Nitric oxide modulation of TNF-α-induced cardiac contractile dysfunction is concentration dependent

JURETA W. HORTON, DAVID MAASS, JEAN WHITE, AND BILLY SANDERS
Department of Surgery, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9160

Horton, Jureta W., David Maass, Jean White, and Billy Sanders. Nitric oxide modulation of TNF-α-induced cardiac contractile dysfunction is concentration dependent. Am J Physiol Heart Circ Physiol 278: H1955–H1965, 2000.— Whereas previous studies suggest that tumor necrosis factor-α (TNF-α) induces cardiac contraction-relaxation deficits, the mechanisms remain unclear. Our recent studies have implicated cardiac-derived nitric oxide (NO). This study examined the detrimental and protective effects of NO donors S-nitroso-N-acetyl-penicillamine (SNAP) or (Z)-1-[N-(3-ammonio-propyl)-N-(n-propyl)aminodiazen-1-ium-1,2diolate (PAPA/NO) on TNF-α-related changes in cardiac contractile function (Langendorff), cellular injury, and intracellular myocyte Ca2+ concentration ([Ca2+]i). Myocytes were incubated in the presence/absence of TNF-α (200–500 pg/ml × 10^6 cells) for 3 h; subsets of myocytes were incubated with one of several concentrations of SNAP or PAPA/NO (0.1, 0.3, 0.5, and 1.5 mM) for 15 min before TNF-α challenge. Supernatant creatine kinase (CK), cell viability (Trypan blue dye exclusion), and myocyte [Ca2+]i (fura 2-acetoxymethyl ester) were measured. In parallel experiments, cardiac function (Langendorff) was examined after TNF-α challenge in the presence or absence of SNAP or PAPA/NO (0.1 and 1.5 mM). TNF-α in the absence of an NO donor impaired cardiac contraction and relaxation and produced cardiomyocyte injury. Pretreating perfused hearts or isolated cardiomyocytes with a low concentration of either SNAP or PAPA/NO decreased TNF-α-mediated cardiac injury and improved contractile dysfunction, whereas high concentrations of NO donor exacerbated TNF-α-mediated cardiac effects. These data provide one explanation for the conflicting reports of beneficial versus detrimental effects of NO in the face of inflammation and suggest that the effects of NO on organ function are concentration dependent; low concentrations of NO are cardioprotective, whereas high concentrations of NO are deleterious.

Sprague-Dawley rats; cardiac contraction and relaxation; Langendorff perfusion; cardiomyocytes in culture; tumor necrosis factor-α-related cardiac depression.

MYOCARDIAL CONTRACTILE DEPRESSION is a common complication of stress-related injury such as hemorrhage, trauma, or sepsis; and myocardial abnormalities include decreases in cardiac output, ejection fraction, and systolic pressure paralleled by increases in preload and ventricular dilatation (10, 36, 38, 39, 42, 46, 47, 49). Myocardial depressant factors have been described in the serum of both septic (9, 48) and burn patients (5) and in experimental models of hemorrhagic shock (11, 33, 34). The use of isolated hearts, ventricular muscle preparations, and cardiomyocytes has confirmed that contractile abnormalities after major hemorrhage, burn trauma, and sepsis are specific to the myocardial contractile elements and not related to changes in either peripheral vascular function or inadequate ven- tricular filling (1, 2, 17, 19, 20–24, 41, 44, 45, 51, 58). Whereas there is abundant evidence that trauma and sepsis alter myocardial function, cardiac contraction and relaxation defects are frequently transient and reversible, implying an absence of myocardial necrosis (38).

