Role of Myocardial Inducible Nitric Oxide Synthase in Contractile Dysfunction and β-Adrenergic Hyporesponsiveness in Rats With Experimental Volume-Overload Heart Failure

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Background—Whereas nitric oxide (NO) has been implicated in the pathophysiology of heart failure (HF), the significance and functional role of different NO synthase (NOS) isoforms in this pathology are controversial. Our aim was to study in the myocardium of rats with volume-overload–induced HF the expression, activity, and localization of endothelial (eNOS) and inducible (iNOS) isoforms and the involvement of iNOS in depressed cardiac contractile properties, intracellular Ca²⁺ ([Ca²⁺]i) transients, and β-adrenergic hyporesponsiveness.

Methods and Results—HF was induced by aortocaval fistula (ACF). Compensated and decompensated subgroups of HF were selected on the basis of daily sodium excretion. ACF induced cardiac hypertrophy in rats with compensated (36%) and decompensated (76%) HF. Whereas in HF rats, cardiac eNOS expression and activity were unchanged, iNOS expression and activity increased 2-fold. iNOS immunostaining was observed in ventricular myocytes of compensated and decompensated HF rats but not in controls. Isoproterenol-positive inotropic and lusitropic effects were markedly attenuated in papillary muscle of HF rats, more pronouncedly in decompensated than in compensated rats. Isoproterenol-induced increases in the rates of [Ca²⁺]i activation and relaxation were also depressed in ACF rats. Selective iNOS blockade by N-(3-(aminomethyl)benzylacetamidine improved the attenuated β-adrenergic responsiveness in HF rats.

Conclusions—Our findings indicate that myocardial iNOS is activated in rats with volume-overload HF and suggest that increased iNOS activity contributes to depressed myocardial contractility and β-adrenergic hyporesponsiveness. (Circulation. 2002;105:236-243.)

Key Words: heart failure ■ nitric oxide synthase ■ contractility

Nitric oxide (NO) is a ubiquitous signaling molecule that features diverse physiological and pathophysiological actions. In addition to its role as an endothelium-derived vasorelaxant, neurotransmitter, and immunomodulator, NO also modulates cardiac function. The heart expresses all 3 NO synthase (NOS) isoforms: neuronal NOS (nNOS) in sympathetic nerve terminals, endothelial NOS (eNOS) in endothelial cells and cardiomyocytes, and inducible NOS (iNOS), which generates higher NO levels than eNOS, in myocytes stimulated with cytokines. The cardiac effects of NO include negative or positive inotropic effects (depending on the concentration) and attenuated β-adrenergic responsiveness.

Increasing evidence suggests that alterations in NO synthesis are of pathophysiological importance in heart failure (HF), although the evidence concerning the expression and function of different NOS isoforms in HF is controversial. In particular, the role of iNOS in HF pathogenesis has not been clarified. Thus, whereas some authors reported iNOS expression only in septic hearts but not in other types of HF, others demonstrated iNOS expression and activity in HF of various origins. Furthermore, some reports support the notion that excessive NO production contributes to β-adrenergic hyporesponsiveness in HF patients, whereas others disagree.

We have previously used rats with aortocaval fistula (ACF) as a rapidly developing volume-overload cardiomyopathy model characterized by cardiac hypertrophy and neurohormonal and renal consequences that resemble those of HF patients. ACF rats can be subdivided into 2 subgroups: those with compensated and decompensated HF. Compared with the compensated subgroup, decompensated rats feature avid renal sodium retention, marked neurohormonal activation, and a higher degree of cardiac hypertrophy and mortality. Recently, the chemokine monocyte chemoattractant protein 1 (MCP1) was found to be upregulated in the...
myocardium of ACF rats, which suggests that proinflammatory agents may contribute to altered cardiac function in this model.16 Although altered NO activity has been implicated in the pathogenesis of the renal hemodynamic abnormalities in ACF rats,17 the contribution of the NO system to the impaired myocardial function in this model has not been studied. In particular, the possibility that iNOS activation contributes to myocardial dysfunction and deterioration into a decompensated state has not been evaluated.

