Myocardial fibrosis blunts nitric oxide synthase-related preload reserve in human dilated cardiomyopathy

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Bronzwaer, Jean G. F., Christophe Heymes, Cees A. Visser, and Walter J. Paulus. Myocardial fibrosis blunts nitric oxide synthase-related preload reserve in human dilated cardiomyopathy. Am J Physiol Heart Circ Physiol 284: H10–H16, 2003; 10.1152/ajpheart.00401.2002.—The purpose of the study was to investigate interactions between myocardial nitric oxide synthase (NOS) and myocardial fibrosis, both of which determine left ventricular (LV) preload reserve in patients with nonischemic dilated cardiomyopathy (DCM). In previous animal experiments, chronic inhibition of NOS induced myocardial fibrosis and limited LV preload reserve. Twenty-eight DCM patients underwent LV catheterization, balloon caval occlusions (BCO; n = 8), intracoronary substance P infusion (n = 8), and procurement of LV endomyocardial biopsies for determinations of collagen volume fraction (CVF), of gene expression of NOS2, NOS3, heme oxygenase (HO)-1, and TNF-α, and of NOS2 protein. CVF was unrelated to the intensity of NOS2, NOS3, HO-1, or TNF-α gene expression or of NOS2 protein expression. Preload recruitable LV stroke work (PR-LVSW) correlated directly with NOS2 gene expression (P = 0.001) and inversely with CVF (P = 0.04). High CVF (>10%) reduced baseline LVSW and PR-LVSW at each level of NOS2 gene expression. In DCM, myocardial fibrosis is unrelated to the intensity of myocardial gene expression of NOS, antioxidative enzymes (HO-1), or cytokines (TNF-α) and blunts NOS2-related recruitment of LV preload reserve.

collagen; diastole; myocardial contraction
The present study investigates interactions between the intensity of myocardial NOS gene expression and myocardial fibrosis in patients with nonischemic DCM in relation to their ability to recruit LV preload reserve. Baseline hemodynamic measures and the change in LVSV during balloon caval occlusion were correlated with the intensity of NOS2 and NOS3 gene expression, intensity of protein expression, and collagen volume fraction (CVF) in simultaneously procured LV endomyocardial biopsies. To reproduce interactions between myocardial NO content and CVF, the hemodynamic response to an intracoronary infusion of substance P (13), which releases NO from the coronary endothelium, was also analyzed in relation to CVF of the LV endomyocardial biopsy.

METHODS

**Patients and study protocol.** Twenty-eight patients with nonischemic DCM were included. The group consisted of 8 women and 20 men (Table 1). At the time of study, heart failure therapy was maintained and consisted of ACE inhibitors (ACEI; n = 28), diuretics (n = 25), and β-blockers (n = 2). Cutoff values for DCM were a LV end-diastolic volume index (LVEDVI) > 102 ml/m² (normal average value = 2SD) and a LV end-diastolic pressure (LVEDP) at the upper limit of normal (LVEDV at the upper limit of normal and a LV ejection fraction (LVEF) ≤ 45%. All patients had higher than normal baseline LV end-diastolic wall stress (LVEDWS) ≥ 85 kdyn/cm² = LVEDWS in a LV with a LVEDV at the upper limit of normal and a LV end-diastolic pressure (LVEDP) at the upper limit of normal (LVEF ≥ 16 mmHg) (38). A control group of three patients was also studied. Two male patients (age 44 and 24 yr, LVEF: 54% and 63%) were described previously (13). A control group of three patients was also studied. Two male patients (age 44 and 24 yr, LVEF: 54% and 63%) were referred for myocarditis, and one female patient (age 72 yr, LVEF 57%) was referred for amyloidosis. Histological examination of the LV endomyocardial biopsies revealed no evidence of myocarditis or amyloidosis nor were there other abnormalities.

