Disruption of Inducible Nitric Oxide Synthase Improves β-Adrenergic Inotropic Responsiveness but Not the Survival of Mice With Cytokine-Induced Cardiomyopathy


Abstract—Transgenic (TG) mice with cardiac-specific overexpression of tumor necrosis factor-α develop congestive heart failure. We have previously reported that short-term inhibition of inducible nitric oxide synthase (iNOS) ameliorates β-adrenergic hyporesponsiveness in TG mice. To examine whether long-term inhibition of iNOS may rescue TG mice from developing congestive heart failure, we disrupted iNOS gene by crossing TG mice with iNOS knockout mice. Myocardial levels of iNOS protein were significantly increased in TG mice compared with age- and sex-matched wild-type (WT) mice. No iNOS protein was detected in TG mice with the disruption of iNOS. Myocardial levels of endothelial NOS were not different among these mice. To examine the effects of iNOS disruption on myocardial contraction, left ventricular pressure was measured. In TG mice, +dP/dt\textsubscript{max} was significantly suppressed, and its β-adrenergic responsiveness was blunted. As in the case with short-term inhibition of iNOS, the disruption of iNOS gene improved β-adrenergic inotropic responsiveness in TG mice but not in WT mice. However, the iNOS disruption did not alter myocardial inflammation, ventricular hypertrophy, or the survival of these mice. These results indicate that although myocardial expression of iNOS plays a key role in the attenuation of β-adrenergic inotropic responsiveness, NO-independent mechanisms might be more important in the development of congestive heart failure. (Circ Res. 2002; 90:959-965.)

Key Words: cytokines • nitric oxide synthase • heart failure • tumor necrosis factor • transgenic mice

Recent studies have shown that myocardial production of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), may play an important role in the pathogenesis of cardiac dysfunction in a variety of cardiovascular diseases.\textsuperscript{1,2} TNF-α not only suppresses myocardial contractility\textsuperscript{3,4} but also promotes ventricular remodeling by provoking ventricular hypertrophy,\textsuperscript{5,6} interstitial fibrosis,\textsuperscript{7} and apoptosis.\textsuperscript{8} To investigate the pathophysiological significance of myocardial production of TNF-α, we made transgenic (TG) mice that overexpress TNF-α specifically in the heart.\textsuperscript{9} These mice developed myocardial inflammation with extracellular matrix remodeling, ventricular hypertrophy with four-chamber dilatation, impaired contractility with diminished β-adrenergic inotropic responsiveness, reactivation of the fetal gene program with downregulation of calcium handling genes, and premature death with congestive heart failure.\textsuperscript{9–12} TG mice also demonstrated a substantial amount of apoptosis in the myocardium; however, this was largely isolated to the interstitial cells.\textsuperscript{13} Several aspects of these results have since been confirmed by another laboratory.\textsuperscript{14} Thus, myocardial production of TNF-α may play a critical role in the pathogenesis of congestive heart failure. However, the mechanisms by which TNF-α damages the myocardium remain undefined.

In addition to proinflammatory cytokines, increased production of NO may also play an important part in the pathogenesis of congestive heart failure.\textsuperscript{15,16} NO is a free radical gas synthesized from L-arginine by a family of nitric oxide synthases (NOSs), including neuronal (nNOS), inducible (iNOS), and endothelial isoforms (eNOS). Both nNOS and eNOS are constitutively expressed, whereas iNOS is induced by inflammation, allograft rejection, and cytokine activation. Although a small amount of NO produced by nNOS and eNOS is supposed to be cardioprotective,\textsuperscript{16} a large amount of NO produced by iNOS may be cardiotoxic by suppressing myocardial contractility\textsuperscript{17} and promoting myocardial apoptosis.\textsuperscript{18} It has been shown that a substantial amount of iNOS is induced in the failing human heart.\textsuperscript{19–22} Because TNF-α is a potent inducer of iNOS,\textsuperscript{15} the negative inotropic effect of TNF-α may be mediated in part by the enhanced production of NO in the myocardium. However, conflicting results have been reported regarding the effects of NOS inhibition on cytokine-induced cardiac dysfunction. Some
investigators reported that negative inotropic effects of cytokines were blocked by NOS inhibition, whereas others did not.