Hence, our aims were to compare the myocardial expression, activity, and immunoreactivity of eNOS and iNOS in compensated and decompensated rats and to test the effects of selective iNOS inhibition on cardiac contraction and intracellular Ca²⁺ ([Ca²⁺]i) transients under basal conditions and after β-adrenergic stimulation.

Methods

Experimental Model

Experiments were conducted on male Wistar rats (Harlan, Jerusalem, Israel) weighing 270 to 320 g maintained on standard rat diet (0.5% NaCl). HF was induced by surgical creation of an ACF between the abdominal aorta and the inferior vena cava (side to side, outer diameter 1.0 to 1.2 mm).14,17 Sham-operated rats served as controls. Daily urine output and sodium excretion were monitored to establish the daily sodium excretion rate (UNaV), on the basis of which ACF rats were divided into 2 subgroups: compensated (UNaV >1200 μEq/24 hours) and decompensated (UNaV <100 μEq/24 hours). Rats were killed 7 to 9 days after surgery by intraperitoneal injection of Nembutal (60 mg/kg), and hearts were removed, weighed, and treated as described below. All animal handling was approved by the Technion ethics committee for experiments in animals.

Myocardial NO System in HF Rats

Western blot analysis was performed on heart tissue stored at −70°C. iNOS and eNOS antibodies (Transduction Laboratories) were diluted 1:250 for eNOS and 1:500 for iNOS. Macrophage and endothelial cell lysates (Transduction Laboratories) were used as positive controls for iNOS and eNOS, respectively. Blots were visualized by an alkaline phosphatase (AP) substrate kit (Bio-Rad Laboratories). Negative images of blots were analyzed densitometrically. Immunohistochemical analysis was performed on paraffin-embedded heart sections according to the streptavidin-biotin-peroxidase technique with a Histostain Plus Kit (Zymed Laboratories). Positive reactions were visualized as brown precipitates. Ca²⁺-dependent and Ca²⁺-independent NOS activities were measured with the [14C]L-citrulline assay.18

Mechanical Performance and [Ca²⁺]i Handling

Isometric twitch was recorded from right ventricular papillary muscles stimulated at 0.5 Hz.19 [Ca²⁺]i transients and myocyte contraction were recorded from enzymatically dissociated ventricular myocytes.20 To test β-adrenergic responsiveness, the effect of isoproterenol (10⁻⁶ to 10⁻⁷ mol/L) was determined.

iNOS Inhibition

Enzymatic activity of iNOS was blocked by 3 consecutive subcutaneous injections (5, 18, and 28 hours before rats were killed) of 0.2% N-(3-(aminomethyl)benzylacetamide (1400W; 1 mg/kg body weight; Calbiochem-Novabiochem Corporation). A similar protocol was shown to prevent vascular leakage caused by endotoxin.21 To further validate the effectiveness of iNOS blockade, the same protocol was applied to normal rats injected with lipopolysaccharide (Escherichia coli serotype 0111:B4, 10 mg/kg IP). iNOS activity of cardiac tissues from endotoxin-injected rats treated with 1400W was reduced by 60% compared with untreated rats, whereas cardiac eNOS activity was unchanged (data not shown).

Results

Cardiac Hypertrophy in HF Rats

The heart/body weight ratio, an index of cardiac hypertrophy, differed (P<0.05) between the 2 HF subgroups, as indicated by a 37% and 76% increase in compensated (0.435±0.007, n=28) and decompensated (0.556±0.016, n=24) rats, respectively, versus control rats (0.316±0.0053, n=29; P<0.01).

