Pathophysiologically Relevant Concentrations of Tumor Necrosis Factor-α Promote Progressive Left Ventricular Dysfunction and Remodeling in Rats

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Background—Although patients with heart failure express elevated circulating levels of tumor necrosis factor-α (TNF-α) in their peripheral circulation, the structural and functional effects of circulating levels of pathophysiologically relevant concentrations of TNF-α on the heart are not known.

Methods and Results—Osmotic infusion pumps containing either diluent or TNF-α were implanted into the peritoneal cavity of rats. The rate of TNF-α infusion was titrated to obtain systemic levels of biologically active TNF-α comparable to those reported in patients with heart failure (~80 to 100 U/mL), and the animals were examined serially for 15 days. Two-dimensional echocardiography was used to assess changes in left ventricular (LV) structure (remodeling) and LV function. Video edge detection was used to assess isolated cell mechanics, and standard histological techniques were used to assess changes in the volume composition of LV cardiac myocytes and the extracellular matrix. The reversibility of cytokine-induced effects was determined either by removal of the osmotic infusion pumps on day 15 or by treatment of the animals with a soluble TNF-α antagonist (TNFR:Fc). The results of this study show that a continuous infusion of TNF-α led to a time-dependent depression in LV function, cardiac myocyte shortening, and LV dilation that were at least partially reversible by removal of the osmotic infusion pumps or treatment of the animals with TNFR:Fc.

Conclusions—These studies suggest that pathophysiologically relevant concentrations of TNF-α are sufficient to mimic certain aspects of the phenotype observed in experimental and clinical models of heart failure. (Circulation. 1998;97:1382-1391.)

Key Words: heart failure ■ remodeling ■ contractility ■ cytokines

Despite repeated observations that patients with heart failure express elevated circulating levels of TNF-α in their peripheral circulation, the clinical and functional significance of this finding remains unknown.1–6 Although the elaboration of TNF-α in patients with heart failure was originally suggested to be a potential biochemical mechanism that was responsible for the cachexia that occurs in this syndrome,7 it is also known that overexpression of this proinflammatory cytokine can produce LV dysfunction, pulmonary edema, and cardiomyopathy in human subjects.7–9 These latter observations have prompted the thought that overproduction of TNF-α may contribute to disease progression in heart failure by virtue of the direct toxic effects that this molecule exerts within the heart and circulation.10–12 Nonetheless, the countervailing point of view that has been raised is that the elaboration of TNF-α in heart failure may simply represent an epiphenomenon; that is, TNF-α is a marker of “disease severity” that has little or no bearing on the progression of the disease process itself.

Central to the validation of the so-called “cytokine hypothesis” for heart failure10,11 is a clear appreciation of the structural and functional effects of pathophysiologically relevant concentrations of TNF-α on the heart. Whereas previous experimental studies have examined either the effects of a single bolus infusion of TNF-α on cardiac structure and function13–17 or alternatively, the effects of a continuous infusion of TNF-α on noncardiac metabolism,18–21 thus far no previous study has examined the effects of continuous infusion of TNF-α on the cardiac structure or function. Accordingly, the purpose of this study was to examine the effects of a continuous infusion of pathophysiologically relevant concentrations of TNF-α on cardiac structure (remodeling) and function in rats. The results of this study suggest that TNF-α, when infused continuously at levels that occur in clinical heart failure, is sufficient to produce changes in cardiac structure and function that mimic certain aspects of the phenotype observed in experimental and clinical models of heart failure.
Methods

TNF-α Infusion Model
Experiments were performed on adult Sprague-Dawley rats (250 to 350 g) of either sex maintained on a diet of standard rat chow and water. Rats were anesthetized with 25 mg/kg pentobarbital IP, followed by implantation of an osmotic minipump (Alzet model 2002, Alza Corp) in the peritoneal cavity. The osmotic minipumps were filled either with recombinant human TNF-α and L-NMMA at 1% rat albumin, which was used as a carrier, or with 1% rat albumin alone (diluent). In preliminary control experiments using TNF-α infusion rates of 0.125 to 7.5 μg · kg⁻¹ · min⁻¹, we established that an infusion rate of 2.5 μg · kg⁻¹ · min⁻¹ resulted in systemic levels of biologically active TNF-α comparable to those reported in patients with heart failure, that is, ~80 to 100 U/mL. Accordingly, this infusion rate was chosen for the studies described below. Control animals received an infusion of diluent at a comparable rate. Animals treated with a continuous infusion of TNF-α or diluent were observed for 15 days, after which they were reanesthetized with 25 mg/kg pentobarbital IP, the osmotic minipumps were surgically removed, and the animals were observed for an additional 15 days as they recovered. Thus, four groups of animals were examined: (1) diluent treatment for 15 days, (2) diluent treatment for 15 days followed by surgical removal of the osmotic minipumps and evaluation for an additional 15 days, (3) TNF-α (2.5 μg · kg⁻¹ · min⁻¹) treatment for 15 days, and (4) TNF-α (2.5 μg · kg⁻¹ · min⁻¹) treatment for 15 days followed by surgical removal of the osmotic minipumps and evaluation for an additional 15 days.

Characterization of the Experimental TNF-α Infusion Model
To characterize the levels of biologically active TNF-α in the experimental model, we examined tail blood samples on days 0 to 15 after implantation of the osmotic infusion pumps in the diluent and TNF-α-treated animals. TNF-α bioactivity was measured with an L929 cell bioassay exactly as we have described previously. The specificity of the cell toxicity in the L929 assay was confirmed with a neutralizing anti-TNF-α antibody, as described. The continuous-infusion model was also characterized hemodynamically by determining the heart rate and tail cuff blood pressure on days 0, 5, 10, 15, 20, 25, and 30 for the diluent- and TNF-α–treated animals. For the hemodynamic studies, the animals were lightly anesthetized with 15 to 25 mg/kg pentobarbital IP and then allowed to recover for 15 to 30 minutes before hemodynamic measurements were performed.