Cardiac contractile deficits after trauma or sepsis have been attributed to complex inflammatory responses that include an imbalance in the synthesis of proinflammatory and antiinflammatory cytokines. In this regard, increasing evidence supports the role of tumor necrosis factor-α (TNF-α) in the cardiac contractile dysfunction that occurs after traumatic injury, and evidence has accumulated indicating that the heart itself synthesizes several inflammatory cytokines in cardiac-related illnesses as well as in stress-related injury (12, 14, 27, 30, 43, 55). Despite evidence that TNF-α plays a pivotal role in cardiac dysfunction after stress-related injury, the mechanisms by which this pleiotropic cytokine alters cardiac mechanical function remain unclear. Several studies by us and others (13, 14, 27, 29, 30, 43, 55) show that TNF-α challenge of left ventricular (LV) muscle preparations or of cardiomyocytes acts as a negative inotrope. Other studies (12, 43, 59) suggest that TNF-α mediates cardiac contractile dysfunction by enhancing cardiac synthesis of nitric oxide (NO) and by activating the sphingomyelinase pathway. However, the role of NO in cardiac dysfunction after stress-related injury remains highly controversial. In this regard, Finkel and colleagues (12) showed that a specific inhibitor of NO synthase ablated the negative inotropic effects of TNF-α and suggested that TNF-α stimulates production of a secondary mediator such as NO, which in turn directly impairs myocyte contractile function. In contrast, Yokoyama and colleagues (59) reported that increased levels of NO did not mediate TNF-α-induced myocardial contractile abnormalities (59). Finally, more recent studies suggest that locally generated NO may interact with the superoxide radical to produce peroxynitrite (ONOO⁻), which in turn promotes peroxidative damage to lipid membrane components and cellular DNA (54).
We propose that the effects of NO on TNF-α-induced cardiac contractile depression are concentration dependent. We hypothesized that low concentrations of NO donors are cardioprotective, whereas high concentrations of NO donors are deleterious. In this present study, we used isolated perfused hearts (Langendorff) as well as isolated cardiomyocytes to determine whether NO donors could modulate TNF-α-mediated dysfunction. All cardiac preparations were treated with TNF-α to produce myocardial contractile abnormalities in a controlled in vitro setting, eliminating the complex inflammatory cascade that may modulate cardiac function in vivo after stress-related injury or cardiac illness. In addition, ventricular muscle preparations and cardiomyocytes were used to determine the modulatory effects of NO donors on TNF-α-mediated cardiac contractile dysfunction.

METHODS

Experimental animals. Adult Sprague-Dawley rats (300–350 g body wt) were used throughout the study. All animals were obtained from Harlan Laboratories (Houston, TX) and were allowed 5–6 days to acclimatize to their surroundings. Commercial rat chow and tap water were available ad libitum. All work was performed under a protocol that was approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Research Advisory Committee, and the work conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health.

Cardiomyocyte isolation. All pipettes, plates, test tubes, and other equipment used for preparation and culture of cardiomyocytes were sterile. Preliminary studies showed that the culture media, cytokine solutions, and other solutions used for preparation and culture of the myocytes were endotoxin free as determined by a chromogenic Limulus amebocyte lysate assay (unpublished data). Rats were heparinized and then decapitated, and the heart of each rat was removed through a medial sternotomy using sterile technique and then decapitated, and the heart of each rat was perfused with calcium-free Tyrode solution using a Langendorff perfusion apparatus. The Langendorff perfusion apparatus. The Tyrode solution was equilibrated with 95% O2-5% CO2 during perfusion of the heart. Perfusion was maintained for 5 min, and ventricular drainage was ensured by placing a 22-gauge needle in each ventricle. Perfusion was then continued for an additional 10 min using a collagenase solution, which contained 80 ml of calcium-free Tyrode, 40 mg of collagenase A (0.05%, Boehringer Mannheim, Indianapolis, IN), and 4 mg of protease (polysaccharide XIV, Sigma Chemical, St. Louis, MO) with continuous oxygenation. After this enzymatic digestion, the heart was removed from the cannula, and the ventricular tissue was separated from the base of the heart in a petri dish containing Tyrode solution with 100 μM calcium, and gentle minced increased cell dispersion over 5 min. The myocyte suspension was then filtered and the cells allowed to settle. The supernatant was removed, and the cells were resuspended in 50 ml of Tyrode; the rinsing and settling step was repeated three times with 10 min between each step and with gentle swirling between each step to allow myocyte separation. The calcium concentration of the rinsing solution was gradually increased during these steps, with calcium concentrations of 100 μM, 200 μM, and finally 1.8 mM. The cell viability was measured (Trypan blue dye exclusion); myocytes with a rodlike shape, clear formed edges, and clear striations were prepared with a final cell count of 5 × 105 cells/ml (18, 28).