Expression, Activity, and Localization of eNOS and iNOS

Ventricular homogenates from all 3 groups were analyzed for eNOS and iNOS protein immunoreactive levels and enzymatic activity by means of Western blotting and the citrulline assay, respectively. A 140-kDa band was observed in all samples blotted with eNOS antibody (Figure 1A), which corresponds to the known molecular mass of eNOS.22 As shown in Figure 1B, both eNOS immunoreactive protein level and activity were similar in control and HF rats. In contrast, although a 130-kDa band, which corresponds to the molecular mass of iNOS,8 was observed in all ventricular homogenates blotted with iNOS antibody (Figure 2A), the intensity of the iNOS bands was markedly increased in HF rats (Figure 2B). Likewise, iNOS activity was also increased in both HF subgroups compared with controls (Figure 2B).
Figure 3 depicts the immunohistochemical localization of eNOS and iNOS in myocardial sections. eNOS-positive staining was observed in myocytes and endothelial cells from control and HF rats (Figures 3B and 3C). In contrast, whereas iNOS staining was undetectable in control hearts (Figure 3D), a strong positive iNOS reaction was evident in myocytes from compensated and decompensated rats (Figures 3E and 3F). Sections treated with nonimmune serum instead of primary antibody were negative (Figure 3A). In summary, whereas eNOS was unaltered, iNOS was activated in volume-overload–induced HF.

β-Adrenergic Responsiveness: Modulation by iNOS Blockade

Papillary Muscles

Because we have demonstrated iNOS activation in ACF rats, and because excessive NO has been implicated in depressed contraction and β-adrenergic responsiveness,12 we analyzed the effects of selective iNOS inhibition on basal and isoproterenol-stimulated ventricular contraction. As seen in Table 1, twitch tension, maximal rate of tension activation (+dT/dt), and relaxation (−dT/dt) were reduced to 50% (compensated) and 20% (decompensated) of the control values. In decompensated rats, these properties were depressed (P<0.05) compared with compensated rats. In all 3 groups, iNOS blockade with 1400W had no effect on basal twitch characteristics.

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>n</th>
<th>Tension, g/mm²</th>
<th>+dT/dt, g/mm² per s</th>
<th>−dT/dt, g/mm² per s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>9</td>
<td>0.99±0.13</td>
<td>1.62±0.09</td>
<td>0.98±0.06</td>
</tr>
<tr>
<td>Sham-operated+1400W</td>
<td>6</td>
<td>0.98±0.12</td>
<td>1.58±0.27</td>
<td>0.79±0.12</td>
</tr>
<tr>
<td>Compensated HF</td>
<td>7</td>
<td>0.45±0.09*</td>
<td>0.69±0.06†</td>
<td>0.34±0.08†</td>
</tr>
<tr>
<td>Compensated HF+1400W</td>
<td>5</td>
<td>0.55±0.09</td>
<td>0.77±0.28</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>Decompensated HF</td>
<td>5</td>
<td>0.23±0.08†</td>
<td>0.33±0.07‡</td>
<td>0.16±0.03‡</td>
</tr>
<tr>
<td>Decompensated HF+1400W</td>
<td>6</td>
<td>0.37±0.09</td>
<td>0.49±0.07</td>
<td>0.20±0.05</td>
</tr>
</tbody>
</table>

Tension indicates maximal twitch tension; +dT/dt, maximal rate of tension activation; −dT/dt, maximal rate of tension relaxation; and n, number of animals. Data are mean±SEM.

*P<0.05, †P<0.01, ‡P<0.001 vs control.
Figure 4 demonstrates the effects of isoproterenol on the isometric twitch in the 3 experimental groups treated with 1400W and in matched groups of untreated rats. Isoproterenol increased twitch tension, \( +d[T]/dt \); and relaxed twitch tension, \( -d[T]/dt \). Results are expressed as mean±SEM.

Figure 4. Mechanical responsiveness to isoproterenol of papillary muscle preparations from sham-operated, compensated, and decompensated rats; modulation by iNOS blockade with 1400W. A, Maximal twitch tension; B, maximal rate of tension activation, \( +dT/dt \); and C, maximal rate of tension relaxation, \( -dT/dt \). Results are expressed as mean±SEM.

