Endomyocardial nitric oxide synthase and the hemodynamic phenotypes of human dilated cardiomyopathy and of athlete’s heart

Jean G.F. Bronzwaer, Christopher Zeitz, Cees A. Visser, Walter J. Paulus

Abstract

Objective: In dilated cardiomyopathy and in athlete’s heart, progressive LV dilatation is accompanied by rightward displacement of the diastolic LV pressure–volume relation. In dilated cardiomyopathy, an increase in diastolic LV stiffness can limit this rightward displacement thereby decreasing LV systolic performance. Because nitric oxide (NO) reduces diastolic LV stiffness, the present study relates diastolic LV stiffness and LV systolic performance to intensity of endomyocardial NO synthase (NOS) gene expression in dilated cardiomyopathy and in athlete’s heart. Methods: Microtip LV pressures, conductance-catheter or angiographic LV volumes, echocardiographic LV wall thicknesses and snap-frozen LV endomyocardial biopsies were obtained in 33 patients with dilated cardiomyopathy and in three professional cyclists referred for sustained ventricular tachycardia. Intensity of LV endomyocardial inducible NOS (NOS2) and constitutive NOS (NOS3) gene expression was determined using quantitative reverse transcription–polymerase chain reaction (RT–PCR). Results: Dilated cardiomyopathy patients with higher diastolic LV stiffness-modulus and lower LV stroke work had lower NOS2 and NOS3 gene expression at any given level of LV end-diastolic wall stress. The intensity of NOS2 and NOS3 gene expression observed in athlete’s heart was similar to dilated cardiomyopathy with low LV diastolic stiffness-modulus and preserved LV stroke work. Conclusions: High LV endomyocardial NOS gene expression is observed in athlete’s heart and in dilated cardiomyopathy with low diastolic LV stiffness and preserved LV stroke work. Favourable effects on the hemodynamic phenotype of high LV endomyocardial NOS gene expression could result from a NO-mediated decrease in diastolic LV stiffness and a concomitant rise in LV preload reserve.

Keywords: Cardiomyopathy; Gene expression; Hemodynamics; Nitric oxide; Ventricular function

Abbreviations: ACE, angiotensin converting enzyme; CVF, collagen volume fraction; HR, heart rate; IL-1β, interleukin-1β; IL-6, interleukin 6; k, left ventricular chamber stiffness constant; LV, left ventricular; LV dP/dtn, peak rate of left ventricular pressure rise; LVEDP, left ventricular end-diastolic pressure; LVEDV, left ventricular end-diastolic volume; LVEDVI, left ventricular end-diastolic volume index; LVEDWS, left ventricular end-diastolic wall stress; LVEF, left ventricular ejection fraction; LVPS, left ventricular peak systolic pressure; LVSW, left ventricular stroke work; NO, nitric oxide; NOS, nitric oxide synthase; NOS2, inducible nitric oxide synthase; NOS3, constitutive nitric oxide synthase; RT–PCR, reverse transcription–polymerase chain reaction; Stiffness-Mod, radial myocardial left ventricular stiffness modulus; TNFi, tumor necrosis factor α

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This article is referred to in the Editorial by J.T. Stark et al. (pages 225–228) in this issue.

1. Introduction

Rightward displacement of the diastolic LV pressure–volume relation underlies progressive LV dilatation and remodeling in congestive cardiomyopathy [1]. Limitation of this rightward displacement leads to a hemodynamic phenotype with elevated LV diastolic stiffness, poor LV...
systolic performance and poor prognosis [2,3]. Because of nitric oxide (NO)’s ability to shift the diastolic LV pressure–volume relationship rightward in patients with dilated cardiomyopathy [4,5], a low intensity of LV endomyocardial NOS gene expression could underlie the development of this hemodynamic phenotype. The objective of the present study therefore was to relate intensity of LV endomyocardial NOS gene expression to LV diastolic stiffness and to LV systolic performance in patients with congestive cardiomyopathy. A rightward displacement of the diastolic LV pressure–volume relation is also observed in athlete’s heart [6]. We therefore investigated the same relationships in professional cyclists referred for malignant ventricular arrhythmias.