Effect of a Continuous Infusion of TNF-α on LV Function
LV function was characterized in vivo by measurement of the extent of LV fractional shortening on days 0, 5, 10, 15, 20, 25, and 30 of the protocol. Echocardiographic images were obtained in lightly anesthetized animals with a 7-MHz neonatal transducer interfaced with an Acuson XP-10 machine. Two-dimensionally targeted M-mode echocardiograms were obtained from short-axis views of the left ventricle at the mid–papillary muscle levels. Images were stored on ½-in VHS tape and were stored for playback. LV cavity measurements were measured at peak systole (LV ESD) and at end diastole (LV EDD) between the endocardial surfaces at the interventricular septum and the posterior wall according to the leading-edge technique of the American Society of Echocardiography. The extent of fractional shortening (%) was determined as [(LV EDD–LV ESD)/LV EDD] × 100.

Effect of a Continuous Infusion of TNF-α on Cardiac Myocyte Function
Isolated cell mechanics were examined in cells that had been obtained from the hearts of animals treated continuously with diluent for 15 days or with TNF-α for 15 days, or treated with TNF-α for 15 days and then allowed to recover for 15 days after removal of the osmotic infusion pumps. Rodent cardiac myocytes were isolated as described previously. Isolated cell mechanics were performed with video edge detection methodology, with experimental conditions and stimulation protocols identical to those we have described previously. To determine whether the negative inotropic effects of TNF-α in isolated cardiac myocytes were sensitive to inhibition of NOS, we studied isolated cardiac cell motion in myocytes from TNF-α–treated rats in the presence and absence of two different NOS inhibitors (30 minutes of pretreatment): L-NMMA or L-NAME (range, 1 to 100 μmol/L for both). On the basis of a recent report from this laboratory that showed that activation of the neutral sphingomyelinase pathway is responsible for mediating the immediate negative inotropic effects of TNF-α in vitro, we also studied cell motion in myocytes from TNF-α–treated rats after pretreating the cells (30 minutes) with 1 μmol/L NOE, which effectively inhibits TNF-α–induced activation of the neutral sphingomyelinase pathway.

Effect of a Continuous Infusion of TNF-α on LV Structure
LV Structure In Vivo
Two-dimensionally targeted M-mode echocardiograms were used to measure LV EDDs and LV wall thickness during infusion with TNF-α or diluent on days 0, 5, 10, 15, 20, 25, and 30 of the protocol. LV EDD and LV posterior wall thickness were obtained at the mid–papillary muscle level by the leading-edge convention of the American Society of Echocardiography. LV mass (LVM) was determined from the echocardiographic images by the uncorrected cube assumption formula: LVM = (LV EDD + PW + AW)³ − (LV EDD)³, where PW and AW are posterior and anterior wall thicknesses (in millimeters), respectively.

LV Morphology
At the time of terminal study, TNF-α– and diluent-treated animals were euthanized with a lethal injection of 50 mg/kg ketamine, and their hearts and lungs were removed. The following parameters were measured: body weight (g), heart weight (wet), lung weight (wet-to-dry ratio), and heart weight/tibial length. Tissues were perfusion-fixed with a buffered sodium cacodylate solution containing 2% glutaraldehyde solution (pH 7.4, 325 osm) for 20 minutes at a perfusion pressure of 100 mm Hg. Four interrelated studies were performed to delineate the effects of a continuous infusion of TNF-α or diluent on myocardial structure. First, hematoxylin–eosin–stained myocardial tissues were examined for the presence or absence of contraction band necrosis, areas of fibrosis, and the presence and absence of leukocytic infiltrates. Morphological analyses were performed by an experienced veterinary pathologist (E.C.) who was blinded with respect to the nature of the protocol that was performed. Second, cardiac myocyte cross-sectional area was calculated from endocardial and epicardial myocardial sections obtained at the LV mid–papillary muscle level according to the methods described by Spina et al. Briefly, hematoxylin–eosin–stained LV myocardial sections were mounted on an inverted microscope (IM-35, Zeiss), and cardiac myocytes were imaged at a magnification of ×1000 with an epifluorescence illuminator equipped with a rhodamine filter. Myocytes in a cross-sectional orientation were digitized and analyzed with an image analysis system (Sigma ScanImage, Jandel). Only those myocytes in which the nucleus was centrally located within the cell were digitized and analyzed so as to ensure that the

Selected Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EDD</td>
<td>End-diastolic dimension</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>L-NAME</td>
<td>Nω-nitro-l-arginine methyl ester</td>
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<tr>
<td>L-NMMA</td>
<td>Nω-monomethyl-l-arginine</td>
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<tr>
<td>LV</td>
<td>Left ventricular</td>
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<tr>
<td>NOE</td>
<td>L-NAME</td>
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<tr>
<td>NO</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>LVM</td>
<td>Left ventricular mass</td>
</tr>
<tr>
<td>PW</td>
<td>Posterior wall thickness</td>
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<tr>
<td>AW</td>
<td>Anterior wall thickness</td>
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LV = left ventricle
NOE = nitric oxide synthase
TdT = terminal deoxynucleotidyl transferase
TNF = tumor necrosis factor