LV pressure and the first derivative of LV pressure (LV dP/dt) were derived from a high-fidelity micromanometer-tipped catheter. In eight patients (Table 1, patients 21–28), a 5-min intracoronary infusion of substance P (20 pmol/min) was performed (13). Substance P causes receptor-mediated coronary endothelial release of NO. At the end of the infusion period, LV hemodynamic and angiographic measures were repeated and compared with baseline values. In eight patients (Table 1, patients 1–8), a conductance catheter (Levcom Sigma) was used for continuous LV volume and pressure measurement. In these patients, a 8-Fr balloon catheter was introduced into the right atrium. Transient pullback of the inflated balloon into the inferior vena cava created multiple, variably preloaded beats. LV endomyocardial biopsies were obtained using a disposable transfemoral bioprobe (Cordis). The study protocol was approved by the local review board (VU-University Medical Center, Amsterdam, The Netherlands), and informed consent for the study protocol was obtained from all patients.

**RT-PCR for NOS2, NOS3, heme oxygenase-1, and TNF-α mRNA.** Snap-frozen biopsies were obtained in 20 patients (Table 1, patients 1–20) for determination of NOS2 (n = 20), NOS3 (n = 20), heme oxygenase (HO)-1 (n = 14), and TNF-α mRNA (n = 10). RNA extraction, internal standard preparation, and oligonucleotides used for RT-PCR and quantitative RT-PCR protocol, using a defined amount of specific RNA mutant as an internal standard, have been extensively described previously (13). For HO-1, the primers chosen were

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Dig, use of digitalis; HR, heart rate; LVPSP, left ventricular (LV) peak systolic pressure; LVEDP, LV end-diastolic pressure; LV dP/dt max, maximal first derivative of LV pressure; LVEDVI, LV end-diastolic volume index; LVEF, LV ejection fraction.
5'-CAGGCAGAATGCTGAGTTC-3' (sense) and 5'-GCTTCACATAGCCGCTCA-3' (antisense), amplifying the 79- to 429-bp region of HO-1. The expression of TNF-α was quantified as the ratio of the target band intensity to the β-actin band intensity.

Quantitative morphometry and immunohistochemistry. The extent of interstitial fibrosis was determined on elastica von Gieson-stained sections of at least three LV endomycardial biopsies placed in 5% formalin with the use of an automated image analyzer (Prodit). Patients were divided into low and high extents of myocardial fibrosis in accordance to the CVF. A CVF = 10% corresponded to the median value of the CVF data and was used as the cutoff value to separate the low from the high extent of myocardial fibrosis. NOS2 protein expression was analyzed by immunohistochemistry using a commercially available antibody (NOS2, BioMol). Antibodies were detected by an indirect peroxidase antibody conjugate technique, and NOS2 staining was graded semiquantitatively on a scale of 0 to 4.

Data analysis. LVSW was derived from the area within the LV pressure-volume diagram (13). Preload recruitable LVSW (PR-LVSW) equaled the slope of a linear curve fit to the LVSW-versus-LVEDP data of the variably preloaded beats induced by transient pullback of the inflated balloon into the inferior vena cava. Baseline LVSW correlated closely (P < 0.001, r = 0.90) with PR-LVSW. This correlation justifies the use of baseline LVSW as a measure of LV preload reserve in the entire patient population.

Circumferential LV end-diastolic wall stress (LVEDWS) was computed using a thick wall ellipsoid model of the LV (23) as follows

\[ \text{LVEDWS} = \frac{P D}{2h} \times [1 - (h/D) - (D^2/3L^2)] \times 1.332 \text{ dyn cm}^{-2} \]

where P is LVEDP, h is LV echocardiographic end-diastolic wall thickness, and D and L are LV end-diastolic diameter and length at the midwall, respectively.

To assess diastolic LV myocardial material properties, a radial myocardial LV stiffness modulus (Stiffness-Mod) (6) was calculated from the microtip LV pressure and the echocardiographic LV wall thickness using the formula

\[ \text{Stiffness-Mod} = \frac{\Delta \sigma_r}{\Delta e_r} = -\Delta P/(\Delta h/h) = -\Delta P/\Delta \ln h \]

and assuming the increment in radial stress (\( \Delta \sigma_r \)) to be equal but opposite in sign to the increment in LV pressure (P) at the endocardium, and the increment in radial strain (\( \Delta e_r \)) to be equal to the increment in wall thickness (\( \Delta h \)) relative to the instantaneous wall thickness. Because \( \Delta h/h = \Delta \ln h \), Stiffness-Mod was equal to the slope of a P-versus-ln h plot.