Langendorff perfused hearts. To examine cardiac contractile function, awake rats were anticoagulated with heparin sodium (1,000 units, Elkins-Sinn, Cherry Hill, NJ) and decapitated with a guillotine. The heart was rapidly removed and placed in ice-cold (4°C) Krebs-Henseleit bicarbonate-buffered solution (in mM: 118 NaCl, 4.7 KCl, 21 NaHCO3, 2.5 CaCl2, 1.2 MgSO4, 1.2 K2HPO4, and 11 glucose). All solutions were prepared each day with demineralized, deionized water and bubbled with 95% O2-5% CO2 (pH, 7.4; po2, 550 mmHg; pCO2, 38 mmHg). A 17-gauge cannula was placed in the ascending aorta and connected via glass tubing to a buffer-filled reservoir for perfusion of the coronary circulation at a constant flow rate. Hearts were suspended in a temperature-controlled chamber maintained at 38 ± 0.5°C, and a constant flow pump (model 911, Holter, Critikon, Tampa, FL) was used to maintain perfusion of the coronary artery by retrograde perfusion of the aortic stump cannula. Coronary perfusion pressure was measured, and effluent was collected to confirm coronary flow rate. Contractile function was assessed by measuring intraventricular pressure with a saline-filled latex balloon attached to a polyethylene tube and threaded into the LV chamber. LV developed pressure (LVPD) was measured with a Statham pressure transducer (model P23 ID, Gould Instruments, Oxnard, CA) attached to the balloon cannula, and the rates of LVP rise (+dP/dt) and fall (−dP/dt) were obtained using an electronic differentiator (model 7P20C, Grass Instruments, Quincy, MA) and recorded (model 7WILN 1, Grass Recording Instruments). Data from the Grass recorder was input to a Del Pentium computer, and a Grass Poly VIEW Data Acquisition System was used to convert acquired data into digital form.

TNF-α-induced negative inotropic effects. Two groups of experiments were performed. Rat recombinant TNF-α (Sigma Chemical) was initially used to determine the effects of this cytokine on cardiomyocyte viability and creatine kinase (CK) release. Cardiomyocytes were plated at 50,000 cells/ml; myocytes were subsequently challenged with either diluent alone or one of several concentrations of TNF-α (200, 300, 400, 500, and 1,000 pg/ml, n = 10 cell preps per TNF-α concentration). Diluent consisted of standard culture media (medium 199 diluted in a balanced salt solution and supplemented with penicillin, streptomycin, and glutamine). Cells were incubated at 37°C in 5% CO2 for several time periods (either 1, 2, 3, or 4 h). After the designated time of cardiomyocyte exposure to TNF-α, microtiter plates were removed from the incubator, the supernatants were harvested to measure CK in the supernatant, and cell viability was measured by Trypan blue dye exclusion. These data provided information regarding the concentration and time-dependent effects of TNF-α on cardiomyocytes in vitro, allowing us to select concentrations of TNF-α for subsequent addition to perfused hearts to assess cardiac performance.

The next set of experiments determined the effects of TNF-α on cardiac contractile function using a Langendorff preparation. On the basis of the isolated cardiomyocyte studies and our previous studies examining TNF-α secretion by isolated cardiomyocytes (55), TNF-α (400 pg/ml) was selected as the concentration for use in the isolated hearts. After the isolated hearts were perfused in a Langendorff mode for 15–20 min, TNF-α was added to the perfused heart via a sidearm port above the heart, ensuring thorough mixing
of the TNF-α with coronary perfusate. Coronary flow rate was maintained constant, and the hearts were perfused with TNF-α-containing buffer in a recirculating manner for 15 min. Additional hearts were included to serve as control (n = 6); in these hearts, an identical volume of diluent was added to the sidearm port above the heart, and perfusion was maintained for an identical time period as described for the TNF-α-challenged hearts. The specificity of TNF-α-induced effects on cardiac contraction and relaxation was determined by use of an anti-TNF-α antibody (anti-TNF-α, lot B24369, Calbiochem, La Jolla, CA). The anti-TNF-α antibody was added to the coronary perfusate as described above and recirculated for ~10 min before the addition of TNF-α (400 pg/ml) in an additional group of hearts (n = 8). The effect of TNF-α on cardiac performance was examined in the presence and absence of anti-TNF-α antibody by measuring LVDP and maximal pressure rise and fall (±dP/dt max) responses to incremental increases in either LV balloon volume (from 0.03 to 0.2 ml), increases in coronary flow rate (from 6 to 15 ml/min), or increases in perfusate calcium (from 1 to 8 mM).