Isolated Ventricular Myocytes

To investigate potential mechanisms underlying the findings described above, we tested the effect of isoproterenol on contraction and [Ca\(^{2+}\)]

isolated ventricular myocytes isolated from untreated and 1400W-treated control, compensated, and decompensated rats. As seen in Table 2, diastolic [Ca\(^{2+}\)], was increased, and the rate of [Ca\(^{2+}\)], relaxation \( (−d[Ca^{2+}] / dt) \) was slower in both HF groups versus control. The rate of [Ca\(^{2+}\)], activation \( (+d[Ca^{2+}] / dt) \) was slower in the decompensated but not in the compensated stage compared with control. Treatment with 1400W increased \( (P<0.05; \text{ie, partially restored}) \) both \( +d[Ca^{2+}] / dt \) and \( −d[Ca^{2+}] / dt \) in decompensated rats. Figure 5 depicts representative [Ca\(^{2+}\)], transients and myocyte shortening traces under basal conditions and during stimulation with a maximal dose of isoproterenol. In isoproterenol-treated myocytes from compensated and decompensated rats, the paced responses were followed by aftercontractions, which probably resulted from delayed afterdepolarizations. As seen in Figure 6, in control myocytes, isoproterenol increased \( +d[Ca^{2+}] / dt \) (Figure 6A), \( −d[Ca^{2+}] / dt \) (Figure 6B), shortening velocity (Figure 6C), and relaxation velocity (Figure 6D) in a dose-dependent manner \( (P<0.001) \). In contrast, as was shown for papillary muscles, \( β \)-adrenergic responsiveness in HF myocytes was attenuated for all 4 parameters studied \( (P<0.01) \) compared with the control group. Moreover, the isoproterenol-induced response was smaller in decompensated HF \( (P<0.001) \) than in compensated HF. Finally, 1400W did not alter the \( β \)-adrenergic responsiveness of control and compensated rats but caused an upward shift \( (P<0.001) \) for all 4 isoproterenol dose-response relations in decompensated HF (Figure 6).

Discussion

The major findings of the present study are as follows: (1) The cardiac immunoreactive level and activity of iNOS but not of eNOS were augmented in rats with compensated and decompensated HF. (2) Ventricular contractility and \( β \)-adrenergic responsiveness were markedly depressed in HF rats, more so in the decompensated than in the compensated stage. (3) Selective iNOS blockade improved the attenuated \( β \)-adrenergic responsiveness, more effectively in decompensated than in compensated rats with HF.

NOS System in HF Rats

Quantitative analyses of eNOS and iNOS immunoreactive level and activity indicate that whereas eNOS was unaltered, myocardial iNOS was increased \( ∼2 \)-fold in both compensated and decompensated HF compared with controls. iNOS immunohistochemical localization suggests that this increase is due, at least in part, to iNOS activation in cardiomyocytes. Our findings are in agreement with previous studies reporting increased iNOS expression and/or activity in failing human myocardium.\(^9,10,23\) De Belder’s group\(^10\) was the first to demonstrate cardiac iNOS activity in patients with idiopathic...
dilated cardiomyopathy. Other studies reported expression of iNOS mRNA and protein in failing myocardium from patients with idiopathic, ischemic, or valvular cardiomyopathy and iNOS protein expression in failing hearts from patients in septic shock but not in other kinds of HF. Furthermore, increased iNOS mRNA and activity were detected in left ventricular tissue of patients with New York Heart Association functional class IV, regardless of the cause of HF. In contrast, Stein et al detected increased expression of eNOS mRNA and protein in patients with idiopathic dilated, ischemic, or postmyocarditis cardiomyopathy and only low iNOS expression in 2 of 30 failing hearts. Similarly, Khadour and coworkers reported increased myocardial eNOS expression and activity in rats and dogs with HF, but no Ca\(^{2+}\)-independent iNOS activity. The reasons underlying these conflicting findings are unknown and may reflect the diverse causes of HF, as well as species differences. The present study demonstrates myocardial iNOS activation in rats with volume-overload–induced HF, a model not considered to be of inflammatory origin. Hence, our observations are in agreement with the “cytokine hypothesis,” which suggests that cytokines play an important pathogenic role in development of HF. This notion is further supported by 2 recent studies demonstrating that iNOS knockout mice display less cardiac dysfunction after myocardial infarction than wild-type controls. Recently, MCP1 expression was found to be markedly upregulated in the myocardium of rats with ACF, which may contribute to iNOS activation. However, such an association remains to be established in future studies.

