index >102 mL/m² (normal average value +2SD) and an LV ejection fraction ≤45% were used as cutoff values for dilated cardiomyopathy. Informed consent was obtained from all patients, and the study was approved by the local review boards. There were no complications.

Study Protocol

LV pressure was measured by using a high-fidelity tip-micromanometer catheter, and right heart pressures were measured by using a Swan-Ganz catheter. Left ventricular endomyocardial biopsies were obtained in 20 patients (Table; patients 1 through 15 and 28 through 32) using a long biopomte-guiding sheath and a disposable transluminal biopsy (Cordis Corp). Baseline LV function (Table) of the 20 patients in whom LV endomyocardial biopsies were obtained was comparable to the baseline LV function of the entire patient cohort.

After diagnostic cardiac catheterization, an intracoronary infusion of substance P (20 pmol/min for a 5-minute period) was performed in 12 patients (Table; patients 16 through 27). At the end of the infusion period, hemodynamic measures were repeated. In 11 patients (Table; patients 1, 2, 4 through 9, 12, 13, and 15), an intravenous infusion of dobutamine was administered; it was progressively titrated upward to a dose of 11±2 μg · kg⁻¹ · min⁻¹ to increase resting heart rate by 20 bpm. The infusion protocols for substance P and for dobutamine were extensively described in a previous study.13 Data from 3 patients who were part of this previous study were included in the present study.

Reverse Transcription–Polymerase Chain Reaction for NOS2 and NOS3 mRNA

Biopsy Procurement

Biopsy samples used for the subsequent quantification of NOS2 and NOS3 mRNA by reverse transcription–polymerase chain reaction (RT-PCR) were immediately frozen in liquid nitrogen and stored at -80°C. In 15 patients (Table; patients 1 through 15), 1 biopsy sample was used, and in 5 patients (Table; patients 28 through 32), 2 biopsy samples from different sites of the LV cavity were used. In these 5 patients, the variability of the NOS2 and NOS3 mRNA concentrations was 7±2% and 15±3%, respectively.

RNA Extraction

Total RNA from 25 LV endomyocardial biopsies was extracted according to Trizol reagent protocol (Life Technologies). Purified RNA was dissolved in water, and the concentration was measured by absorbance at 260 nm. Quantitative RT-PCR for NOS2 and NOS3 was then performed in the presence of a defined amount of specific RNA mutant as an internal standard.

Internal Standard Preparation

The NOS2 and NOS3 internal standards were subcloned into a pSP(64)poly(A) vector (Promega). cRNA was then synthesized in vitro as a sense probe from 10 μg of the PvuI-linearized plasmid using SP6 RNA polymerase in the presence of 10 μCi of α-32P-UTP. The concentration of the transcript was determined after measuring the radioactivity incorporated into the RNA product.

Oligonucleotides Used for RT-PCR

For NOS2, the primers chosen were 5'-AAGACCCAGTGCCC-TGCTTT-3' for sense and 5'-GCACAAACATAGGGTGCC-3' for antisense; they allowed the distinct amplification of NOS2 mRNA (388 bp) and of the internal standard (452 bp). For NOS3, the primers chosen were 5'-TGCTGGCCCCACTGTCCCTC-3' for sense and 5'-TGCAACGGTCTGCAGGACGTTGTT-3' for anti-sense, thus amplifying a DNA fragment of 616 bp for NOS3 and of 680 bp for the internal standard. These primers were chosen to encompass several introns in the coding region without amplifying contaminating genomic DNA.

Quantitative RT-PCR Protocol

Total RNA was reverse-transcribed with a fixed amount of the specific synthetic RNA and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). Single-strand cDNA synthesis was performed in 20 μL of reaction buffer (in mmol/L): Tris-HCl 20 (pH 8.3), KCl 50, MgCl₂ 4, dNTP 1, and DTT 10 and 0.2 μmol/L oligo(dT). The reaction mixture was incubated for 10 minutes at 25°C and then for 60 minutes at 37°C. The resultant cDNA was amplified using 2.5 U of Taq DNA polymerase (Boehringer) and 0.5 μmol/L sense and antisense primers in 50 μL of (in mmol/L) KCl 50, Tris-HCl 10 (pH 8.3), MgCl₂ 4, and dNTP 1 and 0.01% gelatin. A total of 28 amplification cycles were undertaken as follows: denaturation at 94°C for 1 minute, annealing at 62°C for NOS2 and at 63°C for NOS3 for 1 minute, and extension at 72°C for 1 minute. The final extension was performed for 10 minutes. To quantify NOS isoform mRNA levels, a trace amount of [32P]-dCTP was included in the PCR reaction. After PCR amplification, the PCR products were separated on a 5% polyacrylamide gel, and radioactive signals were analyzed using a computer-based imaging system (Fuji BAS 1000, Fuji Medical Systems).