We have recently demonstrated that iNOS is induced in the TG myocardium and that short-term pharmacological inhibition of iNOS ameliorates \( \beta \)-adrenergic inotropic hyporesponsiveness in TG mice. Therefore, the purpose of this study was to investigate whether long-term inhibition of iNOS may prevent TG mice from developing congestive heart failure and prolong survival. To ensure complete long-term inhibition of iNOS, we crossed TG mice with iNOS knockout mice. The results indicate that the disruption of iNOS ameliorated \( \beta \)-adrenergic inotropic hyporesponsiveness in TG mice. However, the survival of TG mice was not improved. Thus, NO-independent mechanisms might be more important in the development of congestive heart failure with cytokine overexpression.

**Materials and Methods**

**Animal Model**

TG mice with cardiac-specific overexpression of TNF-\( \alpha \) and wild-type (WT) littermates were studied. To ensure complete inhibition of iNOS activity, we crossed TG mice with iNOS knockout (iNOS\(^{-/-}\)) mice. Mating TG mice with iNOS\(^{-/-}\) mice yielded TG or WT mice (F1). Then, we mated among TG or WT iNOS\(^{-/-}\) mice to obtain TG or WT mice with iNOS\(^{+/+}\), iNOS\(^{-/-}\), or iNOS\(^{-/-}\) (F2). Mating among F2 generation gave F3, and so forth. To homogenize genetic background that is different between TG mice (FVB) and wild-type mice (C57BL/6), the mice after F4 generation were studied in the present study. The genetic background should be an equal mixture of FVB and C57BL/6 mice, in theory. Furthermore, to minimize the effect of genetic background, littermates were studied in each analysis. Each type of hybrid mice was not apparently different in size, behavior, or reproductive ability. This experiment was reviewed by the Committee of the Ethics on Animal Experiment, Kyushu University Graduate School of Medical Sciences, and carried out under the control of the Guideline for Animal Experiment, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

**Western Blot Analysis**

Tissue samples from the left ventricle were homogenized in Tris buffer containing proteinase inhibitors. The protein samples were then separated in a 7.5% resolving gel, blotted onto a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Laboratory), and blocked with blocking buffer (Block Ace, Dainippon-Pharm) at room temperature for 60 minutes. Immunoblotting was performed using a rabbit polyclonal antibody to murine iNOS (1:500 dilution, N30030/Lot 6, Transduction Laboratories). Immunodetection was accomplished with a horseradish anti-rabbit secondary antibody (1:2000 dilution, Amersham) using an enhanced chemiluminescence kit (Amersham). The data were quantified by the densitometry.

**Left Ventricular Pressure Measurement**

Under anesthesia with 2.5% avertin (14 \( \mu L/g \) body weight, IP, Aldrich Chemical Co), mice were placed in a supine position. A 1.4F micromanometer catheter (Millar Instruments) was inserted into the left ventricle through the right carotid artery. Left ventricular pressure was then recorded at baseline and 2 minutes after low and high doses of isoproterenol (0.02 \( \mu g \) and 0.5 \( \mu g \), IP).

**Echocardiography**

Echocardiographic studies were performed using an ultrasonographic system (ALOKA SSD-5500) as previously reported. After anesthetization with 2.5% avertin (14 \( \mu L/g \) body weight, IP, Aldrich Chemical Co), mice were placed in a supine position. A 7.5-MHz transducer was applied to the left hemithorax. Two-dimensional targeted M-mode imaging was obtained from the short-axis view at the level of the greatest left ventricular dimension at baseline and 2 minutes after isoproterenol injection (0.5 \( \mu g \) IP). M-mode measurements of left ventricular end-diastolic and end-systolic diameter and left ventricular anterior and posterior wall thickness were made using the leading-edge convention of the American Society of Echocardiography. End diastole was determined at the maximal left ventricular diastolic dimension, and end systole was taken at the peak of posterior wall motion. The percentage of left ventricular fractional shortening (LVFS) was calculated as LVFS (\%) = (LVEDD - LVESD)/LVEDD x 100, where LVEDD and LVESD indicate left ventricular end-diastolic and end-systolic dimension, respectively.

