Involvement of inducible nitric oxide synthase in cardiac dysfunction with tumor necrosis factor-α

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Funakoshi, Hajime, Toru Kubota, Yoji Machida, Natsumi Kawamura, Arthur M. Feldman, Hiroyuki Tsutsui, Hiroaki Shimokawa, and Akira Takeshita. Involvement of inducible nitric oxide synthase in cardiac dysfunction with tumor necrosis factor-α. Am J Physiol Heart Circ Physiol 282: H2159–H2166, 2002. First published February 14, 2002; 10.1152/ajpheart.00872.2001.—Transgenic (TG) mice with cardiac-specific overexpression of tumor necrosis factor (TNF)-α develop dilated cardiomyopathy with myocardial inflammation. The purpose of this study was to investigate the role of nitric oxide (NO) in this mouse model of cardiomyopathy. Female TG and wild-type mice at the age of 10 wk were studied. The expression and activity of inducible NO synthase (iNOS) were significantly increased in the TG myocardium, whereas those of endothelial NOS were not altered. The majority of the iNOS protein was isolated in the interstitial cells. The selective iNOS inhibitor (1S,5S,6R,7R)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane hydrochloride (ONO-1714) was used to examine the effects of iNOS induction on myocardial contractility. Echocardiography and left ventricular pressure measurements were performed. Both fractional shortening and the maximum rate of rise of left ventricular pressure were significantly suppressed in TG mice. Although ONO-1714 did not change hemodynamic parameters or contractility at baseline, it significantly improved β-adrenergic inotropic responsiveness in TG mice. These results indicate that induction of iNOS may play an important role in the pathogenesis of cardiac dysfunction in this mouse model of cytokine-induced cardiomyopathy.

cytokine; heart failure; transgenic mice

Tumor necrosis factor (TNF)-α is a proinflammatory cytokine that is involved in a variety of cardiovascular diseases, including endotoxin shock, acute myocarditis, cardiac allograft rejection, myocardial infarction, and congestive heart failure (8, 23). Recent studies have indicated that the heart itself is a source of TNF-α in these disorders (11, 21, 30, 31). To investigate the pathophysiological role of myocardial production of TNF-α, we generated transgenic (TG) mice that overexpress TNF-α specifically in the heart under the control of an α-myosin heavy chain promoter (20). These mice developed ventricular hypertrophy, ventricular dilatation, interstitial infiltrates, interstitial fibrosis, attenuation of adrenergic inotropic responsiveness, and reexpression of atrial natriuretic factor in the ventricle. Furthermore, the mice that died spontaneously demonstrated exceptional dilatation of the heart, organized atrial thrombus, and massive pleural effusion, suggesting that they died of congestive heart failure. Several aspects of these results have since been confirmed by another laboratory (4). These results indicate that myocardial production of TNF-α may play an important role in the pathogenesis of cardiac dysfunction. However, the mechanisms by which TNF-α damages the myocardium remain undefined.

Recent basic and clinical studies have shown that nitric oxide (NO) exerts versatile effects on cardiovascular function (1, 16, 18). NO is a free radical gas synthesized from L-arginine by a family of NO synthases (NOS), including neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS) isoforms. Both nNOS and eNOS are constitutively expressed, whereas iNOS is induced by inflammation, allograft rejection, and cytokine activation (16). Recent studies have indicated that iNOS is increased in the failing human heart (6, 11, 30). A small amount of NO produced by nNOS and eNOS seems cardioprotective by improving myocardial perfusion and inhibiting apoptosis (17). In contrast, a large amount of NO produced by iNOS may be cardiotoxic by suppressing myocardial contractility (18) and promoting apoptosis (17). Because TNF-α is a potent inducer of iNOS (16), the negative inotropic effect of TNF-α may be mediated 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 have reported that negative inotropic effects of cytokines were ameliorated by NOS inhibition (3, 9, 10, 27), whereas others did not (25, 33).

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The present study was thus designed to investigate the role of NO in our mouse model of cardiomyopathy caused by cardiac-specific overexpression of TNF-α. The results indicate that iNOS was highly induced in the myocardium, playing an important role in the pathogenesis of cardiac dysfunction caused by cardiac-specific overexpression of TNF-α.

**METHODS**

**Animal model.** TG mice with cardiac-specific overexpression of TNF-α (19, 20, 22) and wild-type (WT) littermates were studied. All the mice were 10-week-old female mice unless otherwise mentioned. We did not use TG males because they did not tolerate physiological experiments due to the premature development of congestive heart failure (15). 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.