Elevated LV diastolic wall stress has previously been shown to be associated with myocardial gene expression and production of neurohormones [7], natriuretic peptides [8], cytokines [9], stress proteins [10] and growth factors [11]. To detect effects of LV diastolic wall stress on endomyocardial NOS gene expression, the present study also related intensity of LV endomyocardial NOS gene expression to prevailing LV diastolic wall stress. At the time of cardiac catheterization, measurements of LV diastolic stiffness, LV systolic performance and LV wall stress were derived from microtip LV pressures, conductance-catheter or angiographic LV volumes and echocardiographic LV wall thickness. Intensity of endomyocardial inducible NOS (NOS2) and constitutive NOS (NOS3) gene expression was determined on simultaneously procured snap-frozen LV endomyocardial biopsies using quantitative reverse transcription–polymerase chain reaction (RT–PCR).

2. Methods

2.1. Patients

2.1.1. Dilated cardiomyopathy

A total of 33 patients with nonischemic dilated cardiomyopathy underwent diagnostic cardiac catheterization, coronary angiography and LV biopsy procurement. The

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group consisted of 10 women and 23 men (mean age: 50 years; range 23–76). Individual and mean baseline hemodynamic data are listed in Table 1. At the time of study, heart failure therapy was maintained for ethical reasons and consisted of angiotensin-converting-enzyme (ACE) inhibitors (n=33), diuretics (n=31), digitalis (n=15) and β-blockers (n=3; Table 1: patients 10, 19, 24). No patient was using NO-donors. Patients 1–13, 15 (Table 1) were also part of a previous study [4]. A LV end-diastolic volume index (LVEDVI)>102 ml/m² and a LV ejection fraction (LVEF)≤45% were used as cutoff values for dilated cardiomyopathy (normal values: LVEDVI=72±15 ml/m²; LVEF=72±8%). LV endomyocardial biopsies were obtained using a long biopsy-guiding sheath and a disposable transfemoral biopsy (Cordis Corp). Histological examination of the endomyocardial biopsies revealed no evidence of myocarditis.

2.1.2. Athlete’s heart

Three professional cyclists (mean age: 31 years) underwent diagnostic cardiac catheterization, coronary angiography and LV biopsy procurement. They were referred for sustained exercise-induced ventricular tachycardia. Their hemodynamic and echocardiographic studies were consistent with the diagnosis of athlete’s heart as evident from a large LVEDVI (129±11 ml/m²), a normal LVEF (67±9%), a low resting heart rate (55±3 beats/min) and a slightly elevated echocardiographic posterior LV wall thickness (10.3±0.3 mm) (normal value: 9.1±0.5 mm). They took no medications at the time of study. LV endomyocardial biopsies were obtained using a long bioptome-guiding sheath and a disposable transfemoral biopsy (Cordis Corp). Histological examination of the endomyocardial biopsies revealed no evidence of myocarditis.

2.2. Study protocol

Right heart pressures and cardiac outputs were measured using a Swan-Ganz thermodilution catheter. LV pressure and LV dP/dt were derived from a high-fidelity-tip-micromanometer catheter (n=33). In eight patients, a conductance pressure–volume catheter (Leycom Sigma, Zoetermeer, The Netherlands) was inserted into the left ventricle for continuous and on-line LV volume and pressure measurement. In these eight patients, a 8F balloon catheter was introduced from the right femoral vein into the right atrium. Transient pullback of the inflated balloon into the inferior vena cava, allowed for assessment of LV end-diastolic wall stress (EDWS) was calculated from microtip LV pressure (P), angiographic LV end-diastolic major (a) and minor (b) semi-axes and h using the formula [13]

\[ \text{LVEDWS} = (Pb/h)(1 - h/2b - b^2/2a^2) \]

To assess diastolic LV myocardial material properties, a study protocol and there were no complications related to procedure or study protocol.