All results are given as means ± SE. Univariate and multivariate linear regression analysis were performed with the use of the SPSS software package.

RESULTS

Determinants of LV preload reserve. Univariate linear regression analysis showed the intensity of NOS2 gene expression to correlate with baseline LVSW (P = 0.0002, r = 0.75; Fig. 1A) and with PR-LVSW (P = 0.001, r = 0.90), NOS2 immunostaining to correlate with baseline LVSW (P = 0.003, r = 0.63), and CVF to inversely correlate with baseline LVSW (P = 0.02, r = 0.48) and with PR-LVSW (P = 0.04, r = 0.70). Patients with CVF < 10% had higher baseline LVSW (P < 0.01) and higher PR-LVSW (P < 0.03) than patients with CVF > 10%.

Determinants of LV fibrosis. No significant correlations were observed between CVF and the intensity of NOS2, NOS3, HO-1, or TNF-α gene expression or NOS2 immunostaining.

Interaction between NOS, fibrosis, and LV preload reserve. In a multivariate linear regression analysis, in the evaluation of the relation of baseline LVSW to the intensity of NOS3, NOS2, HO-1, and TNF-α gene expression and to the extent of myocardial fibrosis, only the intensity of NOS2 gene expression (P < 0.0005) and the extent of myocardial fibrosis (P = 0.001) significantly correlated with baseline LVSW.

When patients with CVF < 10% were separated from patients with CVF > 10%, the relation between baseline LVSW and NOS2 gene expression was significantly (P < 0.05) shifted upward in patients with CVF < 10% compared with patients with CVF > 10% (Fig. 1B). This upward shift implies a higher LVSW for a given value of NOS2 gene expression in patients with CVF < 10%. In patients with CVF < 10%, a relation was observed between PR-LVSW and NOS2 gene ex-

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pression ($P < 0.01, r = 0.85$). Patients with CVF > 10% fell below the 95% confidence interval of this relation.

When patients with CVF < 10% were separated from patients with CVF > 10%, a significant correlation between baseline LVSW and the intensity of NOS3 gene expression was observed only in patients with CVF < 10% ($P = 0.04, r = 0.64$) (Fig. 2A). In patients with CVF > 10%, NOS3-related augmentation of LVSW was no longer demonstrable. Reduced NOS3-related augmentation of LVSW in patients with a high extent of interstitial fibrosis was also evident during intracoronary infusion of substance P, which induces coronary endothelial release of NO. In patients with CVF > 10%, the substance P-induced increase in LVSW was significantly smaller than in patients with CVF < 10% [ΔLVSW: 10 ± 3 g·m for CVF > 10% vs. 35 ± 5 g·m for CVF < 10% ($P = 0.02$)].

When patients with CVF < 10% were separated from patients with CVF > 10%, a significant correlation between baseline LVSW and the intensity of HO-1 gene expression was observed only in patients with CVF > 10% ($P = 0.04, r = 0.69$; Fig. 2B). In patients with CVF < 10%, these relations were absent.

Interaction between NOS, fibrosis, and LV myocardial stiffness. Multivariate linear regression analysis was used to evaluate the relation of LV myocardial stiffness to the intensity of NOS3, NOS2, HO-1, and TNF-α gene expression and to the extent of myocardial fibrosis. Only the intensity of NOS2 gene expression ($P = 0.001$; Fig. 3A) and the extent of myocardial fibrosis ($P = 0.04$) significantly correlated with the LV Stiffness-Mod. When patients with CVF < 10% were separated from patients with CVF > 10%, the relation between Stiffness-Mod and NOS2 gene expression was significantly ($P < 0.05$) shifted downward in patients with CVF < 10% compared with patients with CVF > 10% (Fig. 3B). This downward shift implies a lower Stiffness-Mod for a given value of NOS2 gene expression in patients with CVF < 10%.

