Role of nitric oxide and cGMP in human septic serum-induced depression of cardiac myocyte contractility

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Kumar, Anand, Rupinder Brar, Peter Wang, Linda Dee, Greg Skorupa, Fadi Khadour, Richard Schulz, and Joseph E. Parrillo. Role of nitric oxide and cGMP in human septic serum-induced depression of cardiac myocyte contractility. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R265–R276, 1999.—Previous studies have demonstrated the existence of a circulating myocardial depressant substance during human septic shock. We have recently identified this substance as a synergistic combination of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). This study utilized an in vitro cardiac myocyte assay to evaluate the potential mechanistic role of nitric oxide (NO) and cGMP in depression of myocardial contractility induced by TNF-α, IL-1β, TNF-α + IL-1β (at low concentrations), and human septic shock serum (HSS). TNF-α, IL-1β, TNF-α + IL-1β, and each of 5 sera from patients with acute septic shock caused depression of both maximum extent and peak velocity of cardiac myocyte shortening and an increase in intracellular cGMP concentration during 30 min of exposure (minimum P < 0.01). NO synthetase (NOS) and guanylate cyclase inhibitors such as N-methyl-L-arginine (L-NMA) and methylene blue prevented these effects; an excess of L-arginine with L-NMA restored them (minimum P < 0.01). In contrast, D-arginine failed to reestablish cytokine-induced myocardial depression and cGMP accumulation prevented by L-NMA. Exposure of myocytes to TNF-α, IL-1β, or TNF-α + IL-1β produced a concentration-dependent increase in intracellular cGMP that paralleled the depression of cardiac myocyte contractility (minimum P < 0.001). In addition, TNF-α, IL-1β, TNF-α + IL-1β, or HSS application to cardiac myocytes resulted in increased NO gas generation, which was inhibited by L-NMA (minimum P < 0.01). Furthermore, unstimulated cardiac myocytes were shown to harbor constitutive but not inducible NOS activity. These data suggest that the sequential generation of NO by a constitutive NOS and cGMP by guanylate cyclase represents an important mechanism of cardiac myocyte depression by TNF-α, IL-1β, TNF-α + IL-1β, and the myocardial depressant substance(s) of septic shock.

myocardial depressant factor; cytokine; heart contractility; cyclic nucleotide; sepsis

SHOCK SECONDARY TO SEPSIS is a serious disorder with significant morbidity and mortality despite appropriate antibiotic and supportive therapy. The typical human cardiovascular response to septic shock is characterized by hypotension, decreased systemic vascular resistance, and elevated cardiac index. Recent studies using bedside radionuclide ventriculography and echocardiography have demonstrated that reversible myocardial depression manifested by reduction of both right and left ventricular ejection fractions and dilation of both ventricles is a common occurrence during human septic shock (44, 45).

Measurements of myocardial cell contraction in the presence of serum from patients with septic shock demonstrate a depression of maximum extent and peak velocity of myocyte shortening that correlates quantitatively and temporally with the depression of ejection fraction seen in the same patients (46, 48). This suggests the presence of a circulating myocardial depressant substance or substances (MDS) during the acute phase of septic shock that may be responsible for human sepsis-induced myocardial dysfunction (46, 48). Previous studies suggest that MDS may represent a protein or proteins in the 10- to 30-kDa-molecular weight range (34, 46, 48). The cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), fit the known physical characteristics of MDS. Serum levels of each are elevated during sepsis and septic shock (12, 25). Using an in vitro assay of cardiac myocyte function, we have recently demonstrated that cardiac myocyte depression can be produced by extremely low concentrations of TNF-α and IL-1β in combination, concentrations that are similar to those found circulating during human septic shock (39). In addition, we have shown that the myocardial depressant activity of human septic serum (HSS) obtained from patients with acute septic shock can be eliminated by the immunabsorption of TNF-α and IL-1β, suggesting a central role for these two cytokines in human septic myocardial depression (39).