2.3. Reverse transcription–polymerase chain reaction

Biopsy procurement, RNA extraction, internal standard preparation, oligonucleotides used for RT–PCR and quantitative RT–PCR protocol were extensively described previously [4]. NOS2 and NOS3 mRNA’s were determined in all patients. Tumor necrosis factor α (TNFα), Interleukin-1β (IL-1β) and Interleukin 6 (IL-6) were determined in a subgroup of 10 patients.

2.4. Quantitative morphometry

In a subgroup of patients (n=20), collagen volume fraction (CVF) was determined on EVG (elastica von Gieson) stained sections of biopsies placed in 5% formalin using an automated image analyzer (Prodit).

2.5. Data analysis

LV volumes were derived from single-plane LV angiograms by use of the area–length method and a regression equation. LV stroke work was derived from the area within the LV pressure–volume diagram. Echocardiographic LV posterior wall thickness (h) measurements were derived from a sector scanner (HP Sonos 5500) using a 3 Mhz transducer. Circumferential LV end-diastolic wall stress (EDWS) was calculated from microtip LV pressure (P), angiographic LV end-diastolic major (a) and minor (b) semi-axes and h using the formula [13]
radial myocardial LV stiffness modulus (Stiffness-Mod) was calculated from the microtip LV pressure and \( h \) using the formula [14,15]

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

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 (\( \Delta \varepsilon_r \)) 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 \( P \) vs. \( \ln h \) plot.

In eight patients, in whom a conductance catheter was inserted into the LV and in whom a transient caval balloon occlusion was performed, a LV chamber stiffness constant (\( k \)) was calculated from an exponential curve fit to the multiple LV end-diastolic pressure–volume points of the variably preloaded beats during caval balloon occlusion using the formula [16]

\[
P = b e^{kV}
\]

where \( P \) is the LV end-diastolic pressure, \( V \) the LV end-diastolic volume and \( b \) the curve-fitting parameter. For these eight patients, ranking of \( k \) and Stiffness-Mod yielded an identical order, thereby validating the use of Stiffness-Mod as a measure of LV stiffness.

All results are given as mean \( \pm \) standard deviation and the level of statistical significance was set at \( P < 0.05 \).

3. Results

3.1. Endomyocardial NOS 2 gene expression and the hemodynamic phenotype of dilated cardiomyopathy

Table 1 summarizes indices of LV systolic and diastolic function observed in the dilated cardiomyopathy study population. Fig. 1 shows representative examples of diastolic portions of LV pressure–volume loops recorded by a LV micromanometer conductance catheter during transient pullback of an inflated balloon into the inferior vena cava. For the dilated cardiomyopathy study population, a significant linear correlation was observed between intensity of NOS2 gene expression and LV diastolic stiffness modulus (Stiffness-Mod) (\( P = 0.003 \); \( r = 0.50 \)). LV stroke work (SW) (\( P = 0.0008 \); \( r = 0.56 \)) and LV ejection fraction (EF) (\( P = 0.004 \); \( r = 0.49 \)) (Fig. 2). In the group of patients in whom a balloon caval occlusion was performed, a significant correlation was also observed between intensity of NOS2 gene expression and LV chamber stiffness constant (\( k \)) (\( P = 0.01 \); \( r = 0.52 \)). In these groups of patients, in whom myocardial collagen volume fraction (CVF) or intensity of myocardial proinflammatory cytokine (TNF\( \alpha \); IL-1\( \beta \); IL-6) gene expression was determined, no correlation was observed between intensity of NOS2 gene expression and CVF or cytokine gene expression. In Fig. 2, most of the data scatter arose from patients with low NOS2 gene expression. In patients with low NOS2 gene expression, the broad range of LV diastolic stiffness-Mod, LVSW and LVEF corresponded with a broad range of LV end-diastolic wall stress (LVEDWS) as shown in Figs. 3 and 4, which plot NOS2 gene expression in function of LVEDWS for different levels of LV diastolic stiffness-Mod or LVEF.