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short axis of the cardiac myocyte was perpendicular to the microscope objective. Third, we determined the relative myocardial volume of the extracellular matrix using the picrosirius red technique. Briefly, LV myocardial sections were embedded in paraffin and stained with picrosirius red according to the methods described by Spina et al. LV myocardial sections were imaged with an inverted microscope (Zeiss Inc) at a final magnification of ×640. Digitized images of the picrosirius red-stained myocardial sections were analyzed on a computer (Matrox Imaging Products), with large epicardial vessels carefully excluded from the analysis. Three random fields were measured from each LV myocardial section; each sample field was 12 288 μm² in area. Final results were expressed as the percent area of myocardium that stained picrosirius red. In addition, we examined the structure of the fibrillar collagen extracellular matrix with scanning electron microscopy. Briefly, LV myocardial samples were flash-frozen in liquid nitrogen and freeze-fractured. The freeze-fractured samples (0.25×0.25 cm) were then dehydrated and critical-point dried (Ladd Research Inc). The samples were mounted on 10×10-mm stubs with conductive adhesive tape (Scotch commercial tape, 3M Inc) and sputter-coated with gold (Hummer II, Technics). The sections were examined in a JOEL JSM-25S scanning electron microscope at an accelerating voltage of 15 kV. Finally, we used two separate methods to quantify the number of cardiac myocyte nuclei with DNA strand breaks in animals treated (15 days) with either diluent or TNF-α: (1) the TdT assay and (2) a recently described PCR-based method for detecting apoptotic cells based on ligation of a double-strand probe to the section, which is more specific for double-strand DNA damage than the TdT assay. TdT labeling was performed with a commercially available kit (TACS In situ Apoptosis Detection Kit, Trevigen, Inc) exactly according to the manufacturer’s suggestions, with streptavidin–horseradish peroxidase as the conjugate and TACS blue label as the reporter system; Kenechotrol nuclear fast red stain (15 to 20 seconds) was used as the counterstain. Immediately after TdT DNA end-labeling, the slides were photographed and the number of apoptotic myocyte nuclei was determined by enumeration of the labeled nuclei per unit area (10 000 μm²) of myocardium. A total of 30 randomly selected fields per heart (mid–papillary muscle) were examined from the endocardium to the epicardium. To determine the fraction of myocyte nuclei that were labeled, we determined the total number of myocyte nuclei per unit area of the myocardium (10 000 μm²); final results were expressed as the number of positively labeled nuclei per 10⁵ myocytes.

Insofar as the TdT technique will label single-strand DNA breaks that are not indicative of apoptosis, as well as double-strand breaks in necrotic tissue, we also used a recently described ligation based method that labels double-strand DNA breaks either with blunt ends or with single base pair overhangs, which are thought to be more characteristic of the double-strand DNA breaks that occur in apoptosis. The ligase method was performed exactly according to the method described by Didenko and Hornsby, using digoxigenin-labeled DNA probes and an alkaline phosphatase reporter system described by Didenko and Hornsby, using digoxigenin-labeled DNA probes and an alkaline phosphatase reporter system. Briefly, LV myocardial sections were flash-frozen in liquid nitrogen and freeze-fractured. The freeze-fractured samples (0.25×0.25 cm) were then dehydrated and critical-point dried (Ladd Research Inc). The samples were mounted on 10×10-mm stubs with conductive adhesive tape (Scotch commercial tape, 3M Inc) and sputter-coated with gold (Hummer II, Technics). The sections were examined in a JOEL JSM-25S scanning electron microscope at an accelerating voltage of 15 kV. Finally, we used two separate methods to quantify the number of cardiac myocyte nuclei with DNA strand breaks in animals treated (15 days) with either diluent or TNF-α: (1) the TdT assay and (2) a recently described PCR-based method for detecting apoptotic cells based on ligation of a double-strand probe to the section, which is more specific for double-strand DNA damage than the TdT assay.

Results

Characterization of the Model: Systemic Levels of TNF-α

Fig 1 depicts the circulating levels of biologically active TNF-α after the implantation of the TNF-α osmotic infusion pumps. Bioactive levels of TNF-α were detectable in the peripheral circulation by day 3, attained maximal levels of 400 to 800 U/mL by day 5 to 7, and then began to decline back down to baseline values by day 15 (P < .001 by ANOVA). Whether the decrease in circulating TNF-α levels represents the characteristics of the pump or whether this represents the formation of rat autoantibodies against the recombinant human TNF-α cannot be determined from the present studies. Post hoc ANOVA testing was used to assess mean differences from baseline in the TNF-α– and diluent-treated animals with a Dunnett’s test or between experimental groups with a Newman-Keuls test. Significant differences were said to exist at P < .05.

Characterization of the Myocardial Cell: Morphology and Function

LV Myocyte Number

Cardiac myocyte volume was computed based on a cylindrical frame of reference using the myocyte cross-sectional area data and the myocyte length computations obtained from the isolated cardiac myocyte experiments. The total LV myocardial volume was computed by dividing LV mass by the specific gravity of muscle tissue (1.06 g/mL). Total myocyte number was computed from the LV myocardial volume and the morphometrically determined isolated myocyte volume. The total number of cardiac myocytes across the LV free wall was determined by stereological principles and was calculated from the myocyte cross-sectional area and the LV wall thickness measured by two-dimensional echocardiography.

Myocardial Rescue Using a Dimeric TNF Antagonist

Previous in vitro studies from this laboratory have shown that a chimeric fusion protein, TNFR:Fc (Immunex Corp) is sufficient to “rescue" isolated contracting cardiac myocytes from the negative inotropic effects of TNF-α. TNFR:Fc is a chimeric fusion protein consisting of the extracellular domain of the type 2 TNF receptor (p75) fused in duplicate to the Fc portion of the IgG1 molecule. To determine whether the effects of TNF-α on LV structure and function could also be rescued in vivo, we treated rats that had received a continuous infusion of 2.5 μg kg⁻¹ min⁻¹ of TNF-α for 7 days with 1.5 mg/kg of TNFR:Fc. The effects of a single injection of TNFR:Fc on cardiac structure and function were monitored by examination of the extent of LV fractional shortening (%) and LV EDD for an additional 8 days after injection of TNFR:Fc (ie, days 7 to 15). To monitor the circulating levels of TNFR:Fc, we used an ELISA (R & D Systems) that is specific for the human p75 TNF receptor and does not cross-react with the rodent p75 TNF receptor.

Statistical Analysis

Each value is expressed as a mean ± SEM. Two-way ANOVA was used to test for mean differences in the extent of LV fractional shortening, LV EDD, heart rate, and blood pressure between the diluent- and TNF-α–treated animals. One-way ANOVA was used to assess differences in cell shortening. When appropriate, post hoc ANOVA testing was used to assess mean differences from baseline in the TNF-α– and diluent-treated animals with a Dunnett’s test or between experimental groups with a Newman-Keuls test. Significant differences were said to exist at P < .05.

