Role of Myocardial Inducible Nitric Oxide Synthase in Contractile Dysfunction and \( \beta \)-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 \( \text{Ca}^{2+} \) transients, and \( \beta \)-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 \( \approx 2 \)-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 pronoucnedly in decompensated than in compensated rats. Isoproterenol-induced increases in the rates of \( \text{Ca}^{2+} \) activation and relaxation were also depressed in ACF rats. Selective iNOS blockade by \( 
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**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 \( \beta \)-adrenergic hyporesponsiveness.

**Key Words:** heart failure \( \bullet \) nitric oxide synthase \( \bullet \) 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 \( \beta \)-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 \( \beta \)-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). 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). 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 M) 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 011: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).

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 ventricular homogenates blotted with iNOS antibody (Figure 1A), 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 1: Summary of Papillary Muscle Isometric Twitch 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>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.
Isoproterenol increased twitch tension, \( \frac{dT}{dt} \), and \( \frac{d}{dt} \) twitch tension relaxation, \( \frac{d[dT]}{dt} \), in untreated control rats in a dose-dependent fashion. Compensated rats responded to isoproterenol to a lesser extent, whereas decompen-sated rats were completely unresponsive. In compensated and decompen-sated rats, iNOS blockade improved (to a different degree) the response of twitch tension, \( \frac{dT}{dt} \), and \( \frac{d}{dt} \) twitch tension relaxation, \( \frac{d[dT]}{dt} \) to isoproterenol. Altogether, these findings demonstrate that ventricular contraction and \( \beta \)-adrenergic responsiveness are depressed in HF rats, more so in the decompen-sated than in the compensated group, and that selective iNOS blockade improves the \( \beta \)-adrenergic response of the failing ventricular muscle.

**Isolated Ventricular Myocytes**

To investigate potential mechanisms underlying the findings described above, we tested the effect of isoproterenol on contraction and [Ca\(^{2+}\)] \(_i\) transients in ventricular myocytes isolated from untreated and 1400W-treated control, compensated, and decompen-sated rats. As seen in Table 2, diastolic [Ca\(^{2+}\)] \(_i\) was increased, and the rate of [Ca\(^{2+}\)] \(_i\) relaxation (\( -\frac{d[Ca^{2+}]}{dt} \)) was slower in both HF groups versus control. The rate of [Ca\(^{2+}\)] \(_i\) activation (\( +\frac{d[Ca^{2+}]}{dt} \)) was slower in the decompen-sated but not in the compensated stage compared with control. Treatment with 1400W increased (\( P<0.05 \); ie, partially restored) both \( +\frac{d[Ca^{2+}]}{dt} \) and \( -\frac{d[Ca^{2+}]}{dt} \) in decompen-sated rats. Figure 5 depicts representative [Ca\(^{2+}\)] \(_i\) transients and myocyte shortening traces under basal conditions and during stimulation with a maximal dose of isoproterenol. In isoproterenol-treated myocytes from compensated and decompen-sated rats, the paced responses were followed by aftercontractions, which probably resulted from delayed afterdepolarizations. As seen in Figure 6, in control myocytes, isoproterenol increased \( +\frac{d[Ca^{2+}]}{dt} \) (Figure 6A), \( -\frac{d[Ca^{2+}]}{dt} \) (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, \( \beta \)-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 decompen-sated HF (\( P<0.001 \)) than in compensated HF. Finally, 1400W did not alter the \( \beta \)-adrenergic responsiveness of control and decompen-sated rats but caused an upward shift (\( P<0.001 \)) for all 4 isoproterenol dose-response relations in decompen-sated 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 decompen-sated HF. (2) Ventricular contractility and \( \beta \)-adrenergic responsiveness were markedly depressed in HF rats, more so in the decompen-sated than in the compensated stage. (3) Selective iNOS blockade improved the attenuated \( \beta \)-adrenergic responsiveness, more effectively in decompen-sated 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 decompen-sated 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 group10 was the first to demonstrate cardiac iNOS activity in patients with idiopathic...
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\(_{380}\)).

+P<0.01, †P<0.001 vs control, ‡P<0.05 vs decompensated HF.

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.\(^9\) In contrast, Stein et al\(^8\) 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\(^24\) 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.\(^25\) 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.\(^26,27\) Recently, MCP1 expression was found to be markedly upregulated in the myocardium of rats with ACF, which may contribute to iNOS activation.\(^16\) 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 decompensated rats. Isometric twitch measurements show that twitch tension, +dT/dt, and −dT/dt were markedly reduced in HF rats, being more depressed in the decompensated than in the compensated stage. These alterations were associated with increased diastolic [Ca\(^{2+}\)] and decreased rates of [Ca\(^{2+}\)] activation (in decompensated rats) and relaxation. Abnormal [Ca\(^{2+}\)] handling was previously described by Hisamatsu et al\(^28\) 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\(^{2+}\)
Figure 6. Summary of effects of isoproterenol on [Ca\textsuperscript{2+}]\textsubscript{i} transients and contraction of myocytes isolated from sham-operated, compensated, and decompensated rats; modulation by iNOS blockade with 1400W. A, Rate of [Ca\textsuperscript{2+}]\textsubscript{i} activation; B, rate of [Ca\textsuperscript{2+}]\textsubscript{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.²⁸ A decreased rate of [Ca²⁺], uptake was also observed in human and canine HF and was shown to result from action potential prolongation and reduced sarcoplasmic reticulum Ca²⁺ sequestration.²⁹ The present study suggests that iNOS activation may contribute to [Ca²⁺],-handling abnormalities in HF and further demonstrates that selective iNOS blockade increases the rates of [Ca²⁺], 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⁺], kinetics. Because the rates of [Ca²⁺], 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,³⁰ 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⁺],-handling abnormalities in HF and further demonstrates that selective iNOS blockade increases the rates of [Ca²⁺], 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⁺], kinetics. Because the rates of [Ca²⁺], 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.

In summary, the present study supports the notion that excessive NO production contributes to β-adrenergic hyperresponsiveness 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.

**Acknowledgments**

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**References**


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