In both Figs. 3 and 4, patients with high LV diastolic stiffness-Mod (>300 kdyne/cm\(^2\)) or low LVEF (<20%) clustered in the upper left hand corner of the graph, patients with intermediate values occupied the middle
3.2. Endomyocardial NOS3 gene expression and the hemodynamic phenotype of dilated cardiomyopathy

For the dilated cardiomyopathy study population, no significant correlations were observed between intensity of NOS3 gene expression and LV diastolic stiffness-Mod, LVSW, LVEF, CVF or intensity of cytokine gene expression. Fig. 5 plots intensity of NOS3 gene expression in function of LVEDWS for different levels of LV diastolic stiffness-Mod. Patients with high (>300 kdyn/cm²) and intermediate LV diastolic stiffness-Mod clustered in the middle portion of the graph and patients with low LV diastolic stiffness-Mod (<150 kdyn/cm²) in the lower portion of the graph. As evident from the insert of Fig. 5, the NOS3/LVEDWS ratio was significantly correlated with the LV diastolic stiffness-Mod \((P=0.0001; \ r=0.58)\). The slope of this linear relation was less steep than the slope of the linear relation between NOS2/LVEDWS versus LV diastolic stiffness-Mod (Fig. 3), consistent with less depression of NOS3 gene expression than of NOS2 gene expression in advanced LV dysfunction. The NOS3/LVEDWS ratio was also significantly correlated with LVEF \((P=0.003; \ r=0.51)\) and with LVSW \((P=0.02; \ r=0.39)\).

3.3. Endomyocardial NOS gene expression and the hemodynamic phenotype of athlete’s heart

All three athletes had slightly elevated LVEDWS because of high LV end-diastolic volume and had low LV stiffness-Mod \((P<0.0001; \ r=0.80)\) or LVEF \((P<0.0001; \ r=0.69)\) to be superior to the correlations shown in Fig. 2. The NOS2/LVEDWS ratio was also more closely correlated with LVSW \((P=0.0002\ and \ r=0.61)\).
diastolic stiffness-Mod. Fig. 3 also shows the data points of the cyclists: the relation between NOS2 gene expression and LVEDWS observed in the athletes coincides with the relation observed in dilated cardiomyopathy patients with low LV diastolic stiffness-Mod and in the insert the athletes fell on the bottom portion of the relation between NOS2/LVEDWS ratio and LV diastolic stiffness-Mod observed in the cardiomyopathy population. The relation between NOS3 gene expression and LVEDWS observed in the athletes also coincided with the relation observed in dilated cardiomyopathy patients with low LV diastolic stiffness-Mod.

4. Discussion

4.1. Endomyocardial NOS and the hemodynamic phenotype of dilated cardiomyopathy

The present study demonstrates lower LV endomyocardial NOS gene expression at a given level of LVEDWS to correspond with a worse hemodynamic phenotype of dilated cardiomyopathy characterized by higher LV stiffness, lower LVSW and lower LVEF. A similar finding was reported by Haywood et al., who found lower endomyocardial NOS2 gene expression in NYHA class IV heart failure than in NYHA class II [17]. Selective downregulation of myocardial gene expression in more advanced stages of LV dysfunction has also been reported for other genes. Lower levels of protooncogenes were observed in peroperative biopsy samples of patients with aortic regurgitation and major LV dysfunction [18] and a fall in expression of gp130 and of interleukin-6 related cardiotrophin-1 was proposed to trigger transition to a maladaptive LV response to hemodynamic overload [19].

Downregulation of endomyocardial NOS gene expression in patients with the worst hemodynamic phenotype could have resulted from altered myocardial expression of proinflammatory cytokines, from reduction of the cardiomyocyte population because of apoptotic cell death, from altered transmission of wall stress to the cardiomyocytes or from altered presence of certain growth factors.