**Pathological Analysis**

After measurement of body and heart weight, tissues were fixed in 10% neutral buffered formalin for hematoxylin and eosin staining or snap-frozen in liquid nitrogen for RNA and protein analysis.

**RNase Protection Assay**

Total RNA was extracted from the left ventricle by an acid guanidium thiocyanate-phenol chloroform method. Multiprobe RNase protection assay (RPA) was performed according to the manufacturer’s protocol (RiboQuant, PharMingen). A custom template set containing murine TNF-\( \alpha \), interleukin-1\( \beta \) (IL-1\( \beta \)), transforming growth factor-\( \beta \) (TGF-\( \beta \)), RANTES, monocyte chemotactic protein-1 (MCP-1), and GAPDH was applied.

**Statistical Analysis**

The results are presented as mean \( \pm \) SD. One-way ANOVA with Student-Newman-Keuls test was used in Figures 1 and 6 and Tables 1 and 2. Two-way ANOVA with repeated measures was used in Figures 2 and 3. Kaplan-Meier survival analysis was used in Figure 4. Mann-Whitney test was used in Figure 5. Differences were considered to be statistically significant at \( P<0.05 \).

**Results**

**Induction of iNOS in TG Myocardium**

Myocardial levels of eNOS and iNOS protein were evaluated by Western blot analysis (Figure 1). There was no difference in myocardial levels of eNOS protein between WT and TG.
mice irrespective of the iNOS disruption. In contrast, iNOS protein was significantly increased in TG mice with intact iNOS gene. As expected, iNOS protein was not detected in TG or WT mice with the iNOS disruption.

**Improved β-Adrenergic Inotropic Responsiveness by iNOS Disruption**

A catheter-tip micromanometer was used to assess in vivo cardiac function. Female mice at the age of 10 weeks were studied. Hemodynamic parameters at baseline are summarized in Table 1. There was no difference in heart rate, systolic blood pressure, or left ventricular end-diastolic pressure (LVEDP) between WT and TG mice. Nevertheless, the maximum rate of left ventricular pressure development (+dP/dt max) was significantly decreased in TG mice (P<0.001), indicating the reduction of baseline contractility. The peak rate of pressure fall (−dP/dt min) was also lower in TG mice, although it did not reach statistical significance (P=0.06). The disruption of iNOS gene did not change baseline heart rate, systolic blood pressure, LVEDP, +dP/dt max, or −dP/dt min in TG or WT mice. Thus, induction of iNOS may not contribute to the development of cardiac dysfunction at baseline.

To evaluate adrenergic hemodynamic responsiveness, graded doses of isoproterenol were administered. As shown in Figure 2, isoproterenol injections increased heart rate in TG and WT mice. Systolic blood pressure remained the same, whereas LVEDP was significantly decreased in a dose-dependent manner. In contrast, the increase in +dP/dt max in response to isoproterenol was significantly blunted in TG compared with WT mice. Although the iNOS disruption did not affect +dP/dt max in WT mice, it significantly improved β-adrenergic inotropic responsiveness in TG mice. Similarly, the adrenergic lusitropic responsiveness was also blunted in TG mice. However, the iNOS disruption did not alter −dP/dt min in WT or TG mice. Thus, the inhibition of iNOS ameliorated β-adrenergic inotropic hyporesponsiveness in TG mice without enhancing baseline contractility or lusitropic responsiveness.