### Contractile Function and [Ca\(^{2+}\)] Handling in HF Rats

In the present study, we compared the mechanical and [Ca\(^{2+}\)] handling properties of the cardiac muscle in compensated and decompen-sated rats. Isometric twitch measurements show that twitch tension, \(+d[Ca^{2+}]/dt\), and \(-d[Ca^{2+}]/dt\) were markedly reduced in HF rats, being more depressed in the decompen-sated than in the compensated stage. These alterations were associated with increased diastolic [Ca\(^{2+}\)] and decreased rates of [Ca\(^{2+}\)] activation (in decompen-sated rats) and relaxation. Abnormal [Ca\(^{2+}\)] handling was previously described by Hisamatsu et al in rats with volume-overload–induced HF 12 weeks after creation of ACF. In agreement with our findings, both the [Ca\(^{2+}\)] transient and the isometric relaxation were prolonged in ACF rats, whereas intracellular Ca\(^{2+}\)

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**Figure 5.** Effect of isoproterenol on [Ca\(^{2+}\)] transients and contraction of myocytes isolated from sham-operated, compensated, and decompen-sated rats. [Ca\(^{2+}\)] levels are represented by ratio of fura 2 fluorescence at 340 and 380 nm, \(R=F_{340}/F_{380}\). Myocyte contraction, represented by cell length, is expressed in micrometers. For each experimental group, representative traces are shown in control (predrug) conditions and in presence of \(10^{-6}\) mol/L isoproterenol. “Aftercontractions” induced by isoproterenol in compensated and decompen-sated HF are indicated by asterisks.

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**TABLE 2. Summary of Myocyte [Ca\(^{2+}\)] Transient Characteristics in Sham-Operated, Compensated, and Decompensated Rats and in Matched Groups Treated With 1400W**

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>n</th>
<th>Diastolic [Ca(^{2+})]</th>
<th>(+d[Ca^{2+}]/dt) s(^{-1})</th>
<th>(-d[Ca^{2+}]/dt) s(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>44</td>
<td>0.583±0.009</td>
<td>8.431±0.584</td>
<td>1.135±0.123</td>
</tr>
<tr>
<td>Sham-operated+1400W</td>
<td>25</td>
<td>0.595±0.014</td>
<td>9.160±0.618</td>
<td>1.192±0.223</td>
</tr>
<tr>
<td>Compensated HF</td>
<td>43</td>
<td>0.688±0.014*</td>
<td>8.231±0.732</td>
<td>0.645±0.071*</td>
</tr>
<tr>
<td>Compensated HF+1400W</td>
<td>21</td>
<td>0.680±0.022</td>
<td>8.354±0.852</td>
<td>0.690±0.070</td>
</tr>
<tr>
<td>Decompensated HF</td>
<td>27</td>
<td>0.712±0.016†</td>
<td>6.778±0.723*</td>
<td>0.628±0.061*</td>
</tr>
<tr>
<td>Decompensated HF+1400W</td>
<td>22</td>
<td>0.703±0.020</td>
<td>8.360±1.350†</td>
<td>0.955±0.145†</td>
</tr>
</tbody>
</table>

Diastolic [Ca\(^{2+}\)] levels represented as the corresponding fluorescence ratios \((R=F_{340}/F_{380})\). +d[Ca\(^{2+}\)]/dt, rate of [Ca\(^{2+}\)] activation; −d[Ca\(^{2+}\)]/dt, rate of [Ca\(^{2+}\)] relaxation; and n, myocyte number. Data are mean±SEM.