**Northern blot analysis.** Total RNA was extracted from the left ventricle (LV) using an acid guanidium thiocyanate-phenol chloroform method. RNA samples (10 μg) were electrophoresed with a formaldehyde-agarose gel and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). The membrane was then hybridized with the following 32P-labeled probes: nNOS, nucleotides 2,582–3,169 (GenBank D14552); iNOS, nucleotides 235–744 (GenBank U43428); and eNOS, nucleotides 2,882–3,287 (GenBank U53142); and 18S rRNA (19, 20). The radioactivity of hybridized bands was quantified by a MacBAS Bioimage Analyzer (Fuji Film; Tokyo, Japan). The results of the cDNA hybridization were normalized to those of the 18S probe to correct for differences in RNA mass and efficiency of transfer. Data were in turn normalized to the mean of the WT samples, arbitrarily set at 1.

**Western blot analysis.** Tissue samples from the LV were homogenized in Tris buffer containing proteinase inhibitors. The protein samples were then separated with a 7.5% resolving gel, blotted onto a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad), and blocked with blocking buffer (Tris-HCl, pH 7.6). Counterstaining was then performed with Mayer’s hematoxylin.

**Plasma levels of nitrate + nitrite.** Plasma levels of nitrate + nitrite (NOx) were measured by the modified chemiluminescence method of Radomski et al. (29) with a NO analyzer (model 270B, Sievers) according to the manufacturer’s instruction.

**Echocardiography.** Echocardiographic studies were performed using an ultrasonicographic system (ALOKA SSD-5500; Tokyo, Japan) as previously reported (15, 19). After anesthetization with 2.5% Avertin (14 μg/g body wt ip, Aldrich Chemical), mice were placed in a supine position. A 7.5-MHz transducer (ALOKA) 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 LV dimension at baseline and 2 min after low and high doses of isoproterenol (0.02 and 0.5 μg ip). M-mode measurements of LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and LV anterior and posterior wall thicknesses were made using the leading edge convention of the American Society of Echocardiography. End diastole was determined at the maximal LV diastolic dimension, and end systole was taken at the peak of posterior wall motion. The percentage of LV fractional shortening (LVFS) was calculated as follows: LVFS (in %) = (LVEDD – LVESD)/LVEDD × 100.

**LV pressure measurements.** After anesthetization with 2.5% Avertin (14 μg/g body wt ip, Aldrich Chemical), mice were placed in a supine position. A 1.4-Fr micromanometer catheter (Millar Instruments) was inserted into the LV through the right carotid artery. LV pressure was then recorded at baseline and 2 min after low and high doses of isoproterenol (0.02 and 0.5 μg ip).

iNOS inhibition with ONO-1714. A novel cyclic amidine analog, [1S,5S,6R,7R]-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane hydrochloride (ONO-1714, Ono Pharmaceutical; Osaka, Japan) (26), was used to inhibit iNOS activity. ONO-1714 was found to be 10-fold selective for human iNOS (inhibitory constant (Ki) = 1.88 nM) over human eNOS (Ki = 18.8 nM). The inhibitory effect of ONO-1714 on iNOS was found to be 451- and 34-fold more selective for iNOS than L-NMMA and aminoguanidine, respectively (26). In terms of the selectivity for human iNOS, ONO-1714 was ~34- and 2-fold more selective for iNOS than L-NMMA and aminoguanidine, respectively. Echocardiography and LV pressure measurements were performed 20 min after anesthetization with 2.5% Avertin (14 μg/g body wt ip, Aldrich Chemical).
after intravenous injections of ONO-1714 (0.1 mg/kg). Phosphate-buffered saline was used as the vehicle.

Statistical analysis. The results are presented as means ± SD. Student’s t-test was used to compare each variable between TG and WT in Fig. 1. One-way ANOVA with Student-Newman-Keuls test was used in Fig. 3. Two-way ANOVA with repeated measures was used in Figs. 4 and 5. Two-way ANOVA without repeated measures was used in Tables 1 and 2. Differences were considered to be statistically significant at \( P < 0.05 \).

RESULTS

Induction of iNOS in the heart. Northern blot analysis demonstrated that both eNOS and iNOS transcripts were present in WT myocardium and significantly upregulated in TG myocardium (\( P < 0.01 \) vs. WT; Fig. 1A). Although we observed nNOS transcripts in the brain homogenate, we did not detect any of them in the myocardium of WT or TG mice (data not shown).