Nitric oxide (NO) and cGMP are known to have significant physiological and pathophysiological roles in the vasculature. The role of NO and cGMP in the heart has not been as well defined. Recent data suggest that NO and cGMP may play a substantial part in physiological regulation of cardiac contractility (2, 3, 9, 17, 28, 30, 31, 36, 41, 42, 55, 61). Investigators have also shown that cytokine and endotoxin-mediated depression of contractility of in vitro myocardial tissue may be produced via an NO- and cGMP-dependent mechanism (4, 5, 8, 21, 22, 37, 40, 53). In contrast, other data argue against a role for NO in either the physiological regulation of cardiac contractility (67) or in endotoxin- or cytokine-driven pathophysiological myocardial depression (18, 68).
Given this conflicting data, this study was designed to evaluate the possible mechanistic role of NO and cGMP in early cardiac myocyte depression induced by TNF-α, IL-1β, low concentrations of TNF-α + IL-1β together, and, in particular, HSS containing MDS activity.

METHODS

Tissue culture and other methods utilized have been previously described (39, 46, 52). Spontaneously beating newborn rat myocardial cells were established using a modification of the technique described by Harary and Farley (29). Standard growth media consisted of 25% HEPES-buffered Medium 199 (GIBCO Laboratories, Grand Island, NY) and 10% heat-inactivated FCS (Sigma Chemical, St. Louis, MO) diluted in an l-arginine (L-Arg)-free balanced salt solution and supplemented with glutamine (Sigma Chemical), penicillin (Sigma Chemical), and streptomycin (Sigma Chemical). Medium 199 and FCS contained 0.33 and 0.21 mM L-Arg, respectively. The final concentration of L-Arg in standard growth media was 0.1 mM.

Latex microbeads were introduced into the culture of spontaneously beating myocytes and affixed themselves to cell membranes. Between 7 and 10 days after plating, the petri dish containing myocardial cells was fastened to the heated (37°C) stage of an inverted-optics, phase-contrast microscope attached to a side-arm video camera. A custom-built electronic tracking system was used to quantitate the movement of a latex bead selected from the many beads attached to the membranes of beating myocytes. The typical maximum initial extent of rhythmic displacement of the bead was between 5 and 8 µm, depending on the length of the myocyte to which it was adherent. The signal was relayed to an intervening electronic instrument (which derived peak contraction velocity from rate of change of bead displacement) and a two-channel strip chart recorder that printed an analog recording of the extent and velocity of bead displacement. To ensure a fixed contraction frequency, a custom-built alternating current electrical pulse generator was utilized to pace myocytes (12 V, maximum 40 mA, 0.7- to 7-ms pulse duration) at 1 Hz. The minimum current and pulse duration required to effectively pace cardiac myocytes were utilized for each experiment.

Each individual assay was performed as follows. After application of fresh growth media, plates were mounted on the microscope stage and myocytes were paced at 60 contractions/min. An appropriate bead was located, and the extent and velocity of myocyte shortening were measured for 5 min (baseline contractility). If the extent of cell contraction was stable (maximum 2.5% variation over 5 min), 1 µl of test solution was added. Test media used either 10% newborn calf serum (NCS) (GIBCO Laboratories) with the addition of designated concentrations of cytokines or 10% HSS in place of NCS. Control studies were performed with 10% NCS alone. After replacement of fresh growth media with test or control media, measurements of maximum extent and peak velocity of myocyte shortening were obtained every 5 min for 30 min. All pipettes, plates, and other equipment used for preparation, culture, or testing of cardiac myocytes was endotoxin-free and disposable. All liquid media contained <1 pg/ml endotoxin. FCS used for growth media contained no detectable endotoxin. NCS used in test media contained 0.48 ng/ml endotoxin. All recombinant cytokines contained <50 pg endotoxin/µg cytokine. Culture media, cytokine solutions, and other test solutions were tested for endotoxin content using a quantitative, chromogenic Limulus amebocyte lysate assay with a detection limit of 5 pg/ml (Whitaker M. A. Bioproducts, Walkersville, MD). TNF-α and IL-1β concentrations in human sera were determined using an enzyme-linked immunosorbent assay (TNF-α by T Cell Science, Boston, MA; IL-1β by Citron Biotechnologies, Pine Brook, NJ) according to the instructions of the manufacturer.