Characterization of the Model: Systemic Levels of TNF-α

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Results
The extent of fractional shortening returned to baseline values by day 20 (5 days after removal of the pump) and was not significantly different from the values obtained in diluent-treated animals by day 30 of the protocol (P=.52).

**Effects of a Continuous TNF-α Infusion on Cardiac Myocyte Function**

Fig 3 shows three salient findings with respect to the studies of isolated cell mechanics in myocytes that were obtained from the hearts of animals treated with diluent or TNF-α. First, compared with myocytes isolated from the diluent-treated rats, there was a significant ~25% to 30% decrease in the extent of cell shortening in the myocytes obtained from the rats treated with TNF-α for 15 days. Second, the decrease in cell shortening in the myocytes from the TNF-α-treated rats was not sensitive to inhibition of NO despite use of two different NO inhibitors, L-NAME and L-NMMA (100 μM each), consistent with a previous report from this laboratory in isolated cardiac myocytes.25 In contrast, the decrease in cell shortening in the myocytes from TNF-α-treated rats was partially sensitive to inhibition by NOE, an inhibitor of the neutral sphingomyelinase pathway,27 consistent with a previous report from this laboratory that showed that activation of the neutral sphingomyelinase pathway was responsible for the negative inotropic effects of TNF-α in isolated cardiac myocytes.29 Third, the extent of cell shortening in the myocytes that had been exposed to a continuous infusion of TNF-α for 15 days and then were allowed to recover for an additional 15 days was not different from control values. One-way ANOVA indicated that there were significant overall differences in the extent of cell shortening within groups (P<.0001); post hoc ANOVA testing (Dunnett’s test) indicated that there were significant differences from control values for cells that had been isolated from the TNF-α-treated rats in either the presence or absence of L-NMMA or L-NAME (P<.05), whereas the extent of cell shortening was not significantly different (P>.05) from control for the cells that had been treated with TNF-α for 15 days and then allowed to recover for 15 days after removal of the TNF-α

**TABLE 1. Hemodynamic Effects of TNF-α**

<table>
<thead>
<tr>
<th></th>
<th>Diluent (n=19)</th>
<th>TNF-α (n=38)</th>
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<tbody>
<tr>
<td>Heart rate, bpm</td>
<td></td>
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<tr>
<td>Day 0</td>
<td>348±16</td>
<td>354±13</td>
</tr>
<tr>
<td>Day 5</td>
<td>370±6</td>
<td>321±10</td>
</tr>
<tr>
<td>Day 10</td>
<td>340±6</td>
<td>391±9</td>
</tr>
<tr>
<td>Day 15</td>
<td>342±9</td>
<td>385±6</td>
</tr>
<tr>
<td>Day 20</td>
<td>379±7</td>
<td>381±6</td>
</tr>
<tr>
<td>Day 25</td>
<td>362±10</td>
<td>335±11</td>
</tr>
<tr>
<td>Day 30</td>
<td>354±9</td>
<td>327±12</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>103±2</td>
<td>107±1</td>
</tr>
<tr>
<td>Day 5</td>
<td>97±6</td>
<td>94±3</td>
</tr>
<tr>
<td>Day 10</td>
<td>101±3</td>
<td>98±5</td>
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<td>Day 15</td>
<td>104±2</td>
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<td>Day 20</td>
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<tr>
<td>Day 25</td>
<td>98±2</td>
<td>96±2</td>
</tr>
<tr>
<td>Day 30</td>
<td>98±2</td>
<td>95±3</td>
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Data are mean±SEM. Two-way ANOVA suggested that there was no significant difference (P>.1 for both) between or within groups with respect to heart rate or mean arterial blood pressure in the diluent- and TNF-α–treated rats.

**Figure 2. Effect of continuous infusion of TNF-α on LV function in vivo.** LV function was studied for 15 days in rats that underwent implantation of intraperitoneal osmotic infusion that contained either diluent (n=20) or TNF-α (n=38). After 15 days, osmotic infusion pumps were removed from diluent- (n=5) and TNF-α–treated (n=13) rats, and animals were allowed to recover for an additional 15 days. LV function was assessed serially at baseline (day 0) and every 5 days for a total of 30 days; echocardiography was used to measure LV fractional shortening (see “Methods” for details).

**Figure 3. Effect of a continuous TNF-α infusion on isolated cell mechanics.** Isolated cell mechanics were studied with video edge detection methodology in animals that had received a continuous infusion of diluent (n=48 cells) or TNF-α (n=62 cells) for 15 days (see “Methods” for details). To determine whether negative inotropic effects of TNF-α were sensitive to NOS inhibition, cells were pretreated (30 minutes) with two different NOS inhibitors: 100 μM L-NAME (n=17 cells) and 100 μM L-NMMA (n=22 cells). To determine whether negative inotropic effects of TNF-α were sensitive to disruption of neutral sphingomyelinase pathway, cells were pretreated (30 minutes) with 1 μM NOE (n=19 cells). Finally, cell motion was examined in hearts of TNF-α–treated (15 days) animals that underwent removal of the osmotic infusion pump and that were allowed to recover for 15 days (n=28 cells).
osmotic pumps. Finally, pretreating the cells with NOE led to a significant improvement in the extent of cell shortening ($P < .05$, Newman-Keuls test) compared with diluent-treated cells from TNF-$\alpha$–stimulated animals; however, the extent of cell shortening was still less than control values ($P < .05$, Dunnett’s test).