Western blot analysis was also performed to see whether the changes in the transcript levels correlated with changes in protein levels. Contrary to the transcript levels, there was no difference in the protein levels of eNOS between WT and TG mice, although iNOS protein was significantly upregulated in TG mice, consistent with the increase in the iNOS transcript (Fig. 1B).

We assessed NOS activities in the myocardium using an arginine-citrulline converting assay. There was no difference in calcium-dependent NOS activity, whereas calcium-independent activity was significantly increased in the TG myocardium (Fig. 1C). Because eNOS activity is calcium dependent and that of iNOS is calcium independent, the result of the arginine-citrulline converting assay was consistent with that of the Western blot analysis. To identify the cell types that express iNOS protein, we stained WT and TG myocardium with an anti-iNOS antibody. As shown in Fig. 2, most of the iNOS-positive cells were infiltrating cells, including macrophages, lymphocytes, and fibroblasts, although some staining was also observed in myocytes and endothelial cells. Polymorphonuclear granulocytes were rarely seen, as previously reported (20).

iNOS inhibition by ONO-1714. We used the selective iNOS inhibitor ONO-1714 to investigate the pathophysiological significance of iNOS induction in TG myocardium. To confirm its efficacy in mice, we first injected two different doses of ONO-1714 into the retroorbital venous plexi of WT mice (0.1 or 0.3 mg/kg) while monitoring LV pressure with a catheter-tipped micromanometer. The systolic LV pressure was not affected by 0.1 mg/kg of ONO-1714, whereas it was significantly elevated by the dose of 0.3 mg/kg (data not shown). Thus we chose the dose of 0.1 mg/kg in the present study to minimize the inhibition of eNOS by ONO-1714.

To see whether this dose of ONO-1714 inhibits iNOS activity, we then administered lipopolysaccharide (LPS; 10 mg/kg ip) to WT mice in the presence or

Fig. 1. Expression and activity of nitric oxide synthase (NOS) in the ventricle of tumor necrosis factor (TNF)-α transgenic mice (TG) and age-matched wild-type (WT) mice. A: Northern blot analysis; B: Western blot analysis; C: NOS activity measured by an arginine-citrulline converting assay. eNOS, endothelial NOS; iNOS, inducible NOS. Values are means ± SD; \( n = 5 \) each. *\( P < 0.01 \) vs. WT mice.
absence of ONO-1714 (0.1 mg/kg). Blood samples were collected 6 h after the injection. As shown in Fig. 3, plasma levels of NOx were significantly increased after LPS injection in WT mice \((P < 0.001)\). This increase was significantly attenuated by concomitant injection of ONO-1714 \((P < 0.05)\), presumably due to the inhibition of iNOS. Although this dose of ONO-1714 did not change systolic blood pressure, it significantly decreased plasma levels of NOx even in the absence of LPS in WT mice \((P < 0.05)\). We also measured plasma levels of NOx in TG mice (Fig. 3). Despite the induction of iNOS in the myocardium, plasma levels of NOx were no greater than those in WT mice. Because TG mice were able to increase plasma levels of NOx in response to LPS, the reason why TG mice did not achieve a higher NOx concentration in plasma is probably because iNOS induction and TNF-α overexpression were limited to the myocardium. ONO-1714 decreased plasma levels of NOx as it did in WT \((P < 0.05)\).

**Echocardiography.** Echocardiographic parameters at baseline are summarized in Table 1. Wall thickness was significantly greater in TG than WT mice \((P < 0.005)\), reflecting the ventricular hypertrophy in TG mice (biventricular weight/body wt: 3.9 ± 0.2 mg/g in WT mice; 4.6 ± 0.2 mg/g in TG mice, \(P < 0.001)\). Although end-diastolic diameter was not different between WT and TG mice, end-systolic diameter was significantly larger and fractional shortening was significantly lower in TG mice \((P < 0.005)\). Because there were no differences in heart rate or in end-diastolic diameter between WT and TG mice, the difference in fractional shortening indicates reduced baseline contractility in TG. Treatment with ONO-1714 did not change the baseline echocardiographic parameters in either group. Thus inhibition of iNOS did not ameliorate cardiac dysfunction at baseline in TG mice.