Effects of NO donors on TNF-α-mediated cardiomyocyte viability and cardiac contractile function. In the above studies, we elected to examine the effects of TNF-α on cardiomyocyte integrity and cardiac performance in the absence of other inflammatory stimuli by preparing hearts and myocytes from control, unchallenged rats. We have previously shown that burn trauma promotes cardiac contractile dysfunction as well as an inflammatory cascade that includes TNF-α, interleukin-1, interleukin-6, and NO release by several cell populations (4, 13, 14, 55). Since the above studies confirmed that exogenous TNF-α altered cardiac contraction and relaxation, we examined the subsequent effects of pretreating cardiomyocytes and perfused hearts with a NO donor followed by TNF-α challenge. In the first approach, cardiomyocytes were isolated and plated as described above (50,000 cell/ml); before the addition of TNF-α to the cardiomyocytes, cells were pre-treated with one of several concentrations of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) at concentrations of either 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, or 1.5 mM or the NO donor (Z)-1-[N-(93-ammonio-propyl-N-(n-propyl)amino) diazen-1-ium-1,2-dio]late (PAPA/NO, Fisher Scientific, Pittsburgh, PA) at a concentration of either 0.1, 0.3, 1.0, or 1.5 mM). The NO donor was added to the cardiomyocytes (n = 8 wells per experimental condition), and after 30 min of exposure of the cardiomyocytes to either SNAP or PAPA/NO, the cells were examined by phase-contrast microscopy to determine that cells maintained normal rodlike morphology with clear striations and intact sarcolemma. After it was confirmed that the NO donor alone did not alter cardiomyocyte integrity, TNF-α (400 pg/ml) was added to each microtiter well. Cardiomyocytes were then incubated for an additional 3 h (5% CO2 incubator at 37°C). The supernatants were then harvested to determine by Trypan blue dye exclusion. These preliminary studies established that the high concentration of SNAP and PAPA/NO (1.5 mM) exacerbated TNF-α-mediated effects on myocyte viability whereas the lower concentrations (0.1 and 0.3 mM) decreased TNF-α-mediated myocyte injury.

A subsequent protocol examined the protective or detrimental effects of pretreating isolated hearts with an NO donor (either SNAP or PAPA/NO) before the addition of TNF-α to the cardiac perfusate. To determine whether an NO donor could modulate TNF-α-induced effects on cardiac contractility, SNAP (final concentration of either 0.1 or 1.5 mM, n = 8 per experimental condition) or PAPA/NO (final concentration either 0.1 or 1.5 mM, n = 7 per experimental condition) was added to cardiac perfusate; the hearts were then reperfused in a recirculating manner with NO donor-containing buffer for 10–15 min; TNF-α (400 pg/ml) was then added to the perfusate and perfusion continued for an additional 15 min. Finally, LV function was assessed by increasing LV volume or preload by adding saline to the intraventricular balloon placed in the cardiac chamber. In this manner, the ability of an NO donor to modulate TNF-α-mediated cardiac contractile effects was studied in an environment free of the neurohormonal modulation that likely occurs after stress-related injury such as burn trauma or sepsis.

Intracellular Ca2+ concentration measurement. Because previous studies have suggested that NO mediates TNF-α-mediated cellular effects by modulating intracellular Ca2+ homeostasis, intracellular calcium concentration ([Ca2+]i) was measured at room temperature with constant low stirring in a Hitachi F-2000 Fluorescence Spectrophotometer. Fura 2-acetoxyethyl ester (AM)-loaded myocytes were suspended in calcium-free saline and placed in a 1-ml quartz cuvette; a magnetic stirring bar in the bottom of the cuvette maintained the cells in suspension. The spectrophotometer was equipped with a 150-W xenon lamp, an interference filter with a 10-nm bandpass was used to establish the excitation wavelengths (340/380 nm), and the emission light was collected through a 510-nm filter with a 10-nm bandpass at a response time of 0.5 s. The calibration procedure included measuring fluorescence ratios of different calcium concentration buffers. [Ca2+], was measured as a ratio (R) of two fluorescent signals (F1 and F2) generated from the two excitation wavelengths (340 nm and 380 nm); autofluorescence of myocytes that had not been loaded with fura 2-AM was subtracted as described by the formula

\[ R = \frac{F_{\text{1,cell}} - F_{\text{1,bkgd}}}{F_{\text{2,cell}} - F_{\text{2,bkgd}}} \]

and applied to the following equation of Grynkiewicz et al. (15)

\[ [\text{Ca}^{2+}] = K_d B (R - R_{\text{min}})/(R_{\text{max}} - R) \]

where \( K_d \) represents the dissociation constant of the complex Ca-fura 2 (224 nm at 37°C), and B is the ratio between the calcium-free and calcium-saturated 380-nm (F_{\text{min}}/F_{\text{max}}) signals. Studies were included to examine the effects of TNF-α on fura 2 leakage from cardiac myocytes as well as intracellular compartmentalization of fura 2 as previously described (3, 37).

Statistical analysis. All values are expressed as means ± SE. Statistical comparison of group values included an analysis of variance and multiple comparison procedure (Newman-Keuls). Relative changes in contractile performance to altered coronary flow rate were compared, as well as differences or similarities between performance-flow relationships achieved in control and TNF-α-challenged hearts. Probability values ≤0.05 were considered significant.