Effect of N-methyl-l-arginine and methylene blue on contractility of cardiac myocytes exposed to TNF-α, IL-1β, TNF-α + IL-1β, and HSS. TNF-α (50 ng/ml) (Sigma Chemical), IL-1β (500 ng/ml) (Sigma Chemical), TNF-α (0.05 ng/ml) + IL-1β (2.0 ng/ml), and HSS were tested. The TNF-α concentrations match those used in a previous series of studies (39). The specified individual concentrations of TNF-α and IL-1β were used because they are within the range of concentrations found circulating during human septic shock (13, 25, 64). Similarly, human septic shock is commonly associated with the very low combined concentrations of TNF-α and IL-1β used in the experiment (13, 25, 64). HSS was obtained from 5 patients with acute septic shock and marked reversible myocardial depression (mean left ventricular ejection fraction 26 ± 7% (SD) during septic shock and 55 ± 7% either before septic shock or after recovery; absolute mean decrease 28 ± 10%). Each HSS sample had previously been confirmed to exhibit marked myocardial depressant activity in this in vitro assay. The primary test solutions consisted of either standard test media (10% NCS) plus the specified concentrations of TNF-α and/or IL-1β or standard media using 10% HSS in place of 10% NCS. Additional test solutions were composed of primary test solution plus 1) 10 µM N-methyl-l-arginine (L-NMA), 2) 10 µM L-NMA + 25 mM L-Arg, 3) 10 µM L-NMA + 25 mM d-arginine (d-Arg) (not done with HSS), or 4) 2.5 µM methylene blue (MeB). In the case of TNF-α + IL-1β, L-NMA at 1 and 5 µM were also run. Furthermore, a panel of control samples containing 1) 10 µM L-NMA, 2) 25 mM L-Arg, 3) 10 µM L-NMA with 25 mM L-Arg, 4) 25 mM d-Arg, and 5) 2.5 µM MeB were also tested for myocardial depressant activity.

To minimize variability, complete sets of experiments (control and all test solutions) were done on the same day using cardiac myocytes from the same litter.

cGMP and cAMP content of cardiac myocytes. Two sets of experiments were performed. Increasing concentrations of TNF-α [0, 0.125, 0.05, 0.2, 0.8, 3.2, 12.5, 25, 50, and 100 ng/ml (n = 4 each)], IL-1β [0, 2, 8, 32, 125, and 1,000 ng/ml (n = 4 each)], and TNF-α + IL-1β [0.0, 0.003:0.125, 0.0125:0.5, 0.05:2, and 0.2:8 ng/ml (n = 4 each)] were placed on cardiac myocytes for 30 min. Following a rapid wash with calcium- and magnesium-free phosphate-buffered saline, myocytes were frozen by direct exposure to liquid nitrogen and then stored at −70°C. In addition, randomly chosen plates from each test and control group of the previously described contractility experiments (TNF-α, IL-1β, TNF-α + IL-1β, or HSS and/or combinations of L-NMA, L-Arg, d-Arg, and MeB) were similarly frozen and stored (n = 4 each).