To evaluate adrenergic inotropic responsiveness of the myocardium, graded doses of isoproterenol were administered in the presence or absence of ONO-1714. As shown in Fig. 4, left, isoproterenol treatment increased heart rate, decreased end-diastolic diameter, and augmented fractional shortening in a dose-dependent manner in WT mice. Treatment with ONO-1714 did not affect any of these parameters. The comparable results in TG mice are summarized in Fig. 4, right. Although the effect of β-adrenergic stimulation on heart rate and end-diastolic diameter in TG mice did not differ from that in WT mice, that of fractional shortening was significantly blunted in TG mice \((P < 0.01)\). Treatment with ONO-1714 did not alter changes in heart rate or in end-diastolic diameter in response to isoproterenol. However, it significantly enhanced isoproterenol-induced fractional shortening in TG mice \((P < 0.05)\).

**LV pressure measurements.** To confirm the results of echocardiography, we also performed LV pressure measurements using a catheter-tipped micromanometer. As summarized in Table 2, there were no differ-
ences in heart rate, systolic blood pressure, or LV end-diastolic pressure (LVEDP) between WT and TG mice. However, the maximum rate of rise of ventricular pressure (+dP/dt max) was significantly lower in TG mice (P < 0.05), suggesting the presence of reduced ventricular contractility at baseline in TG mice. The peak rate of pressure fall (−dP/dt min) was also reduced in TG mice, suggesting impaired relaxation. ONO-1714 did not change the baseline heart rate, systolic blood pressure, LVEDP, +dP/dt max, or −dP/dt min in either group.

As shown in Fig. 5, isoproterenol injections increased heart rate with a slight elevation in systolic blood pressure in TG mice as well as in WT mice. LVEDP was decreased in a dose-dependent manner. The increase in +dP/dt max in response to isoproterenol was blunted in TG mice compared with WT mice. Although ONO-1714 did not affect +dP/dt max in WT mice, it significantly improved β-adrenergic inotropic responsiveness in TG mice. These results were consistent with those of echocardiography. Thus inhibition of iNOS ameliorated β-adrenergic inotropic hypersensitivity in TG mice without improving cardiac dysfunction at baseline. Although NO has been shown to improve diastolic properties of the failing human heart (13), ONO-1714 did not deteriorate relaxation or distensibility of TG myocardium.

**DISCUSSION**

Recent studies have shown that myocardial production of proinflammatory cytokines, especially TNF-α, may play an important role in the pathogenesis of cardiac dysfunction in a variety of cardiovascular diseases (8, 23). However, the mechanisms by which the cytokines cause myocardial dysfunction remain undefined. Because TNF-α is a potent inducer of iNOS (16), it has been postulated that negative inotropic effects of TNF-α may be mediated by enhanced production of NO in the myocardium. Indeed, some studies have demonstrated that negative inotropic effects of TNF-α were almost completely blocked by NOS inhibition (9, 10). However, subsequent studies did not observe the same effects, suggesting that negative inotropism of TNF-α is NO independent (25, 33). Because all the studies were performed in acute settings, it was not clear whether NO contributes to the cardiac dysfunction provoked by chronic exposure to proinflammatory cytokines. Therefore, in the present study, we investigated NO in TG mice with cardiac-specific overexpression of TNF-α (20). The results demonstrated that iNOS inhibition improved β-adrenergic inotropic responsiveness, although it did not change myocardial contractility at baseline.

As expected from the previous reports (16), iNOS was induced in the myocardium of TNF-α TG mice. Increased iNOS activity in the myocardium was confirmed by an arginine-citrulline assay. Immunohistochemical staining indicated that most of iNOS was produced in the infiltrating interstitial cells. Previous reports have demonstrated that TNF-α induces iNOS in cardiac myocytes (3) and that isolated myocytes from the failing heart make enough NO to cause cardiac dysfunction (2). However, we observed little iNOS staining in the myocytes of TNF-α TG mice. Because the TNF-α TG mice we studied were too young and myocardial dysfunction was too mild to develop overt congestive heart failure, it is possible that cardiac myocytes might express high levels of iNOS if we had studied older mice with pulmonary congestion or systemic hypotension. Nonetheless, it is conceivable that

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<th>Table 1. Echocardiographic parameters at baseline</th>
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<td><strong>WT + Vehicle</strong></td>
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<td><strong>HR, beats/min</strong></td>
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<td><strong>AWT, mm</strong></td>
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<td><strong>EED, mm</strong></td>
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Values are means ± SD; n = no. of mice. WT, wild type; TG, transgenic; HR, heart rate; AWT, anterior wall thickness; PWT, posterior wall thickness; EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening. *P < 0.005, TG vs. WT mice.