RESULTS

Only cardiomyocytes with well-defined cell borders and cellular striations were included for study. Our myocyte isolation procedure routinely produces a population of cardiomyocytes with >85% viability as determined by Trypan blue dye exclusion. Myocytes maintained in culture under the conditions described in this study in the absence of either TNF-α or NO remain viable over the 4-h incubation period with little change in the percentage of viable rod-shaped cells. Addition of TNF-α to cardiomyocytes produced a time-dependent and concentration-dependent change in cell viability; as shown in Fig. 1, cell viability decreased significantly.
as the concentration and time of TNF-α exposure was increased (P < 0.05). Whereas previous studies in our laboratory have shown that cardiomyocytes harvested from animals with stress-related injury such as burn trauma or burn complicated by sepsis produced cardiac TNF-α concentrations far in excess of 400 pg/ml, this concentration produced moderate but well-defined cellular changes and thus was selected for the subsequent studies described here.

As shown in Fig. 2 and Tables 1 and 2, the addition of TNF-α (400 pg/ml) to isolated perfused hearts produced significant decreases in cardiac contraction and relaxation as indicated by the fall in LVDP as well as decreases in the rate of +dP/dtmax and −dP/dtmax and produced LV function curves that were shifted downward and to the right of controls (Fig. 2). These responses occurred despite a constant coronary flow rate and constant heart rate. Reperfusion of the TNF-α-treated hearts with cytokine-free buffer restored cardiac contractile function within 120–180 s (data not shown). To further determine the specificity of TNF-α-induced effects on cardiac contractility, an additional group of hearts (n = 6) was pretreated by adding the neutralizing polyclonal rabbit anti-rat TNF-α antibody to the perfusate in a recirculating manner for 10 min before the addition of TNF-α. As also shown in Fig. 2, the anti-TNF-α strategy ablated TNF-α-mediated cardiac contraction and relaxation deficits. In this manner, the specificity of the TNF-α-induced effects on cardiac contractile function was confirmed.

The next series of studies were included to determine the effects of the NO donors SNAP and PAPA/NO on cardiomyocyte viability and CK in the presence and absence of TNF-α. Our preliminary data showed that low concentrations of SNAP and PAPA/NO (0.1–0.3
mM) in the absence of TNF-α produced no changes in cell viability or CK release, whereas high concentrations of the NO donors (1.5 mM) decreased cell viability by 10–12% (data not shown). We then examined the effects of SNAP and PAPA/NO on TNF-α-mediated cellular injury. As shown in Fig. 3, 90% of the myocytes incubated for 3 h in buffer alone (indicated as control) remained viable. In contrast, exposure of cardiomyocytes to TNF-α (400 pg/ml) for 3 h reduced cell viability to 52%. Pretreating myocytes with low concentrations of SNAP (0.3 mM) or PAPA/NO (0.1 mM) before TNF-α exposure reduced TNF-α-mediated cell injury and death, whereas the higher concentrations of NO donors (1.5 mM) failed to provide cellular protection (Fig. 3). CK measured in the supernatants from myocytes incubated in buffer alone for 3 h (indicated as control) was 96 ± 3 U/l (Fig. 4); TNF-α challenge of cardiomyocytes for 3 h produced a significant rise in supernatant CK levels (555 ± 15 U/l, P < 0.05). Low concentration of either SNAP (0.3 mM) or PAPA/NO (0.1 mM) reduced TNF-α-mediated CK release into the supernatant; in contrast, pretreatment of the cardiomyocytes with high concentrations of NO donor (1.5 mM) before TNF-α challenge failed to prevent TNF-α-mediated cardiac injury (Fig. 4).

The next set of studies examined the cardiac contraction and relaxation responses to TNF-α in the presence of NO donors; the concentrations of NO donors selected for the Langendorff studies were those shown in the cardiomyocyte experiments to provide cellular protection. TNF-α in the absence of an NO donor produced significant cardiac contraction and relaxation deficits (Figs. 5 and 6); cardiac function was reduced at each level of preload in TNF-α-challenged hearts compared with that measured in hearts perfused with buffer alone and indicated as controls. These differences in cardiac contraction and relaxation occurred despite identical levels of coronary flow rate and heart rates in all hearts. Hearts from additional animals (n = 8/group) were pretreated with a single concentration (0.1 mM) of either SNAP (Fig. 5) or PAPA/NO (Fig. 6); NO donor was added to the perfusate and recirculated for 10 min before the addition of TNF-α (400 pg/ml). TNF-α was recirculated for 14–20 min, and function was then measured. The low concentration of either SNAP or PAPA/NO significantly reduced TNF-α-mediated cardiac contraction and relaxation deficits. Addition of either SNAP or PAPA/NO (1.5 mM) before the addition of TNF-α exacerbated TNF-α-mediated cardiac contraction and relaxation defects (Figs. 5 and 6).