To confirm the results obtained by pressure measurement, we performed echocardiographic studies in another group of 10-week-old female mice. As summarized in Table 2, both end-diastolic and end-systolic diameters were significantly larger and fractional shortening was significantly lower in TG (P<0.01). The presence or absence of iNOS gene did not affect baseline echocardiographic parameters in WT or TG mice. Isoproterenol injection increased heart rate, decreased end-diastolic diameter, and improved fractional shortening, as shown in Figure 3. Although the increase in heart rate in TG mice was not different from that in WT mice, the improvement of fractional shortening was significantly blunted in TG mice (P<0.01). The iNOS disruption did not affect β-adrenergic chronotropic or inotropic responsiveness in WT mice. However, it significantly improved fractional shortening after isoproterenol injection in TG mice (P<0.005). These echocardiographic results are consistent with those obtained by pressure measurement in vivo and those of our previous study with short-term inhibition of iNOS by a selective inhibitor, ONO-1714.27

**iNOS Disruption Does Not Improve Survival**

Shown in Figure 4 are survival function curves of TG mice with or without intact iNOS gene. Male TG mice died

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**TABLE 1. Hemodynamic Parameters at Baseline**

<table>
<thead>
<tr>
<th></th>
<th>WT/iNOS ++</th>
<th>WT/iNOS −−</th>
<th>TG/iNOS ++</th>
<th>TG/iNOS −−</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>428±16</td>
<td>432±36</td>
<td>393±30</td>
<td>421±14</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>89.0±7.7</td>
<td>91.3±7.4</td>
<td>87.7±7.1</td>
<td>86.2±10.1</td>
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<tr>
<td>LVEDP, mm Hg</td>
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<td>1.6±1.8</td>
<td>2.0±1.9</td>
<td>2.1±2.5</td>
</tr>
<tr>
<td>+dP/dt max, mm Hg/s</td>
<td>597±156</td>
<td>6050±867</td>
<td>4900±441*</td>
<td>4743±822*</td>
</tr>
<tr>
<td>−dP/dt min, mm Hg/s</td>
<td>−454±412</td>
<td>−4750±691</td>
<td>−4225±392</td>
<td>−4143±929</td>
</tr>
</tbody>
</table>

TG indicates TNF-α transgenic mice; WT, wild-type mice; HR, heart rate; SBP, systolic blood pressure; and LVEDP, left ventricular end-diastolic pressure. Values are mean±SD. *P<0.001 vs WT/iNOS ++.
younger than females as reported previously: the mean survival time was 15.8 weeks (95% CI: 11.5–20.0, n=51) in TG male mice and 36.9 (33.1–40.7, n=77) in TG female mice. None of WT mice with or without iNOS gene died spontaneously before the age of 6 months. We hypothesized that the iNOS disruption might delay the development of congestive heart failure in TG mice. However, the results indicated that the disruption of iNOS gene did not prolong the survival of TG mice: the mean survival time with iNOS disruption was 19.3 weeks (13.5–25.0, n=52) in TG male mice and 36.7 (31.0–42.3, n=84) in TG female mice. All of the TG mice that died spontaneously presented exceptional dilatation of the heart, increased lung weight, and pleural effusion, suggesting that they died of congestive heart failure.

Effects on Ventricular Hypertrophy and Inflammation

We examined gross pathology and histology of the heart of TG and WT mice at the age of 10 weeks. All the mice were females. As shown in Figure 5, TG ventricles weighed significantly more than those of WT mice. The iNOS disruption did not affect ventricular weight in WT or TG mice. TG mice that died spontaneously (8.1±3.0 weeks old) demonstrated marked ventricular hypertrophy irrespective of the iNOS disruption. Thus, the induction of iNOS did not participate in the development of ventricular hypertrophy in TG mice.

As previously reported, TG mice had more infiltrating interstitial cells in myocardium than WT mice. The disruption of iNOS gene did not alter the histology of the heart in TG or WT mice (data not shown). To evaluate myocardial expression of cytokines, multiprobe RPA was performed (Figure 6). Transcription levels of TNF-α, IL-1β, RANTES, and MCP-1 were significantly upregulated in the TG myocardium as previously reported. The iNOS disruption did not affect the expression of these cytokines in TG or WT mice. These results indicate that the disruption of iNOS may not affect the myocardial interstitial infiltration provoked by TNF-α overexpression.