Data Analysis

LV volumes were derived from single-plane LV angiograms by using the area-length method and a regression equation. The duration of LV electromechanical systole (LVEST), which indicates the time to onset of LV relaxation, was measured as the interval from the Q wave on the ECG to the moment of the peak rate of fall of LV pressure (LV dp/dtmin). LV stroke work was derived from the area within the LV pressure-volume diagram. Single comparison data were analyzed with a Student t test for paired data.

Results

NOS Gene Expression and LV Function at Rest

The Table summarizes indices of baseline LV function in the dilated, nonischemic cardiomyopathy study population. Fig-
Figure 1 shows representative RT-PCR amplification products of NOS2 and NOS3 mRNA derived from LV endomyocardial biopsies obtained from 4 patients (Table; patients 1, 2, 6, 8). In the subgroup of patients (n = 20) in whom LV endomyocardial biopsies were obtained, a significant linear correlation was observed between intensity of NOS2 mRNA expression and LV stroke volume (P = 0.001; r = 0.74), LV ejection fraction (P = 0.0007; r = 0.77), or LV stroke work (P = 0.0002; r = 0.82) when the analysis was limited to those patients who, at the time of the study, were using LV preload reserve, as evident from elevated LV end-diastolic pressure (LVEDP > 16 mm Hg) (Figure 2). Limiting the analysis to patients who were using LV preload reserve did not improve the correlation between intensity of NOS3 mRNA expression and LV stroke volume or LV stroke work.

LV Preload Reserve and Coronary Endothelial NO

To investigate the myocardial effects of NO on LV preload reserve, an intracoronary infusion of substance P was performed.

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Mean ± SD 85 ± 12 120 ± 27 24 ± 9 917 ± 288 148 ± 37 30 ± 10

HR indicates heart rate; LVSP, left ventricular peak systolic pressure; LVEDVI, left ventricular end-diastolic volume index; and LVEF, left ventricular ejection fraction. Other abbreviations are as in text.
in 12 patients. This resulted in a significant fall in LV peak systolic pressure from 121 ± 6 to 111 ± 7 mm Hg (P=0.0009), in LV end-systolic pressure from 64 ± 6 to 58 ± 6 mm Hg (P=0.01) and in LVEDP from 25 ± 3 to 18 ± 2 mm Hg (P<0.0001) and in LV end-diastolic pressure from 25 ± 3 to 18 ± 2 mm Hg (P<0.0001) and in LVEDP from 21 ± 3 to 7 ± 2 mm Hg (P=0.01). There were no significant changes in LV dP/dt max, heart rate, LV end-diastolic volume, LV ejection fraction, LV stroke volume, or LV stroke work, except in those patients who, at the time of the study, had elevated LV filling pressures (LVEDP >16 mm Hg). In these patients, intracoronary substance P significantly increased LV end-diastolic volume from 220 ± 26 to 240 ± 29 mL (P=0.04), LV stroke volume from 72 ± 13 to 91 ± 16 mL (P=0.06), and LV stroke work from 67 ± 11 to 90 ± 15 g · m (P=0.03) (Figure 5).

NOS Gene Expression and LV Response to \( \beta \)-Adrenoreceptor Stimulation

Intravenous infusion of dobutamine caused a significant increase in heart rate from 82 ± 1 to 100 ± 3 bpm (P=0.0001) and in LV dP/dt max from 924 ± 120 to 1356 ± 175 mm Hg/s (P<0.0001), a significant decrease in LVEST from 430 ± 19 to 328 ± 16 ms (P=0.0002), and no change in LV peak or end-systolic pressures. LV endomyocardial NOS2 mRNA expression was inversely correlated with the dobutamine-induced decrease in LVEST (P=0.04; \( r = 0.63 \)) and the ratio of the dobutamine-induced decrease in LVEST divided by the dobutamine-induced increase in LV dP/dt max (expressed as a fraction of baseline LV dP/dt max value) (P=0.004; \( r = 0.78 \)) (Figure 6). The dobutamine-induced changes in heart rate and in LV dP/dt max were unrelated to LV endomyocardial NOS2 mRNA. The dobutamine-induced changes in heart rate, LV dP/dt max, LVEST, and the ratio of \( \Delta \)LVEST/\( \Delta \)LV dP/dt max were also unrelated to LV endomyocardial NOS3 mRNA expression.

Discussion

The present study provides the first evidence that LV endomyocardial NOS2 and NOS3 gene expression varies with the severity of LV dysfunction in patients with dilated, nonischemic cardiomyopathy. In these patients, higher LV endomyocardial NOS2 and NOS3 gene expression was accompanied by higher LV stroke volume and higher LV stroke work.