**Effects of a Continuous Infusion of TNF-$\alpha$ on LV Structure**

**LV Structure**

A second important finding of this study was that stimulation with TNF-$\alpha$ provoked a time-dependent increase in LV dilation (remodeling). Fig 4 shows that LV EDD increased by day 5 of the TNF-$\alpha$ infusion and was significantly different from control values by day 5 to 15 of the protocol. In contrast, there was no significant difference in the LV EDD from day 0 to 15 of the infusion in the diluent-treated animals. Interestingly, the LV EDD remained significantly elevated in the TNF-$\alpha$–stimulated animals after the infusion pumps were removed and did not completely return to baseline by day 30 of the protocol. However, we cannot discount the possibility that if the animals had been followed up for a longer period of time, there might have been a complete return of LV EDD to control values. Two-way ANOVA indicated that there were significant differences between groups with respect to LV EDD from day 0 to 30 of the protocol ($P < .001$); post hoc ANOVA testing (Dunnett’s test) indicated that the LV EDD in the TNF-$\alpha$–stimulated rats was significantly different ($P < .01$) from baseline values for days 5 to 30 of the infusion and was significantly greater ($P < .05$) than the LV EDD in diluent-treated animals.

To delineate the potential mechanism(s) for the TNF-$\alpha$–induced LV remodeling, we compared LV mass, LV end-diastolic volume, and LV posterior wall thickness in the diluent- and TNF-$\alpha$–treated animals. Table 2 shows that compared with diluent-treated animals, there was a significant increase ($P < .05$) in LV end-diastolic volume in the TNF-$\alpha$–treated animals, whereas LV mass was not different in the two groups of animals. The TNF-$\alpha$–induced increase in LV end-diastolic volume resulted from an increase in LV chamber dimension (Fig 4) and a decrease in LV wall thickness (Table 2). Interestingly, although LV end-diastolic volume returned toward baseline values after the removal of the TNF-$\alpha$ infusion pumps, LV wall thickness remained significantly decreased after the pump was removed. Two

**TABLE 2. LV Structure in Diluent- and TNF-$\alpha$–Stimulated Rats**

<table>
<thead>
<tr>
<th></th>
<th>Diluent (n=5)</th>
<th>TNF-$\alpha$ (n=6)</th>
<th>TNF-$\alpha$/Recovery (n=7)</th>
<th>$P$, ANOVA</th>
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<tr>
<td><strong>Body weight, g</strong></td>
<td>300±7</td>
<td>288±8</td>
<td>298±63</td>
<td>.52</td>
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<tr>
<td><strong>Heart weight, mg</strong></td>
<td>1.4±0.01</td>
<td>1.3±0.1</td>
<td>1.22±0.1</td>
<td>.23</td>
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<tr>
<td><strong>Heart weight/body weight, ×100</strong></td>
<td>0.5±0.03</td>
<td>0.4±0.03</td>
<td>0.4±0.03</td>
<td>.51</td>
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<td><strong>Heart weight/tibial length, ×100</strong></td>
<td>27.5±1.0</td>
<td>24.6±1.2</td>
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<td>.18</td>
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<tr>
<td><strong>Lung weight, wet/dry</strong></td>
<td>3.8±0.3</td>
<td>4.4±0.4</td>
<td>4.2±0.3</td>
<td>.51</td>
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<tr>
<td><strong>LV mass, mg</strong></td>
<td>486±27</td>
<td>477±21</td>
<td>425±21</td>
<td>.42</td>
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<tr>
<td><strong>LV volume, cm$^3$</strong></td>
<td>409±37</td>
<td>553±29*</td>
<td>404±37†</td>
<td>.02</td>
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<td><strong>LV posterior wall thickness, mm</strong></td>
<td>1.7±0.3</td>
<td>1.2±0.1*</td>
<td>1.2±0.1*</td>
<td>.004</td>
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<td><strong>Myocyte cross-sectional area, µm$^2$</strong></td>
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<tr>
<td>Endo</td>
<td>185±5</td>
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<td>Epi</td>
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<td>Average</td>
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<td>177±1*</td>
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</tr>
<tr>
<td><strong>Myocyte volume, µm$^3$</strong></td>
<td>16442±1412</td>
<td>19659±1196</td>
<td>18198±1165</td>
<td>.001</td>
</tr>
<tr>
<td><strong>Total number of LV myocytes, ×10$^6$</strong></td>
<td>26.3±2.5</td>
<td>29.4±2.7</td>
<td>22.2±3.6</td>
<td>.28</td>
</tr>
<tr>
<td><strong>Myocytes across the LV wall, ×10$^6$</strong></td>
<td>126±5</td>
<td>94±7*</td>
<td>96±6*</td>
<td>.008</td>
</tr>
<tr>
<td><strong>Relative LV collagen content, %</strong></td>
<td>11.8±0.8</td>
<td>6.5±0.4*</td>
<td>7.1±0.2*</td>
<td>.001</td>
</tr>
</tbody>
</table>

Data are mean±SEM. LV mass and posterior wall thickness were determined from two-dimensional echocardiography (see “Methods”). Myocyte cross-sectional area was determined by standard morphometric analyses (see “Methods”). LV myocyte volume was computed from the myocyte length, as measured from the isolated cardiac myocytes, and the myocyte cross-sectional area. The total number of myocytes was determined as LV volume/myocyte volume. The total number of myocytes across the LV wall was determined as LV wall thickness/myocyte diameter.

* $P < .05$ vs diluent-treated controls.
additional analyses were undertaken to determine the mechanism for the TNF-α-induced LV wall thinning. First, we calculated the total number of myocytes across the transmural thickness of the LV wall. This analysis showed that there was a significant (P<.05) decrease in the number of myocytes across the LV wall when the TNF-α-treated animals were compared with the diluent-treated controls (Table 2). Second, we examined the relative volume of LV myocardial collagen in the diluent- and TNF-α-stimulated animals. As shown in Table 2, there was a significant decrease (P<.05) in the fractional area occupied by picrosirius staining in the LV myocardial sections taken from the TNF-α–stimulated animals (6.5±0.4%) compared with diluent-treated controls (11.8±0.8%). Thus, the decrease in wall thickness in the TNF-α–stimulated animals was attended by a decrease in the number of myocytes across the wall as well as a reduction in the fibrillar collagen weave within the LV myocardial wall. Interestingly, both the total number of myocytes across the LV wall and the relative LV collagen content in the myocardium remained significantly diminished in the animals that were allowed to recover after removal of the TNF-α infusion pumps (Table 2).