Fig. 2. A: linear relation between baseline LVSW and the intensity of NOS3 gene expression is observed in patients with low-level myocardial fibrosis (CVF < 10%) but not in patients with high-level myocardial fibrosis (CVF > 10%). B: linear relation between baseline LVSW and the intensity of heme oxygenase (HO)-1 gene expression is observed in patients with low-level myocardial fibrosis (CVF < 10%) but not in patients with high-level myocardial fibrosis (CVF > 10%).

Fig. 3. A: linear relation between the LV stiffness modulus (Stiffness-Mod) and intensity of NOS2 gene expression in the DCM study population and in control patients (○). B: the relation between the LV Stiffness-Mod and NOS2 gene expression is shifted downward in patients with low-level myocardial fibrosis (CVF < 10%).
DISCUSSION

**NO, fibrosis, and LV preload reserve.** Patients with DCM are highly dependent on LV preload reserve to augment cardiac output during exercise. The present study identified high LV endomyocardial NOS2 and low CVF to be important for recruitment of LV preload reserve in these patients.

During exercise, patients with DCM are highly dependent on LV preload reserve because of the blunted myocardial inotropic response to catecholamines (14). The enhancement of PR-LVSW in DCM results from a rightward displacement of the diastolic LV pressure-volume relation (15). Limitation of this rightward displacement is accompanied by a restrictive LV diastolic distensibility and PR-LVSW (7, 13). A high extent of myocardial fibrosis could similarly limit LV distensibility and PR-LVSW (36).

The findings of the present study are consistent with NO being physiologically involved in the LV hemodynamic response to a rise in preload and with myocardial fibrosis effectively countering this physiological response. A physiological role for NO in the LV response to preload augmentation was recently established in experiments that measured the intramyocardial NO concentration using a microporphyrinic sensor in the LV wall of the beating rabbit heart (26). In these experiments, a LV volume load due to release of a caval occluder resulted in a prompt rise in intramyocardial NO concentration. Pretreatment with a NOS inhibitor (27) reduced both PR-LVSW and LV diastolic distensibility, and L-arginine resulted in opposite effects.

The present study investigated relations between myocardial fibrosis and the intensity of NOS gene expression not only because NO could reduce the collagen turnover of fibroblasts but also because myocardial fibrosis could affect myocardial NOS gene expression. Fibrosis of the failing myocardium is indeed accompanied by disruption of the collagen weave around individual myocytes leading to malalignment or slippage. This disruption of the collagen weave alters myocardial gene expression because of an altered cascade of stress signals descending from the collagen fibers to sarcomembranous integrins, cytoskeletal proteins, and nuclear membranes. Both a previous study (30) and the present study failed to observe a relation between the extent of myocardial fibrosis and NOS2 gene expression.

The present study also failed to observe a relation between the extent of myocardial fibrosis and NOS3 gene expression or HO-1 gene expression, which is upregulated in the failing myocardium and could augment the bioavailability of NO by protection against oxidative stress (28). The lack of relations between myocardial fibrosis and NOS3 or antioxidative enzymes such as HO-1 does not exclude the involvement of NOS3 or antioxidative enzymes in the development of myocardial fibrosis but suggests excessive myocardial fibrosis in DCM to result more from upregulation of stimulatory pathways such as angiotensin II, endothelin, and aldosterone than from downregulation of inhibitory pathways (36).

**Upregulation of NOS2.** A potential mechanism for the observed upregulation of NOS2 could be a compensatory rise for reduced expression of NOS3 (9). Recent experiments in NOS3 knockout mice indeed observed a superinduction of NOS2 triggered by the oxidative stress of an ischemia-reperfusion episode (16). This superinduction of NOS2 provided an important protective effect against ischemia-reperfusion injury with preservation of systolic and diastolic LV performance. Superior hemodynamic status in the presence of myocardial NOS2 upregulation was also observed in the present study, and this result confirmed the findings of a recent clinical study that failed to observe a change in LV dP/dt max in DCM patients during intracoronary N G-monomethyl-L-arginine infusion despite NOS2 gene expression (8, 22). In addition, recent experimental findings have demonstrated that the heart can tolerate high levels of NOS2 activity without detrimental functional consequences (12).