Myocardial NOS Gene Expression in Heart Failure

Experimental evidence on endomyocardial NOS3 expression and activity in heart failure provides support for the idea that
intensity of NOS3 gene expression varies with the severity of LV dysfunction. In a pacing-induced heart failure dog model, NOS3 activity increased after 2 weeks of pacing, as evident from enhanced endothelium-dependent relaxation of isolated coronary artery rings. This increase in NOS3 activity could have resulted from higher coronary blood flow because of increased myocardial metabolic demand or from higher mechanical stress because of LV cavity dilatation. In the same pacing-induced heart failure dog model, cardiac NO production was reduced after 4 weeks of pacing, and this reduction was accompanied by a switch in myocardial substrate use, a rise in LV end-diastolic pressure, and a fall in LV stroke work.

Clinical studies reporting on myocardial NOS3 gene expression and activity in patients with dilated cardiomyopathy used right ventricular tissue obtained by transvascular biopsy, right ventricular tissue excised during cardiopulmonary bypass, or LV tissue from explanted hearts at the time of cardiac transplantation. Detection of upregulation of myocardial NOS3 as a result of mechanical stress applied to the left ventricle requires LV and not right ventricular tissue, as illustrated in spontaneously hypertensive rats in which NOS3 activity was upregulated in LV but not right ventricular tissue. Both low and high intensities of NOS3 gene expression have been observed in the LV tissue of end-stage explanted cardiomyopathic human hearts. These conflicting results are explained by the present study, which observed that the intensity of NOS3 gene expression varied with the severity of LV dysfunction. The present study determined NOS2 and NOS3 gene expression in LV tissue samples procured by LV transvascular biopsy. The use of LV transvascular biopsies allowed LV endomyocardial NOS gene expression to be assessed not only in patients with end-stage heart failure but also in patients with compensated heart failure. In the present study, most patients were on ACE inhibitor therapy. ACE inhibitor therapy could have augmented NOS3 expression, as evident from the Trial on Reversing Endothelial Dysfunction (TREND), which showed improved coronary endothelial-dependent vasodilator responses during chronic quinapril therapy. The 2 patients in the present study who were on β-blocker therapy had high-
intensity NOS3 gene expression. In failing human myocardium, NOS3 expression is more abundant in patients on $\beta$-blocker therapy, probably because of increased transcription and subcellular targeting to plasmalemmal caveolae of NOS3 as a result of reduced myocardial cAMP content. In patients with dilated cardiomyopathy, upregulated myocardial NOS2 activity or gene expression was reported by most investigators but not all. In a previous study, upregulated myocardial NOS2 gene expression was more frequently observed in patients in NYHA class II than in NYHA class IV. This finding corresponds with the present observation of higher NOS2 gene expression in patients with moderate LV dysfunction. The concordance between both studies, despite the use of LV tissue in the present study and right ventricular tissue in the previous one, supports regulation of myocardial NOS2 expression not by local mechanical stresses but by humoral factors, such as cytokines and neurohormones. No differences in the intensity of NOS2 gene expression were observed in patients receiving digitalis.

**Low Myocardial NOS Gene Expression in Severe LV Dysfunction**

In the present study, both NOS2 and NOS3 gene expression were lower in patients with low LV stroke volume and low LV stroke work. The parallel reduction of NOS2 and NOS3 mRNA in patients with severe LV dysfunction could have resulted from faster degradation of mRNA in biopsies from this patient group. The procedure of biopsy procurement was, however, identical for all patients and, therefore, not responsible for the observed difference. In cardiac myocytes, regulators of gene expression of NOS isoenzymes usually produce opposite effects on NOS2 and NOS3 mRNA. Cytokines increase NOS2 mRNA, and interferon-$\gamma$ and interleukin-1$\beta$ decrease NOS3 mRNA. CAMP stimulates NOS2 mRNA stability and downregulates transcription of NOS3 mRNA. These regulators are, therefore, probably not involved in the observed parallel reduction of gene expression of NOS isoenzymes in patients with low LV stroke work. A possible explanation for the parallel reduction of gene expression of NOS isoenzymes in severe LV dysfunction could be depletion of the myocyte population because of apoptotic cell death triggered by high myocardial concentrations of NO or catecholamines. Such a depletion of the viable myocyte population could explain both the parallel reduction of gene expression of NOS isoenzymes and the impairment of LV contractile performance.