Discussion

In the present study, we demonstrated that the disruption of iNOS gene improved β-adrenergic inotropic responsiveness in transgenic mice with cardiac-specific overexpression of TNF-α. However, the iNOS disruption did not alter myocardial inflammation, ventricular hypertrophy, or the survival of these mice. These results indicate that although myocardial expression of iNOS plays a key role in the attenuation of β-adrenergic inotropic responsiveness in cytokine-induced
cardiomyopathy, NO-independent mechanisms might be more important in the development of congestive heart failure.

Effects of iNOS Inhibition on Myocardial Contractility

One of the characteristics of the failing human heart is attenuated inotropic responsiveness to β-adrenergic stimulation. This finding has generally been attributed to downregulation of β-adrenergic receptors and/or alterations in G proteins. Chung et al have demonstrated that TNF-α uncouples the β-adrenergic receptor from adenylyl cyclase via an effect on the G inhibitory protein. Recent studies suggest that enhanced production of NO may be an additional mechanism that may contribute to β-adrenergic hyporesponsiveness of the failing heart. 

Our previous report demonstrated that short-term inhibition of myocardial NO by overexpression of eNOS inhibitor ONO-1714 augments the positive inotropic response to β-adrenergic stimulation in TG but not in WT mice. In the present study, we disrupted iNOS gene to investigate long-term effects of iNOS inhibition on the development of cytokine-induced cardiomyopathy. As in the case with short-term iNOS inhibition, the disruption of iNOS gene improved β-adrenergic inotropic responsiveness in TG but not in WT mice, suggesting that myocardial induction of iNOS may play a pivotal role in the development of β-adrenergic inotropic hyporesponsiveness in cytokine-induced cardiomyopathy.

In contrast, the iNOS disruption did not affect the baseline contraction or relaxation in WT or TG mice. It has been reported that inotropic effects of NO may be biphasic. High levels of NO are shown to suppress myocardial contractility, whereas low levels of NO seem to exert positive inotropic effects. Because TNF-α is a potent inducer of iNOS, we hypothesized that myocardial dysfunction of TG mice was mediated by induction of iNOS in the myocardium. However, the disruption of iNOS gene did not improve the baseline contractility. Furthermore, NO is shown to improve diastolic properties of the failing human heart. Thus, we had expected that the iNOS disruption might deteriorate relaxation or distensibility of TG myocardium. However, the disruption of iNOS gene did not affect diastolic properties in TG or WT mice.

Therefore, we conclude that the induction of iNOS does not play a major role in maintaining baseline inotropy or lusitropy in this mouse model of cardiomyopathy with TNF-α overexpression. These findings are consistent with early work in isolated cardiomyocytes with cytokine-induced iNOS expression, which exhibited no change in baseline contractile shortening but a significant alteration in β-adrenergic response that was restored with L-arginine. Because positive effects were observed only after β-adrenergic stimulation, it is important to evaluate myocardial function not only at baseline but also under stressed conditions to assess the effects of iNOS inhibition.

Recent studies have clearly demonstrated that stimulation of β-adrenoceptor increases NO production via eNOS and inhibits myocardial contractility in a NO-dependent manner. The disruption of eNOS gene is shown to augment both inotropic and lusitropic responsiveness to β-adrenergic stimulation, which is in contrast with the results of the present study that disrupted iNOS gene. To be precise, the disruption of iNOS gene did not change β-adrenergic inotropic or lusitropic responsiveness in WT mice, in which cardiac contractility was preserved and iNOS was not upregulated. In contrast, the iNOS disruption improved inotropic but not lusitropic responsiveness in TG mice, in which cardiac contractility was impaired and iNOS was upregulated. Chronotropic responsiveness to β-adrenergic stimulation was not affected by eNOS or iNOS disruption. These results indicate that eNOS is involved in the physiological negative-feedback control of inotropic and lusitropic responsiveness to β-adrenergic stimulation, whereas iNOS affects only adrenergic inotropic but not lusitropic responsiveness of the failing heart with iNOS induction. Although both eNOS and iNOS produce NO in the myocardium, their effects on cardiac contractility are not the same.
Effects of iNOS Inhibition on Developing Heart Failure