Similar results were observed when ventricular performance-coronary flow relationships were examined. LVP and ±dP/dt max responses to incremental increases in coronary flow were significantly lowered in TNF-α-treated hearts compared with those calculated for

### Table 1. Effects of TNF-α and TNF-α plus NO donor SNAP on cardiodynamic function

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TNF</th>
<th>Control + 0.1 mM SNAP</th>
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<th>Control + 1.5 mM SNAP</th>
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</thead>
<tbody>
<tr>
<td>LVP, mmHg</td>
<td>97 ± 3</td>
<td>67 ± 1*</td>
<td>97 ± 2</td>
<td>109 ± 2</td>
<td>64 ± 2†</td>
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<tr>
<td>+dP/dt max, mmHg/s</td>
<td>2,123 ± 53</td>
<td>1,360 ± 31*</td>
<td>2,220 ± 170</td>
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<td>−dP/dt max, mmHg/s</td>
<td>1,891 ± 52</td>
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<td>743 ± 81*</td>
</tr>
<tr>
<td>dP/dt, mmHg/s</td>
<td>1,800 ± 54</td>
<td>1,200 ± 30*</td>
<td>1,767 ± 180</td>
<td>1,883 ± 116</td>
<td>1,033 ± 120†</td>
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</tr>
<tr>
<td>TTP, ms</td>
<td>84.5 ± 16</td>
<td>89.3 ± 4.7</td>
<td>85.7 ± 2.1</td>
<td>90.6 ± 4.9</td>
<td>81.0 ± 3.8</td>
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<td>RT 90, ms</td>
<td>84.3 ± 1.7</td>
<td>82.0 ± 6.0</td>
<td>85.0 ± 3.7</td>
<td>86.0 ± 4.7</td>
<td>86.7 ± 6.7</td>
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<td>Time to max +dP/dt, ms</td>
<td>53.0 ± 14</td>
<td>58.3 ± 4.4</td>
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<td>CPP, mmHg</td>
<td>63.3 ± 6.2</td>
<td>54.0 ± 4.2</td>
<td>75.3 ± 10.4</td>
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<td>CVR, mmHg</td>
<td>12.6 ± 1.2</td>
<td>10.2 ± 6.0</td>
<td>15.1 ± 2.1</td>
<td>14.9 ± 0.9</td>
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<td>HR, beats/min</td>
<td>273 ± 27</td>
<td>265 ± 3</td>
<td>296 ± 3</td>
<td>300 ± 8</td>
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All values are means ± SE. LVP, left ventricular pressure; +dP/dt, rate of increase in LVP; −dP/dt, rate of decrease in ventricular pressure; DPa, developed pressure at 40 mmHg; TTP, time to peak pressure; RT 90, time to 90% relaxation; CPP, coronary perfusion pressure; CVR, coronary vascular resistance; HR, heart rate; TNF, tumor necrosis factor; SNAP, S-nitroso-N-acetyl-penicillamine. * Significant differences in TNF-related responses compared with respective control groups; † significant differences among control groups.

### Table 2. Effects of TNF-α and TNF-α plus NO donor PAPA/NO on cardiodynamic function

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<tr>
<td>CVR, mmHg</td>
<td>12.6 ± 1.2</td>
<td>10.2 ± 6.0</td>
<td>15.1 ± 2.1</td>
<td>14.9 ± 0.9</td>
<td>15.9 ± 0.7</td>
<td>17.8 ± 2.3*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>273 ± 27</td>
<td>265 ± 3</td>
<td>296 ± 3</td>
<td>300 ± 8</td>
<td>263 ± 13</td>
<td>263 ± 13</td>
</tr>
</tbody>
</table>

All values are means ± SE. PAPA/NO, (z)-(1-N-(3-ammonio-propyl)-N-(n-propyl)laminodiazin-1-ium-1,2-dioate. * Significant differences in TNF-related responses compared with respective controls; † significant differences among control groups.
controls (P < 0.05). Low concentrations but not high concentrations of the NO donors SNAP (Fig. 7) and PAPA-NO (Fig. 8) improved TNF-α-mediated decreases in ventricular responses to increased coronary flow rate.