Fig. 4. Echocardiographic parameters after the injection of isoproterenol. **A:** HR, heart rate [in beats/min (bpm)]; **B:** EDD, end-diastolic diameter; **C:** FS, fractional shortening. Values are means ± SD. *P < 0.05 vs. vehicle.
overexpression of TNF-α alone may not be potent enough to induce iNOS in cardiac myocytes in vivo.

In contrast, eNOS protein or activity was not changed by TNF-α overexpression, although eNOS transcript level was significantly upregulated. This finding was contrary to a previous report that showed downregulation of eNOS in human umbilical vein endothelial cells treated with TNF-α (34). The disparity between our results and those of previous studies might be due to differences in the experimental models (e.g., in vivo vs in vitro measurements) and/or the cell types examined.

We used the selective iNOS inhibitor ONO-1714 to inhibit NOS activity in the present study. ONO-1714 is one of the most potent and selective iNOS inhibitors currently available; ONO-1714 is 10-fold more selective for iNOS than for eNOS, whereas L-NMMA is 0.3-fold and aminoguanidine is 4.8-fold (26). We chose intravenous injection of ONO-1714 at the dose of 0.1 mg/kg because this dose of ONO-1714 did not change systemic blood pressure or heart rate but significantly inhibited NOS activity. However, the dose of ONO-1714 used in the present study also decreased plasma levels of NOx even in the absence of LPS injection (see Fig. 3). This suggests that eNOS activity was also inhibited to some extent without consequent changes in systemic blood pressure or, alternatively, that there was some constitutive iNOS activity in the untreated WT mice. Indeed, Fig. 1C demonstrated that calcium-independent NOS activity was present in the myocardium of WT mice.

One of the characteristics of the failing human heart is hyporesponsiveness 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. (5) demonstrated that calcium-activated protein kinase C uncouples the β-adrenergic receptor from adenylyl cyclase via an effect on 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. For instance, Hare et al. (12) reported that inhibition of cardiac NO by L-NMMA augments the positive inotropic response to β-adrenergic stimulation in patients with heart failure due to idiopathic cardiomyopathy but not in control subjects with normal LV function. In a canine model of heart failure with rapid ventricular pacing, we observed that NOS inhibition potentiated the positive inotropic response to β-adrenergic stimulation without affecting impaired sartorius mechanics at baseline (32). Furthermore, in the explanted recipient heart after cardiac transplantation, Drexler et al. (7) clearly demonstrated that β-adrenergic responsiveness was inversely related to iNOS activity and augmented by NOS inhibition with L-

### Table 2. Hemodynamic parameters at baseline

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<th>WT + Vehicle</th>
<th>WT + ONO-1714</th>
<th>TG + Vehicle</th>
<th>TG + ONO-1714</th>
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<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>HR, beats/min</td>
<td>395 ± 56</td>
<td>385 ± 36</td>
<td>371 ± 59</td>
<td>382 ± 48</td>
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<tr>
<td>MAP, mmHg</td>
<td>66.3 ± 1.2</td>
<td>65.5 ± 2.5</td>
<td>65.7 ± 1.3</td>
<td>65.0 ± 2.2</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>74.8 ± 1.8</td>
<td>77.2 ± 5.0</td>
<td>79.2 ± 3.6</td>
<td>76.4 ± 2.6</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>1.4 ± 1.2</td>
<td>1.0 ± 1.2</td>
<td>1.4 ± 1.2</td>
<td>1.0 ± 1.2</td>
</tr>
<tr>
<td>+dP/dt\text{max}, mmHg/s</td>
<td>7,060 ± 733</td>
<td>6,960 ± 712</td>
<td>6,400 ± 969*</td>
<td>6,200 ± 565*</td>
</tr>
<tr>
<td>−dP/dt\text{min}, mmHg/s</td>
<td>−5,680 ± 593</td>
<td>−5,480 ± 576</td>
<td>−4,760 ± 727*</td>
<td>−5,040 ± 329*</td>
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Values are mean ± SD; n = no. of mice. MAP, mean arterial pressure; SBP, systolic blood pressure; LVEDP, left ventricular end-diastolic pressure. +dP/dt\text{max}, maximum rate of rise of left ventricular pressure; −dP/dt\text{min}, peak rate of left ventricular pressure fall. *P < 0.05, TG vs. WT mice.