Table 2 also summarizes the morphological findings in the diluent- and TNF-α–treated animals at the time they were killed (15 days). As shown, there was no significant difference in body weight, heart weight, ratio of heart weight to body weight, ratio of heart weight to tibial length, or lung weight (wet/dry ratio) when the TNF-α–treated animals were compared with diluent-treated animals.

**LV Histology**

Fig 5A and 5B shows representative hematoxylin-eosin–stained histological myocardial sections from rats that had been treated with diluent for 15 days, and Fig 5C and 5D show representative hematoxylin-eosin–stained histological myocardial sections from rats that had been treated with TNF-α for 15 days. There were no obvious differences in myocardial histology at the level of light microscopy between the two groups in any of the animals examined. Specifically, there was no evidence of replacement fibrosis or contraction band necrosis in any of the myocardial sections that were examined in the diluent- (n=5) and TNF-α–treated (n=6) animals. Importantly, leukocytic infiltrates were not detected in any of the myocardial sections that were examined or in any of the sections of brain or lung that were examined in the TNF-α–treated animals. There was evidence of focal inflammation in the liver and spleen at sites at which the osmotic infusion pump came into contact with these organs; however, there was no evidence of inflammation in the histological samples of liver and spleen that did not come into contact with the infusion pump. TNF-α stimulation led to an small but significant (P<.05) increase in the average LV myocyte cross-sectional area; however, as shown in Table 2, this increase was largely confined to myocytes in the epicardium.

Fig 5C and 5D show representative scanning electron micrographs from hearts that had been treated with diluent for 15 days, whereas Fig 5G and 5H shows representative scanning electron micrographs from rats that had been treated with TNF-α for 15 days. In the myocardial samples from the diluent-treated animals, we observed a fine weave of collagen around the myocytes as well as a meshlike weave in the interstitial space. However, in the myocardial samples obtained from the animals that had received a chronic infusion of TNF-α, the collagen weave appeared to be significantly disrupted and the fine fibrillar nature of the collagen weave was absent in many areas of the LV myocardium, in keeping with the decrease in the picrosirius staining in the LV myocardial sections taken from the TNF-α–stimulated animals reported in Table 2.

**TNF-α–Induced DNA Damage**

To determine whether TNF-α–induced apoptosis was responsible, at least in part, for the observed effects of this cytokine on LV remodeling, we examined the frequency of DNA strand breaks in diluent- and TNF-α–treated myocytes by two separate techniques: the TdT staining method and the double-strand DNA ligase–based method.34 Fig 6A through 6D shows representative TdT staining in diluent and TNF-α–treated animals, photographed at ×100 and ×400, respectively; E and F, representative photomicrographs of myocardial sections from TNF-α–treated animals, photographed at ×100 and ×400, respectively. C and D, Representative scanning electron micrographs of LV myocardial sections taken from diluent-treated rats; G and H, representative scanning electron micrographs of LV myocardial sections taken from TNF-α–treated rats at a final magnification of ×7500.
Figure 6. Effect of continuous TNF-α infusion on DNA strand breaks. TdT labeling of nuclei was performed in myocardial sections from hearts in animals treated with diluent (n=2) or TNF-α (n=2) for 15 days. A and B, TdT staining in myocardial sections from diluent- and TNF-α-treated animals, respectively, photographed at ×125; C and D, representative photomicrographs of TdT staining of same myocardial sections from diluent- and TNF-α-treated animals, respectively, photographed at ×250 (see “Methods” for details). Black arrows denote nuclei that stained positively with TdT. E and F, Representative DAPI-stained myocardial sections examined for DNA double-strand breaks by a double-strand DNA ligase-based method using digoxigenin-labeled DNA probes and an alkaline phosphatase reporter system that stains black when double-strand DNA breaks are present (see “Methods” for details). Positive ligase staining, if present, would appear as a dark spot within DAPI-labeled nucleus. G, Frequency of TdT-labeled nuclei in diluent- and TNF-α-treated animals.

TdT staining in the myocardium of the diluent- and TNF-α–treated animals. Fig 6G depicts the number of TdT-labeled nuclei in myocardial sections obtained from the diluent- and TNF-treated hearts. As shown, the frequency of positive TdT staining was low in both diluent- and TNF-α–treated animals (<0.002%), in keeping with the observation that the total number of myocytes (computed) was not statistically different in the hearts of the diluent- and TNF-α–treated animals (Table 2). However, there was a significant (P<.01), 3.2-fold increase in the TNF-α–treated relative to diluent-treated animals. Because TdT labeling may be seen in single-strand DNA breaks, which are not indicative of apoptosis but also occur in necrotic tissue,34 we used the double-strand DNA ligase–based method to assess the double-strand DNA breaks in myocardial sections from diluent- and TNF-α–treated animals. Fig 6E and 6F depicts representative DAPI-stained myocardial sections by the ligase-based method. As shown, there was no evidence of DNA labeling in any of the myocardial sections that were examined, suggesting that the double-strand DNA breaks characteristic of apoptosis were not present. Although the reason(s) for these discrepant findings between the two techniques is not clear, one likely possibility is that the increased TdT labeling in the myocytes from the TNF-treated animals represents increased DNA damage, as opposed to increased double-strand damage characteristic of apoptosis. This interpretation is in keeping with the observation that TNF-α can lead to the generation of reactive oxygen species that are known to produce DNA damage.39