cGMP and cAMP concentrations were measured using the Biotrak cAMP enzyme-immunoassay system (dual range) according to the protocol of the manufacturer (Amersham International). Frozen tissue culture samples in 35-mm tissue culture petri dishes were rapidly thawed by the application of a 1-ml volume of room-temperature assay buffer (0.05 M sodium acetate, pH 5.8) containing 4 mM EDTA for phosphodiesterase inhibition. Cells were scraped (as an intact tissue sheet) and immediately immersed in boiling water for 10 min to denature and precipitate protein (15). Following centrifugation at 3,000 g (4°C) for 10 min, 100 µl of supernatant were utilized for the nonacetylation protocol instructions of the manufacturer.
Determination of NO synthetase presence and activity. Routinely cultured neonatal rat cardiac myocytes were grown to confluence. Between day 7 and day 10, when myocytes appeared to exhibit the density and robust spontaneous beating typical of cells used for assay of contractility, they were incubated in 30 min with 10% NCS alone; 10% NCS with 50 ng/ml TNF-\(\alpha\), 500 ng/ml IL-1\(\beta\), or 0.05 ng/ml TNF-\(\alpha\) + 2.0 ng/ml IL-1\(\beta\); and 10% HSS in place of 10% NCS (n = 3 each). Subsequently, myocytes were washed with phosphate-buffered saline, scraped, spun into a cell pellet (−0.25 g each), frozen with liquid nitrogen, and stored at −75°C. A similar number of myocytes were also harvested before the 30-min incubation with test or control media. Calcium-dependent and -independent NO synthetase (NOS) activity was determined by measuring the conversion of L-[\(^{14}\)C]Arg to L-[\(^{14}\)C]citrulline (detection limit <0.1 pmol citrulline/mg protein−1·min−1) as described by Schulz and colleagues (53, 54).

To determine whether detectable NO was being produced by cardiac myocytes exposed to TNF-\(\alpha\), IL-1\(\beta\), both cytokines together, and HSS, cardiac myocytes were grown to confluence in 25-ml tissue culture flasks in standard growth media. Cytokine test media solutions consisted of standard test media (10% NCS) with 50 ng/ml TNF-\(\alpha\), 500 ng/ml IL-1\(\beta\), or 0.05 ng/ml TNF-\(\alpha\) + 2.0 ng/ml IL-1\(\beta\). Another three test solutions contained 10% HSS from three patients in place of 10% NCS. Additional test solutions contained the same cytokine or HSS concentrations with 10 \(\mu\)M L-NMA. The positive control was standard media with 10% NCS ± 10 \(\mu\)M carbamylcholine (Sigma Chemical). Headspace NO gas concentration was measured using a modification of the techniques described by Archer et al. (1) and Brien et al. (10). After washing with phosphate-buffered saline, the test solution was introduced. The flask headspace was flushed for 5 min with an NO-free gas mixture of 75% nitrogen, 20% oxygen, and 5% carbon dioxide, and an airtight rubber cap was placed on top. Ten milliliters of headspace gas were aspirated from the flask before and after 30-min incubation with the test solutions (n = 4 each). NO in the sample was determined by chemiluminescence using a NO gas analyzer (model 280A; Sievers Instruments, Boulder, CO) with a detection limit of 5 parts per billion. NO gas production was assessed by calculating the difference between headspace NO gas concentration before and 30 min after introduction of the test solution.

Statistical analysis. By comparing the maximum extent and peak velocity of shortening at each 5-min interval to the baseline value, changes were referenced to initial contractility. Data for the change in maximum extent and peak velocity of cardiac myocyte shortening (percentage change from baseline) were pooled and plotted as a function of time for each control and test solution concentration. Linear regression analysis was utilized to fit a line for each resulting plot. The slopes of these lines were compared with the slope of the line for the control solution by a two-tailed Student’s t-test to determine whether these slopes were significantly different. In this manner, increased depressant activity was indicated by a more negative value for slope of the regression line. Where appropriate, a Bonferroni adjustment for multiple comparisons was made as indicated in the legends to Figs. 1–7.

For studies of cGMP content derived from cardiac myocytes exposed to increasing concentrations of TNF-\(\alpha\) and/or IL-1\(\beta\), individual cGMP values were plotted as a function of cytokine concentration. Logistic regression was utilized to fit a line to the data and the slope of that line compared with a theoretical zero slope by Student’s t-test. A significant slope indicated the existence of a concentration-dependent effect on intracellular cGMP content.

Multivariate analysis using data for all time points and concentrations was used to determine whether a concentration-dependent reversal of TNF-\(\alpha\)-mediated depression was caused by increasing concentrations of L-NMA.