Excessive production of NO by iNOS may be involved in the remodeling process of the failing heart by provoking cardiac myocyte apoptosis. Indeed, the iNOS disruption reduced apoptotic cardiac myocyte death and improved left ventricular function and mortality rate after myocardial infarction. Furthermore, apoptosis by TNF-α is shown to be mediated by induction of iNOS in cardiac myocytes. Thus, we had expected that the iNOS disruption would reduce apoptosis in the heart of TG mice and prolong survival. On the contrary, another study has demonstrated that NO inhibits TNF-α–induced apoptosis by reducing the generation of ceramide. NO is also shown to attenuate hypertrophic effects of adrenergic agonists on cardiac myocytes. Furthermore, blockade of iNOS activity may affect other inflammatory pathways, including the cyclooxygenase system. Therefore, it is also possible that the disruption of iNOS might accelerate ventricular hypertrophy and apoptosis and aggravate congestive heart failure. However, in the present study, neither hypothesis was supported. The iNOS disruption did not change the survival or ventricular hypertrophy of TG mice, which indicates that the effects of iNOS induction on ventricular remodeling and heart failure in this mouse model of cardiomyopathy are neutral.

We observed a marked difference in the survival rate between male and female TNF-α transgenic mice. Because the extent of myocardial expression of TNF-α has been shown to be comparable in both sexes, this is probably due to higher expression of TNF receptors in the myocardium of male transgenic mice. The sex difference in the expression of myocardial TNF receptors is also documented in wild-type FVB mice. Echocardiographic analysis has demonstrated that impairment of baseline myocardial contractility and attenuation of β-adrenergic inotropic responsiveness are more severe in male transgenic mice. In the present study, we examined only female mice for physiological, pathological, and biochemical analyses. This is because more than half of male transgenic mice had died before the age of 10 weeks when these analyses were performed. As a result, we are not certain whether the disruption of iNOS gene improves β-adrenergic inotropic responsiveness even in male transgenic mice with advanced myocardial dysfunction. In any case, the iNOS disruption did not improve the survival in either sex.

It is well recognized that TNF-α induces iNOS in various types of cells and tissues as we observed in the present study. Conversely, Kalra et al recently reported that NO provokes myocardial expression of TNF-α through a CGMP-dependent pathway. Thus, TNF-α and NO may construct a positive-feedback loop, which facilitates inflammatory reactions in the myocardium. However, in the present study, we have demonstrated that myocardial expression of cytokines are not affected by the disruption of iNOS gene. This is partly because the expression of TNF-α in TG mice is driven by α-myosin heavy chain promoter that is supposed to be NO-independent.

The results of the present study are similar to those of endotoxemia in iNOS knockout mice. The disruption of iNOS gene ameliorated endotoxin-induced myocardial dysfunction but did not affect induction of proinflammatory cytokines or the survival of these mice. These results suggest that although negative inotropic effects of proinflammatory cytokines are mediated by the iNOS induction, at least in part, cytopathic effects of these cytokines are predominantly brought by NO-independent pathways. Furthermore, Heger et al have recently reported that a massive cardiac-specific overexpression of iNOS in transgenic mice is not associated with deleterious effects on cardiac hemodynamics and energetics and does not result in heart failure, supporting the view that iNOS induction may only be one factor of a complex set of pathomechanisms leading to heart failure.

In summary, the disruption of iNOS gene ameliorated β-adrenergic inotropic hyporesponsiveness in TG mice. However, the survival of TG mice was not improved. Thus, myocardial expression of iNOS may play a minimal role in the development of congestive heart failure in cytokine-induced cardiomyopathy. Mechanisms by which TNF-α provokes premature cardiac death still remain undefined.

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