Myocardial Rescue Using a Dimeric TNF Antagonist

Previous studies from this laboratory have shown that a dimeric soluble TNF receptor (TNFR:Fc; Immunex, Corp) antagonizes the negative inotropic effects of TNF-α in vitro. To determine whether TNFR:Fc was sufficient to antagonize the negative inotropic effects of TNF-α in vivo, we treated rats with this soluble TNF antagonist on day 7 after implantation of the osmotic infusion pumps, at a time when the TNF-α–induced negative inotropic effects were maximal. Levels of TNFR:Fc (measured as circulating human sTNFR2 levels) were detectable starting on the day of the injection (1234±557 pg/mL SC) and remained elevated until day 3 (1045±644 pg/mL) after injection (ie, days 7 to 12 of the protocol), after which time immunologically detectable levels of sTNFR2 were no longer present. The salient finding shown by Fig 7A is that administration of TNFR:Fc resulted in a time-dependent improvement in LV fractional shortening. LV fractional shortening was not significantly improved at 6 and 12 hours after the administration of TNFR:Fc (data not shown); however, LV fractional shortening was significantly improved by 24 hours (ie, day 8) and had returned completely to baseline values observed on day 0, within 48 hours after administration of TNFR:Fc. The observation that a specific antagonist for TNF-α was sufficient to antagonize the negative inotropic effects of TNF-α suggests that the observed LV dysfunction was not secondary either to endotoxin contaminating the recombinant proteins used here or to rat autoantibodies formed against the recombinant human cytokine. Fig 7B shows that administration of TNFR:Fc did not lead to a significant decrease in LV EDD. One-way ANOVA indicated that there were significant overall changes in the extent of LV fractional shortening and LV EDD (P<.05) in the rats exposed to a continuous infusion of TNF-α; post hoc ANOVA testing (Newman-Keuls test) indicated that LV fractional shortening improved significantly 24 hours after the injection and was no different from baseline values 48 hours after TNFR:Fc admin-
Discussion

The major conclusion to be drawn from this experimental study is that sustained, pathophysiologically relevant circulating concentrations of TNF-α are sufficient to provoke deleterious changes in LV structure and function in rats. The major finding with respect to myocardial function was that a continuous infusion of TNF-α led to a time-dependent depression in LV function that was evident at the level of the intact ventricle as well as in the isolated cardiac myocyte itself. As shown in Fig 2, stimulation with TNF-α led to a decrease in LV fractional shortening in the absence of significant changes in arterial blood pressure and heart rate (Table 1), suggesting that the TNF-α–induced effects were not secondary to alterations in LV loading conditions. The observation that isolated cell shortening (Fig 3) was depressed by 25% to 30% in the myocytes isolated from TNF-α–treated animals further suggested that the cytokine-induced effects were not secondary to altered LV loading conditions. Interestingly, the TNF-α–induced shortening of myocyte shortening was not sensitive to inhibition with L-NAME or L-NMMA, whereas it was sensitive, at least in part, to disruption of the neutral sphingomyelinase pathway with NOE (Fig 3), consistent with previous reports from this laboratory that have implicated this novel NOS-independent pathway as a molecular signaling mechanism for the TNF-α–induced myocardial depression. However, it is important to note that the TNF-α–induced depression in cell motion was not reversed completely with NOE, as we had observed in vitro, implying that continuous stimulation with TNF-α may produce negative inotropic effects through an alternative and as yet undetermined mechanism. Furthermore, it also bears emphasis that our studies do not exclude a potentially important role for NO-mediated blunting of β-adrenergic receptor signaling as a mechanism for TNF-α–induced myocardial depression. In keeping with previous reports from this and other laboratories, the negative inotropic effects of TNF-α were fully reversible. Fig 2 shows that neither the TNF-α–induced depression in LV fractional shortening nor the cytokine-induced depression in isolated cardiac myocyte shortening was evident 15 days after the TNF-α osmotic infusion pumps were removed. Moreover, the effects of TNF-α on LV fractional shortening were completely reversible in the animals that were treated with TNFR:Fc, a specific TNF-α antagonist that reverses the negative inotropic effects of TNF-α in isolated contracting cardiac myocytes in vitro. Although the above-mentioned studies have implicated a direct role for TNF-α as a negative inotrope, it should be emphasized that these studies do not vitiate a potentially important contributory role for other “downstream” proinflammatory cytokines, such as IL-1β and IL-6, in terms of modulating LV function.

The major finding with respect to myocardial structure was that continuous infusion of TNF-α induced a time-dependent increase in LV remodeling (Fig 4). However, in contrast to the findings observed with myocardial function, the effects of TNF-α on LV structure were not fully reversible after removal of the osmotic infusion pumps (Fig 4) or on treatment with a specific TNF-α antagonist, TNFR:Fc (Fig 6B). Fig 4 shows that TNF-α stimulation produced an increase in LV dilation that was accompanied by a decrease in transmural LV wall thickness; however, LV mass did not change significantly during TNF-α infusion (Table 2). Although this study was not designed to determine the precise mechanism for LV wall thinning, the data suggest that TNF-α–induced degradation of the fibrillar collagen matrix may contribute to alterations in the spatial arrangement of myocytes within the LV wall. Two distinct lines of evidence support this possibility. First, continuous stimulation with TNF-α led to an decrease in the volume of fibrillar collagen, as assessed by picrosirius red staining (Table 2) and scanning electron microscopy (Fig 5), consistent with the known effects of TNF-α on activation of matrix metalloproteinases that are capable of degrading extracellular matrix proteins. Second, the TNF-α–induced decrease in LV wall thickness was accompanied by a decrease in the number of myocytes (calculated) across the transmural thickness of the LV wall. This decrease in number of myocytes across the thickness of the LV wall did not appear to be secondary to obvious tissue necrosis, as suggested by the absence of contraction bands in the cardiac myocytes, replacement fibrosis, or significant leukocytic infiltration in histological specimens of myocardium from TNF-α–treated animals. Moreover, the decrease in number of transmural myocytes across the thickness of the LV wall did not appear to be secondary to obvious TNF-α–induced myocyte apoptosis. That is, when we used two separate techniques to assess the extent of DNA damage in myocardial sections from TNF-α–treated animals, the frequency of DNA strand breaks ranged from 0% to 0.002%, which would not explain the observed decrease in myocyte number across the LV wall. Furthermore, the total numbers of myocytes were not different in the diluent- and TNF-α–treated hearts. Thus, we postulate that TNF-α–induced remodeling in this model system is secondary, at least in part, to degradation of the fibrillar collagen matrix with resultant alterations in the spatial alignment of myocytes within the LV wall.