**Myocardial NOS Gene Expression and LV Preload Reserve**

In the present study, linear correlations were observed between LV stroke volume or LV stroke work and intensity of myocardial NOS2 or NOS3 gene expression. Patients with dilated cardiomyopathy are highly dependent on preload recruitable LV stroke work to compensate for reduced inotropic reserve. This enhancement of preload recruitable LV stroke work results from a rightward displacement of the diastolic LV pressure-volume relation. In the present study, an intracoronary infusion of substance P, which releases NO from the coronary endothelium, induced an acute rightward displacement of the LV end-diastolic pressure-volume relation and an increase in LV stroke volume and LV stroke work when LV filling pressures were elevated. This short-term, NO-induced increase in LV diastolic distensibility, LV stroke volume, and LV stroke work supports a myocardial action of NO to mediate the observed correlations between myocardial NOS gene expression and LV stroke volume or LV stroke work. A similar myocardial action of NO on LV preload

**Figure 5.** Effects of intracoronary infusion of substance P (SP) on LV end-diastolic volume (LVEDV) (A), LV stroke volume (LVSV) (B), and LV stroke work (LVSW) (C) in patients with elevated LV filling pressures (LVEDP $>$ 16 mm Hg). *$P$ < 0.05; †$P$ = 0.06.

**Figure 6.** Inverse correlation between intensity of NOS2 mRNA expression and dobutamine-induced abbreviation of LV electromechanical systole time ($\Delta$LVEST) divided by dobutamine-induced increase in LV $dP/dt_{\text{max}}$. When endomyocardial NOS2 mRNA was higher, the dobutamine-induced abbreviation of LV contraction was larger for similar dobutamine-induced rise in LV $dP/dt_{\text{max}}$. $r$ = 0.78, $P$ = 0.004.
reserve was recently observed in isolated guinea pig hearts. In this preparation, adding N\textsuperscript{\textnd}-monomethyl-L-arginine, a specific inhibitor of NOS, to the coronary perfusate resulted in a leftward displacement of the diastolic LV pressure-volume relation and a reduction of LV stroke volume. Similar actions of NO or its second messenger, cGMP, on diastolic myocardial properties have been observed in isolated rat cardiomyocytes\textsuperscript{28,29} and in isolated rabbit cardiomyocytes.\textsuperscript{30}

In isolated rat cardiomyocytes, an increase in diastolic cell length was observed after exposure to a cGMP analogue\textsuperscript{28} or a NO donor.\textsuperscript{29} In isolated rabbit cardiomyocytes, exposure to lipopolysaccharides\textsuperscript{30} altered cell volume through an NO- and cGMP-mediated mechanism. Beneficial effects of NO on diastolic LV properties could result not only from short-term cGMP-mediated myocardial actions but also from long-term effects on the LV interstitium because of altered extracellular matrix metalloproteinase activity and collagen turnover.\textsuperscript{31}

Myocardial NOS Gene Expression and β-Adrenoceptor Stimulation

The present study also demonstrated a significant correlation between the dobutamine-induced abbreviation of LV contraction and NOS2 mRNA expression. In a previous study using isolated muscle strips from explanted human cardiomyopathic hearts, a similar correlation was observed between NOS2 activity measured by citrulline formation and β-agonist–induced changes in the timing of isometric force decline.\textsuperscript{11} In the present study population, no relation was found at baseline between the duration of LV contraction and myocardial NOS mRNA expression, despite the elevated adrenergic drive and the presence of such a relation during intravenous infusion of dobutamine. This suggests that the myocardial effects of elevated plasma catecholamines in heart failure may be offset by a simultaneous reduction in myocardial β1-receptors and/or a simultaneous increase in myocardial G\textsubscript{o}-proteins.

Study Limitations

The myocardial presence of NOS was established in the present study by demonstrating NOS mRNA by quantitative RT-PCR and not by directly demonstrating NO or cGMP in the myocardium. This would provide more definite proof of NOS presence and activity because of earlier reports of posttranscriptional and posttranslational modification of NOS.\textsuperscript{24} In the present study, the effects of NO on LV stroke volume or on LV stroke work were reproduced during intracoronary infusion of substance P, which releases NO from the coronary endothelium. Intracoronary infusion of specific NOS antagonists, which was not performed in the present study, could provide further evidence for NO contributing to LV preload reserve in these cardiomyopathic hearts. To address the issue of homogeneous endomyocardial expression of NOS isoenzymes, multiple biopsies were obtained from different LV sites in 5 patients. In these patients, the variability of NOS2 and NOS3 mRNA concentrations in the different biopsy samples was, respectively, 7±2% and 15±3%.

Conclusions

In the present study, which was the first to analyze gene expression of NOS isoenzymes in LV transvascular biopsies from dilated cardiomyopathy patients, the intensity of NOS2 and NOS3 gene expression was linearly correlated with LV stroke volume and LV stroke work. Intracoronary infusions of substance P, which releases NO from the coronary endothelium, revealed that these correlations could have resulted from a NO-mediated rightward shift of the LV end-diastolic pressure-volume relation and a concomitant increase in LV preload reserve. Future studies should be directed at identifying the mechanisms responsible for the fall in gene expression of NOS isoenzymes in patients with severe cardiomyopathic LV dysfunction, because the present study suggests NO exerts a beneficial hemodynamic effect in the failing human heart through maintenance of the Frank-Starling response.

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