Fig. 5. Hemodynamic parameters after the injection of isoproterenol in the presence or absence of ONO-1714. A: HR; B: systolic blood pressure (SBP); C: left ventricular end-diastolic pressure (LVEDP); D: maximum rate of rise of left ventricular pressure (+dP/dt\text{max}); E: peak rate of left ventricular pressure fall (−dP/dt\text{min}). Values are means ± SD. *P < 0.001 vs. vehicle.
NMMA. These findings are consistent with the present results, indicating that enhanced iNOS activity in the failing heart may be responsible at least in part for the impaired inotropic responsiveness to β-adrenergic stimulation but not for the decreased myocardial contractility at baseline.

Only inotropic, not chronotropic, responsiveness was attenuated in TNF-α TG mice. Because we did not measure the plasma or myocardial concentrations of isoproterenol after each injection, we are unable to rule out the possibility that the blunted inotropic responsiveness could be the consequence of increased degradation and/or decreased peritoneal resorption of isoproterenol in TNF-α TG mice. However, if this is the case, it is difficult to explain why only the inotropic but not the chronotropic responsiveness was blunted in TNF-α TG mice. Furthermore, ONO-1714 augmented inotropic responsiveness without affecting the adrenergic chronotropism. If the effect of ONO-1714 was mediated by modulation of isoproterenol utilization, it should have augmented the chronotropic responsiveness as well. Thus the attenuation of β-adrenergic inotropic responsiveness in TNF-α TG mice appears to come about at the myocyte level through undefined iNOS-dependent mechanisms at least in part.

In contrast, the contractile state at baseline was not back to that of WT mice by ONO-1714. Thus there seems to be an intrinsic difference in the contractile state between WT and TG mice that is NO independent. Indeed, the myocardial dysfunction in TNF-α TG mice is accompanied with activation of the fetal gene program, including the isoform shift of myosin heavy chain, induction of atrial natriuretic peptide in the ventricle, and downregulation of sarcoplasmic reticulum proteins (19). TG mice also developed myocardial hypertrophy and interstitial fibrosis (22), which may not be immediately reversed by iNOS inhibition. Therefore, it might not be surprising that acute inhibition of iNOS did not improve the myocardial dysfunction at baseline. However, it may be too judicious to conclude that myocardial induction of iNOS was irrelevant to myocardial contractility at baseline. It is possible that the inhibition of iNOS by ONO-1714 might be inadequate to restore myocardial dysfunction at baseline. Additionally, although we have shown that this dose of ONO-1714 inhibits systemic production of NO in response to LPS injection, we did not provide any evidence that it indeed inhibited cardiac iNOS activity in vivo. Furthermore, myocardial dysfunction observed in the present study was not severe enough to develop overt congestive heart failure. If we had studied older TG mice with distinct cardiac dysfunction, the results might have been different. However, once the mice develop congestive heart failure, they do not tolerate general anesthesia for physiological measurements. Thus we studied only young mice with compensated hemodynamics in the present study. Taken together, we are unable to conclude that the iNOS induction in the myocardium does not affect the baseline contractility from the present results alone.

As is the case with other organ-specific TNF-α overexpression models (4, 24, 28), our mice developed marked infiltration of inflammatory cells in the myocardium. Because most of iNOS was localized in infiltrating interstitial cells, it is plausible that NO produced by those inflammatory cells may have made myocytes irreversible to β-adrenergic stimulation. However, in patients with idiopathic dilated cardiomyopathy, most of iNOS was localized in cardiac myocytes in the failing heart (11, 30), where interstitial inflammatory cells, including macrophages and T lymphocytes, are increased (14). Therefore, the mechanism by which iNOS was induced in the myocardium might be different between idiopathic dilated cardiomyopathy and TNF-α TG mice. To distinguish direct effects of TNF-α on cardiac myocytes from those mediated by recruited inflammatory cells, further studies are needed.

In conclusion, this study demonstrates that acute inhibition of iNOS potentiates the positive inotropic response to β-adrenergic stimulation in TG mice with cardiac-specific overexpression of TNF-α. This finding suggests that increased production of NO by upregulated iNOS predominantly in interstitial inflammatory cells may be of functional significance in the pathogenesis of cytokine-induced cardiomyopathy.

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