Finally, we examined the effects of TNF-α, in the presence and absence of SNAP, on cardiomyocyte [Ca²⁺]i levels. After exposure of the myocytes to TNF-α for 3 h, cells were loaded with fura 2-AM, and [Ca²⁺]i was measured. [Ca²⁺]i in TNF-α-treated cardiomyocytes was significantly higher (198 ± 12 mM, P < 0.05) than that measured in myocytes incubated in buffer alone for an identical time period (96 ± 4 mM). Pretreatment of the cardiomyocytes with low concentration SNAP (0.3 mM) but not higher concentration of SNAP (1.5 mM) ablated the TNF-α-mediated rise in [Ca²⁺]i (112 ± 8 and 170 ± 11 mM, respectively).

DISCUSSION

TNF-α challenge in experimental animals impairs LV systolic pressure and acts as a negative inotrope in both LV muscle preparations and isolated cardiomyocytes (16, 40, 43, 56, 57). Other studies have reported that a monoclonal antibody against TNF-α effectively attenuated sepsis-related cardiopulmonary dysfunction (13, 16, 25, 57). This present study extends that previous work to examine the modulating effects of the NO donors on TNF-α-mediated cardiac contractile dysfunction. Whereas our previous studies have confirmed that cardiomyocytes secrete abundant TNF-α in response to either endotoxemia or burn trauma (13, 14, 55), both sepsis and burn trauma initiate a complex inflammatory cascade that include the synthesis of numerous cytokines and mediators by diverse cell populations. We considered examining the effects of NO donors on hearts harvested from either septic or burn injured animals, but we reasoned that stress-related inflammatory responses would complicate assessment of the modulating effects of NO; our use of an in vitro model of TNF-α-mediated cardiac dysfunction in the presence or absence of a NO donor eliminated this concern.

In this present study, TNF-α challenge in cardiomyocytes altered cell viability and increased CK supernatant levels, indicating TNF-α-mediated myocyte injury.
Furthermore, addition of TNF-α to buffer-perfused hearts impaired contraction and relaxation, supporting previous reports of the cardiodepressive effects of TNF-α. A major focus of this study was to examine the modulating effects of NO on TNF-α-mediated organ dysfunction, and our study confirmed that pretreatment of either cardiomyocytes or isolated perfused hearts with low concentrations of the NO donors SNAP or PAPA/NO provided significant cardiac protection against TNF-α challenge, whereas higher concentrations of the NO donors exacerbated TNF-α-induced cardiac contractile depression. TNF-α-mediated cardiac contractile depression was evident from decreased LVDP as well as decreases in the rate of LV pressure rise and fall; furthermore, TNF-α-impaired LVP and ±dP/dt responses to increases in either preload or coronary flow rate. The rapid onset of contraction and relaxation deficits after TNF-α challenge in isolated hearts confirmed the direct effects of this cytokine on cardiac contractile function. In addition, the specificity of TNF-α-induced cardiac dysfunction was confirmed by our finding that a monoclonal antibody to TNF-α ablated TNF-α-related dysfunction.

This study was not designed to characterize the mechanisms for the concentration dependency of NO to protect against TNF-α-mediated cardiac dysfunction; however, we did consider that NO may exert a protective or deleterious effect by modulating oxidant-mediated responses. Recent studies by others show that inflammatory-related production of oxygen radicals is paralleled by the production of reactive nitrogen; and NO can influence oxygen radical reactions in several ways (52–54, 60). NO may protect against oxidant-mediated injury by rapidly interacting with the superoxide radical to produce the unreactive nitrite ion (NO₃⁻), thus serving as a scavenger of the superoxide radical and reducing oxidative injury (6, 7, 26, 31, 35, 50). However, NO-reactive O₂ species (ROS) interactions also produce peroxynitrite (ONOO⁻) and peroxynitrous acid, extremely potent and reactive oxidants. Increased NO synthesis (shown to occur via the induc-
NO synthase occurs in several types of inflammation and sepsis; and under conditions of inflammation and excess NO production, NO may exacerbate ROS injury through peroxynitrite formation (60). Detrimental effects of peroxynitrite have been attributed to oxidation of sulfhydryl groups and thiol esters as well as nitration and hydroxylation of aromatic compounds such as tyrosine or tryptophan (53). In addition, peroxynitrite has been shown to react with various cellular enzymes, suppressing catalytic activity, oxidizing ascorbic acid, and further compromising antioxidant capacity of the subject with sepsis or inflammation (32). Finally, peroxynitrite-mediated DNA single strand breakage and subsequent protease-activated receptor activation has been shown to alter the energetic status of cells and to promote cell death and apoptosis. Whereas numerous studies have suggested that the peroxynitrite anion is a cytotoxic agent, Lefer and colleagues (32) showed that nanomolar concentrations of peroxynitrite inhibited ische-

Fig. 5. TNF-α challenge in absence of nitric oxide (NO) donors produced significant cardiac contractile depression as indicated by downward and rightward shift of left ventricular function curves (n = 6–7 hearts/group). Low concentration, but not high concentration, SNAP significantly ameliorated cardiac contractile depression. All values are means ± SE. *Significant difference among groups at P < 0.05.