In experimental preparations, proinflammatory cytokines are potent stimulators of myocardial NOS2 gene expression and in patients with dilated nonischemic cardiomyopathy, increased myocardial expression of proinflammatory cytokines has been observed [20]. In a subset of patients, intensity of myocardial gene expression of proinflammatory cytokines was therefore determined. The lack of correlation between cytokines and NOS2 also argues against apoptotic cell death as the cause of the downregulated NOS2 in patients with the worst hemodynamic phenotype. An increase in apoptotic cell death should however be ruled out by looking at specific markers of apoptosis in the biopsy samples, which was not performed in the present study.

The remodeling process of progressive LV cardiomyopathic dysfunction involves increased extracellular matrix degradation and turnover because of upregulation of certain matrix metalloproteinases and downregulation of tissue inhibitors of metalloproteinases [21,22]. This leads to disruption of the collagen weave around individual myocytes and to malalignment or slippage of myocytes [23]. Disruption of the collagen weave could alter transmission of LV wall stress to the cardiomyocytes and disturb wall stress-induced gene expression because of an altered cascade of signals originating from the collagen fibers and descending to sarcolemmal integrins, to cytoskeletal proteins and to nuclear membranes [1]. The lack of correlation in the present study between CVF and intensity of NOS2 gene expression, argues against myocardial fibrosis as the cause of the observed downregulation of NOS2 in patients with the worst hemodynamic phenotype.

Previous studies demonstrated abundant myocardial expression of TGFβ in patients with dilated cardiomyopathy [24]. TGFβ is known to reduce mRNA stability of NOS2 [25] and a high myocardial level of TGFβ is therefore a potential mechanism for the fall in NOS2 mRNA observed in patients with the worst hemodynamic phenotype. Variability in the relative increase of myocardial NOS and TGFβ expression after an elevation of LVEDWS could contribute to the variability of the hemodynamic phenotype of patients with dilated cardiomyopathy in analogy to the variable response of TGFβ to experimental LV pressure or volume overload [26].

In the present study, the intensity of endomyocardial NOS gene expression was higher in cardiomyopathy patients with preserved LVSW and low diastolic LV stiffness-Mod. Patients with this hemodynamic phenotype had an intensity of endomyocardial NOS gene expression comparable to professional athletes. This finding confirmed recent observations in dilated cardiomyopathy patients which failed to demonstrate cardiodepressant effects related to myocardial NO production or to the presence of NOS2 gene expression [27]. Absence of a NO- or NOS2-induced cardiodepressant effect [28,29] was also observed in isolated muscle strips of explanted cardiomyopathic human hearts. In these muscle strips, a NOS inhibitor left baseline isometric tension development unaltered [30]. High myocardial concentrations of NO could also favorably affect the hemodynamic phenotype by blunting growth promoting effects of neurohormones on cardiomyocytes [31] and by preventing expression of a phenotype with depressed Ca++ ATPase activity and LV diastolic dysfunction [32].
4.2. Endomyocardial NOS and the hemodynamic phenotype of athlete’s heart

The present study observed similar levels of endomyocardial NOS expression in patients with mild LV dysfunction and in athletes. In dogs, exercise is known to augment activity of coronary endothelial NOS3 both acutely [33] and chronically as a result of increased NOS3 gene expression [34]. Endomyocardial expression of NOS2 has not been reported previously in athletes or in exercising animals but endomyocardial expression of heat shock proteins and of atrial natriuretic peptide was observed in rats subjected to repetitive daily episodes of treadmill running [35,36]. The observed upregulation of endomyocardial NOS2 gene expression in athletes could therefore have resulted from a myocardial stress response to repetitive exercise-related mechanical stretching. In arterial smooth muscle, cyclic stretch was recently reported to enhance expression of NOS2 and of insulin-like growth factor I (IGF-I) [37]. Transcardiac IGF-I production is also increased in physiological hypertrophy of athlete’s heart [38] and the observed upregulation of NOS2 could counteract mitogenic effects of this enhanced IGF-I production.