*P<0.01, †P<0.001 vs control, ‡P<0.05 vs decompensated HF.
Figure 6. Summary of effects of isoproterenol on \( \text{[Ca}^{2+} \text{]}_i \) transients and contraction of myocytes isolated from sham-operated, compensated, and decompensated rats; modulation by iNOS blockade with 1400W. A, Rate of \( \text{[Ca}^{2+} \text{]}_i \) activation; B, rate of \( \text{[Ca}^{2+} \text{]}_i \) relaxation; C, myocyte shortening velocity; and D, myocyte relaxation velocity. For all panels, \( P<0.01 \) for compensated (Comp.) versus sham-operated; \( P<0.001 \) for decompensated (Decomp.) versus sham-operated; and \( P<0.001 \) for decompensated versus compensated.
release, uptake, and maximal number of [3H]-ryanodine binding sites were lower than in controls. A decreased rate of [Ca2+]i uptake was also observed in human and canine HF and was shown to result from action potential prolongation and reduced sarcoplasmic reticulum Ca2+ sequestration. The present study suggests that iNOS activation may contribute to [Ca2+]i-handling abnormalities in HF and further demonstrates that selective iNOS blockade increases the rates of [Ca2+]i activation and relaxation in rats with compensated HF but not in those with compensated HF. Because iNOS activity was similarly increased in both HF subgroups, this finding may suggest that compensated rats are more sensitive to the depressant effect of NO on [Ca2+]i, kinetics. Because the rates of [Ca2+]i activation and relaxation are regulated by intracellular cAMP levels, this could involve cAMP as well as phosphodiesterase II–related mechanisms. The mechanism underlying the differential effect of iNOS blockade in compensated and decompensated HF remains to be established in future studies.

Attenuated β-Adrenergic Responsiveness in HF Rats: Modulation by iNOS Blockade
In agreement with related reports, the inotropic and lusitropic effects of isoproterenol were markedly attenuated in HF rats. Importantly, in HF myocytes, isoproterenol frequently generated aftercontractions, which probably resulted from the underlying [Ca2+]i-overload—induced delayed afterdepolarizations, which constitutes the cellular basis of ventricular arrhythmias. Supporting the notion that excessive NO production contributes to the depressed β-adrenergic responsiveness in HF, selective iNOS blockade improved the response to isoproterenol in compensated and decompensated rats but had no effect in control rats. In decompensated rats, iNOS blockade caused an upward shift of isoproterenol dose-response relations for twitch tension, +dT/dt, −dT/dt, the rates of [Ca2+]i, activation and [Ca2+]i, relaxation, and myocyte shortening and relaxation velocities. The alleviating effect of 1400W was less pronounced in compensated rats and was evident at the papillary muscle level for twitch tension and −dT/dt. Thus, although iNOS blockade affected compensated and decompensated rats to a different extent via mechanisms yet to be elucidated, it clearly improved the attenuated β-adrenergic responsiveness in volume-overload HF. Several studies reported improved β-adrenergic responsiveness of the failing myocardium after nonselective blockade of NO activity. Perfusion of myocytes isolated from dogs with rapid pacing—induced HF with NG-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, augmented the inotropic response to isoproterenol. In addition, in humans with left ventricular failure, intracoronary administration of the nonselective inhibitor NG-monomethyl-L-arginine (L-NMMA) improved the β-adrenergic response to dobutamine. However, the experimental strategies used in these studies did not allow the authors to conclude whether their findings were due to suppression of eNOS or iNOS activity. Drexler’s group has shown that L-NMMA enhanced the β-adrenergic inotropic responsiveness of myocardial strips from patients with terminal HF, an effect that was inversely related to cardiac iNOS activity but not to total NOS activity. Thus, Drexler’s findings along with ours suggest that the enhancing effect of NO blockade on β-adrenergic responsiveness was due to iNOS inhibition.

In summary, the present study supports the notion that excessive NO production contributes to β-adrenergic hyporesponsiveness in HF and demonstrates that this effect is apparently due to activation of iNOS. These findings contribute to the understanding of the mechanisms activated in HF and may therefore aid in the development of new therapeutic strategies.

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References


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