Fig. 6. Effects of TNF-α challenge on left ventricular function was examined in absence or in presence of NO donor PAPA/NO (n = 6–7 hearts/group). TNF-α challenge alone produced significant cardiac contractile dysfunction. Low concentrations of PAPA/NO, but not high concentration, reduced TNF-α-mediated cardiac contractile dysfunction. All values are means ± SE. *Significant difference among groups at P < 0.05.
mia-reperfusion-mediated leukocyte-endothelial/adherence activation and attenuated the myocardial contractile dysfunction that is typical of ischemia reperfusion.

Whereas these previous studies have described both the protective and detrimental effects of NO in models of inflammation and sepsis, this present study focused on the effects of NO in an environment free from inflammation and free from the modulating effects of systemic mediators and cytokines. We elected to use the pleiotropic cytokine TNF-α as a model of myocardial depression because this cytokine has been implicated in a variety of cardiac illnesses as well as in several models of stress-related injury. In this study, we selected a cytokine concentration range that was 1) comparable to that measured in the systemic circulation after burn trauma (8), and 2) consistent with TNF-α levels produced by cardiomyocytes after lipopolysaccharide challenge (55).

Whereas numerous chemical and biochemical interactions within the cells likely determine the protective versus deleterious effects of NO in the setting of inflammation, it is clear from our present study that...
low concentrations of NO, but not high concentrations, provided myocardial protection. It is possible that in the intact subject, stress-related injury such as burn trauma decreases endothelial NO synthase, reducing available NO and initiating leukocyte adherence and activation; this period is followed by increased inducible NO synthase activity, and the increased NO levels likely overwhelmed endogenous scavenging systems (52). Thus the use of NO donors during early periods of NO scarcity could prevent leukocyte activation and the subsequent inflammatory cascade.

A potential problem with the choice of SNAP, one of the NO donors in our study, is that SNAP is a thiol-containing compound. The thiols are well-recognized scavengers of reactive nitrogen oxides, and thus the effects of SNAP could be related not only to its role as a NO donor but also to the potential scavenging capacity of the thiol-containing breakdown products (52). In addition, several studies have described the potential toxicity of thiol-containing compounds, and thus the cardiodepressive effects of high concentrations of SNAP could be related to increased concentrations of thiol-containing breakdown products of SNAP. To address this concern, we examined the protective and detrimental effects of another NO donor, PAPA/NO, a nonthiol-containing compound. In our study, both NO donors had similar effects on TNF-α-mediated cardiac injury as well as TNF-α-mediated cardiac contraction and relaxation defects. These data eliminated our concerns regarding the thiol breakdown products of SNAP.

Whereas specific cellular and intracellular mechanisms by which TNF-α and NO exert their effects on cardiac contractile function were not addressed in this present study, the TNF-α-related effects on cardiac function could be related to direct cytotoxicity because addition of TNF-α to isolated cardiomyocytes increased supernatant CK levels and produced a concentration by a specific anti-TNF-α antibody, cardiac contractile dysfunction. Another concern was that endotoxin contamination of the recombinant rat TNF-α could have contributed to the changes in cardiomyocyte viability or myocardial contractile dysfunction. In addition, we considered that endotoxin contamination of the recombinant TNF-α could promote NO synthesis by coronary endothelial cells via NO synthase; alternatively, endotoxin contamination could promote additional synthesis of TNF-α by either the isolated cardiomyocytes or within the perfused hearts. However, endotoxin contamination of the recombinant TNF-α was <0.1 ng/ml. Finally, the use of endotoxin-free glassware and materials in our laboratory and a failure to detect measurable endotoxin levels in the perfusate suggest that the effects observed in our study were not endotoxin mediated.

In summary, the results of this study confirm that the effects of NO donors on cardiomyocyte integrity and myocardial function are concentration dependent. Low concentrations of NO donors produce cardioprotection, whereas high concentrations of NO donors exacerbate cytokine-mediated myocardial contractile depression. Further studies examining the specific cellular and molecular mechanisms by which NO and TNF-α alter cellular integrity and function are warranted.

This study was supported by the National Institute of General Medical Sciences Burn Center Grant GM-21681. Address for reprint requests and other correspondence: J.W. Horton, Dept. of Surgery, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9160 (E-mail: jureta.horton@email.swmed.edu).

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