TNF-α as a Mediator of Disease Progression in the Failing Heart

The search for the potential mechanism(s) that are responsible for the development and progression of heart failure has been practically exhaustive; nonetheless, a full description of the biological mechanisms that are responsible for the transition to heart failure has not been forthcoming. germane to this discussion is the recent insight that heart failure may be caused, at least in part, by the overexpression of biologically active molecules such as neurohormones, which by virtue of their toxic effects are sufficient to produce increased LV remodeling and progressive LV dysfunction (the neurohormonal hypothesis). More recently, it has become clear that, in addition to the classic neurohormones, overexpression of a second class of biologically active molecules called cytokines may also be sufficient to contribute to disease progression by virtue of the toxic effects that these proteins exert in the myocardium.
Thus far, two major classes of cytokines have been identified in heart failure: vasoconstrictor cytokines, such as endothelin-1 and big endothelin,47–48 and vasodepressor “proinflammatory” cytokines, such as TNF-α, IL-1, and IL-6.11 Although there is now substantial evidence to suggest that overexpression of endothelin-1 in the heart may lead to progressive cardiac decompensation through myocytolysis, replacement fibrosis,49 and peripheral vasoconstriction,50 the potential role for vasodepressor cytokines such as TNF-α, IL-1, and IL-6 has remained largely speculative, in large measure because of the lack of appropriate model systems to study the sustained effects of these cytokines on LV structure and function. In this regard, the results of the present study suggest that a continuous infusion of TNF-α at levels that are known to exist in clinical heart failure patients leads to progressive LV dysfunction, progressive LV remodeling, degradation of the extracellular matrix, and cardiac myocyte hypertrophy (Table 2).51,52 However, further studies will be necessary to delineate the potential pathophysiological role of downstream cytokines, such as IL-1 and IL-6.

One of the more interesting findings in the present study, particularly in light of recent in vitro findings regarding TNF-α–induced apoptosis in isolated cardiac myocytes53 and other cultured mammalian cells,54 was the striking absence of double-strand DNA breaks characteristic of apoptosis in myocardial sections from animals treated with TNF-α. Although the reasons for the discrepancy between the present in vivo study and previous in vitro study in juvenile and adult myocytes53 are not clear, there are several potential explanations. First, in the previous in vitro report in myocytes, there was substantial ongoing myocyte apoptosis in the control cell cultures treated with diluent alone (≈5% of cells), whereas we observed that ≈0% to 0.001% of the nuclei from myocytes from normal myocardium contained detectable DNA strand breaks, depending on the technique used. Accordingly, one explanation for the discrepant findings between the two studies may be that TNF-α alone may not be sufficient to trigger the apoptotic machinery in normal robust cardiac myocytes, whereas this cytokine may be sufficient to trigger apoptosis in cells that have been primed to undergo apoptosis after cell injury, such as might occur during the process of primary myocyte cell isolation and/or cell culture. Second, the concentrations of TNF-α used in the aforementioned in vitro study were ≈40-fold higher than those used in the present study and ≈30-fold higher than are observed in clinical heart failure. Third, there were differences in the methodologies used to detect apoptosis in the two different studies. Thus, although the present study does not exclude an important role for TNF-α in triggering apoptotic cell death in adult cardiac myocytes, it does suggest that in vivo, TNF-α alone may not be sufficient to trigger apoptosis in the short term and that perhaps other factors that occur with longer-term (ie, >2 weeks) cytokine stimulation of cardiac myocytes, such as oxidative stress, upregulation of proto-oncogenes, or long-term exposure to other peptide growth factors or cytokines,55 may be necessary to “prime” the cells to undergo apoptosis. Alternatively, it is possible that the higher concentrations of TNF-α that may potentially occur with local intramyocardial expression of TNF-α are necessary to trigger apoptotic cell death in myocytes. Additional studies will be necessary to test these interesting possibilities.

Conclusions

The relatively recent insight that heart failure may progress as a result of the overexpression of toxic molecules, such as neurohormones, has prompted the search for additional portfolios of biologically active molecules that might contribute to the inexorable progression of heart failure. In an attempt to delineate a biochemical mechanism for the cardiac cachexia that occurs in patients with advanced heart failure, Levine et al1 made the important observation that patients with advanced heart failure express elevated levels of TNF-α (originally called “cachectin”) in their peripheral circulation. However, in the ensuing years since this observation, there has been increasing speculation that TNF-α might also directly contribute to the progression of heart failure by virtue of the direct toxic effects that this molecule exerts on the heart and the circulation.10–12 Although direct correlations between short-term effects of TNF-α in rats in vivo and the long-term effects of TNF-α in vivo in humans with heart failure are not appropriate, the results of the present study suggest that pathophysiologically relevant levels of TNF-α are at least sufficient to mimic some aspects of the so-called “heart failure phenotype,” including progressive LV dysfunction, LV remodeling, fibrillar collagen degradation, and cardiac myocyte hypertrophy. On a more pragmatic level, the results of this study are important for a second reason. That is, this study shows that a genetically engineered TNF antagonist can reverse some (but not all) of the effects of pathophysiologically relevant concentrations of TNF-α. These latter observations raise the intriguing possibility that neutralizing TNF-α with a specific antagonist may lead to clinical improvements in patients with advanced heart failure. Ongoing research efforts are being directed at this interesting possibility.

Acknowledgments

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Pathophysiologically Relevant Concentrations of Tumor Necrosis Factor-α Promote Progressive Left Ventricular Dysfunction and Remodeling in Rats

Biykem Bozkurt, Scott B. Kribbs, Fred J. Clubb, Jr, Lloyd H. Michael, Vladimir V. Didenko, Peter J. Hornsby, Yukihiro Seta, Hakan Oral, Francis G. Spinale and Douglas L. Mann

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