The myocardial upregulation of NOS2 observed in the present study could also be part of the reexpression of the fetal gene program frequently observed in hypertrophied or failing myocardium. NOS2 is indeed abundantly expressed in the fetus and gets downregulated before birth (2).

Myocardial cytokines could be important determinants of LV preload reserve because of their ability to induce NOS2 (30) and to increase activity of matrix metalloproteinases with a concomitant acceleration of collagen breakdown (33). In the present study, myocardial TNF-α gene expression appeared unrelated to LVSW, myocardial NOS2 gene expression, or the extent of myocardial fibrosis.

**Therapeutic implications.** The present study shows recruitment of LV preload reserve in patients with DCM to relate to the high intensity of myocardial NOS gene expression and to the low extent of myocardial fibrosis. ACEI, β-blockers, and spironolactone have all been shown to both upregulate myocardial NOS gene expression and to induce regression of endomyocardial fibrosis. Some of the benefits of ACEI, β-blockers, and spironolactone in the treatment of chronic heart failure could therefore be ascribed to their ability to maintain LV preload reserve of the cardiomyopathic heart because of upregulation of NOS3 activity and prevention of myocardial fibrosis. ACEI augment coronary endothelial NO activity acutely as evident from intracoronary enalaprilat infusion during pacing tachycardia (21) and chronically as evident from the Trial on Reversing Endothelial Dysfunction, which showed improved endothelial-dependent vasodilator responses during long-term quinapril therapy (18). Apart from beneficial effects on LV preload reserve, ACEI-induced upregulation of myocardial NO content could also...
favourably modify substrate utilization and mitochondrial respiration of failing myocardium (17, 29, 35). In patients with hypertensive heart disease, 6 mo of lisinopril therapy induced significant regression of myocardial fibrosis and improvement of diastolic LV function (5). DCM patients on β-blocker therapy had a higher intensity of NOS3 gene expression in their endomyocardial biopsies (11, 13). β-Blocker therapy could also favourably affect myocardial fibrosis through reduction of angiotensin II-related activation of matrix metalloproteinases (31). In patients with heart failure, spironolactone improved endothelial dysfunction by increasing NO bioactivity (10), and, in the Randomized Aldactone Evaluation Study, serum levels of markers of cardiac fibrosis were significantly reduced by spironolactone therapy (39).

Study limitations. LV endomyocardial NOS gene expression was derived from a single biopsy sample, which therefore did not take into account spatial heterogeneity of gene expression (4). In a previous study (13), however, multiple biopsies from different LV sites were obtained in the same patient and the variability of endomyocardial NOS2 and NOS3 gene expression was low (7% and 15%). In the present study, LVSW and Stiffness-Mod were correlated with endomyocardial NOS mRNA and protein and not with measures of endomyocardial NO activity, such as myocardial cGMP concentration or transcardiac nitrite/nitrate production. Only the latter could have accounted for posttranscriptional and post-translational modification of NOS, for substrate deficiency, or for the reduced bioavailability of NO because of elevated oxidative stress.

The LV endomyocardial biopsies used for determination of interstitial myocardial fibrosis were considered representative of the whole LV myocardium and therefore assumed homogeneity in LV myocardial structure.

In conclusion, in the cardiomyopathic heart, a high intensity of myocardial NOS gene expression and low-level myocardial fibrosis appeared to be essential for maintaining LV preload reserve. Part of the benefit of ACEI, β-blockers, and spironolactone in the treatment of chronic heart failure could be related to their ability to upregulate NOS2 gene expression and to regress myocardial fibrosis. Upregulation of myocardial NOS2 and regression of myocardial fibrosis are important targets for prevention of progression of cardiomyopathic LV dysfunction.

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REFERENCES