The upregulation of endomyocardial NOS2 gene expression in athletes challenges exclusive control of NOS2 gene expression by immune mediators [39,40] and supports a ‘constitutive’ expression of NOS2 [41] susceptible to upregulation by mechanical stress. The abundant myocardial expression of NOS2 in the fetus [42], in hypertrophied [43] and in senescent [44] myocardium also supports the notion of a ‘constitutive’ myocardial expression of NOS2, which gets upregulated during adult life as part of a stress-induced reexpression of the fetal gene programme. An identical myocardial response to exercise- and overload-related stretch was recently also demonstrated by the different ACE genotypes, which induced a similar distribution of LV hypertrophy in response to training and to arterial hypertension [45].

5. Study limitations

The present study correlated the hemodynamic phenotype with endomyocardial NOS mRNA and not with endomyocardial NOS protein, myocardial cGMP concentration or transcardiac nitrite/nitrate production, which would have provided more definite proof of endomyocardial NO activity because of posttranscriptional and post-translational modification of NOS [39]. A previous study in transplant recipients however showed elevated myocardial cGMP and NOS2 protein immunostaining to correlate with NOS2 mRNA expression [46] and in the same patient population higher transcardiac nitrite/nitrate production was recently demonstrated in the presence of upregulated NOS2 gene expression [47]. The limited procurement of endomyocardial tissue by transvascular biopsy did not allow for simultaneous NOS2 immunostaining and determination of myocardial cGMP concentration. The use of transvascular biopsies however provided the advantage of obtaining endomyocardial tissue also from patients with mild LV dysfunction and not only from patients with the worst hemodynamic phenotype referred for cardiac transplantation. It was precisely in the group of patients with mild LV dysfunction that the level of endomyocardial NOS gene expression corresponded to the level observed in athletes.

LV endomyocardial NOS gene expression was derived from a single biopsy sample, which therefore did not take into account spatial heterogeneity of gene expression [41]. In a previous study however, multiple biopsies from different LV sites were obtained in the same patient and in this study [4] the variability of endomyocardial NOS2 and NOS3 gene expression was low (7 and 15%). Spatial nonuniformity of LV wall thickness changes could have influenced Stiffness-Mod calculations but in the subgroup of patients, in whom balloon caval occlusions were obtained, Stiffness-Mod correlated closely with k, an index of global diastolic LV function. The concurrent use of certain medications could also have influenced intensity of endomyocardial NOS gene expression. All cardiomyopathy patients were on ACE inhibitor therapy, which is known to upregulate coronary endothelial NOS3 expression [48] and some patients were on β-blocker therapy, which also upregulates endomyocardial NOS3 [49]. ACE inhibitor therapy could explain the better maintenance of NOS3 than of NOS2 gene expression in the patients with the worst hemodynamic phenotype.

The number of athletes investigated in the present study was limited (n = 3). They came to medical attention because of a history of sustained ventricular arrhythmias. Myocarditis or another cardiomyopathic process could have caused these arrhythmias and could also have influenced their pattern of NOS gene expression. At the time of study however, their hemodynamic phenotype was consistent with athlete’s heart [6] as evident from an enlarged LV end-diastolic volume, a normal LV ejection fraction, a low heart rate and a slight increase in LV wall thickness. Histological examination of their biopsies also failed to reveal any evidence of myocarditis. A major inflammatory or cardiomyopathic contribution to their pattern of NOS gene expression therefore seems unlikely.

6. Conclusions

Dilated cardiomyopathy patients with preserved LVSW and low LV diastolic stiffness have LV endomyocardial NOS2 and NOS3 gene expression equal to professional cyclists and higher than patients with low LVSW and high LV diastolic stiffness. A NO-mediated decrease in LV diastolic stiffness and a concomitant rise in LV preload reserve explains the beneficial effect of higher endo-
myocardial NOS2 and NOS3 gene expression on the hemodynamic phenotype of dilated cardiomyopathy.

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