All other analyses used Student’s t-test with a Bonferroni adjustment for multiple comparisons where appropriate (as indicated in the legends to Figs. 1–7).

RESULTS

Contractility, NO production, and cyclic nucleotide content of TNF-\(\alpha\)-stimulated cardiac myocytes. Figure 1A shows cardiac myocyte contractility (expressed as change in maximum extent of myocyte shortening) as a function of time for cells exposed to control media, TNF-\(\alpha\), TNF-\(\alpha\) + L-NMA, TNF-\(\alpha\) + L-NMA + L-Arg, TNF-\(\alpha\) + L-NMA + D-Arg, or TNF-\(\alpha\) + MeB. The same data are also shown in Fig. 1B, where the slopes of the regression lines fit to the data for each test group seen in Fig. 1A are plotted. Control media (10% NCS) caused relatively little decrease of contractility (decreased maximum extent of cardiac myocyte shortening). TNF-\(\alpha\) resulted in marked depression compared with the control (P < 0.005). The combination of TNF-\(\alpha\) with L-NMA had depression similar to control (P < 0.001 vs. TNF-\(\alpha\)). L-Arg in combination with TNF-\(\alpha\) and L-NMA reestablished depression compared with either control (P < 0.001) or TNF-\(\alpha\) + L-NMA (P < 0.001). D-Arg with TNF-\(\alpha\) and L-NMA did not reestablish depression. Similar to L-NMA with TNF-\(\alpha\), MeB with TNF-\(\alpha\) reversed TNF-\(\alpha\)’s depressant activity (P < 0.01 vs. TNF-\(\alpha\)). Data for peak velocity of cardiac myocyte shortening were entirely parallel and similarly significant (not shown).

Intracellular cGMP concentrations of cardiac myocytes from the preceding experiment are shown in Fig. 1C. TNF-\(\alpha\) resulted in a significant increase of intracellular cGMP concentration compared with control media (P < 0.005). The addition of L-NMA prevented this increase (P < 0.005 vs. TNF-\(\alpha\)). L-Arg (but not D-Arg) combined with TNF-\(\alpha\) and L-NMA reestablished the increased cGMP concentration (P < 0.001 vs. control, P < 0.001 vs. TNF-\(\alpha\) + L-NMA). MeB with TNF-\(\alpha\) also prevented the increase in cGMP seen with TNF-\(\alpha\) (P < 0.005 vs. TNF-\(\alpha\)). Intracellular cAMP levels were similarly assessed but did not show any significant response to the interventions (not shown).

In addition, it having been previously demonstrated that TNF-\(\alpha\) produces a concentration-dependent decrease in cardiac myocyte contractility (39), the effect of increasing concentrations of TNF-\(\alpha\) on intracellular cGMP concentration of cardiac myocytes was assessed (Fig. 1D). TNF-\(\alpha\) induced a highly concentration-dependent increase in cGMP concentration (\(r^2 = 0.63, P < 0.001\)), which paralleled the previously described decrease in cardiac myocyte contractility (39).

NO production by myocytes stimulated with standard media alone, carbamylcholine (positive control), and TNF-\(\alpha\) is displayed in Fig. 2. Significantly more NO...
was detected in headspace gas above myocytes incubated with TNF-α for 30 min than myocytes not exposed to TNF-α (P < 0.01) or myocytes exposed to TNF-α + L-NMA (P < 0.01).

Contractility, NO production, and cyclic nucleotide content of IL-1β-stimulated cardiac myocytes. Figure 3A shows cardiac myocyte contractility (expressed as change in maximum extent of myocyte shortening) as a function of time for cells exposed to control media, IL-1β, IL-1β + L-NMA, IL-1β + L-NMA + L-Arg, IL-1β + L-NMA + D-Arg, or IL-1β + MeB. This data is also shown in Fig. 3B, where the slopes of the regression lines fit to the data for each group are plotted. Control media (standard media) again caused relatively little decrease of maximum extent of cardiac myocyte shortening. IL-1β resulted in marked depression of maximum extent of cardiac myocyte shortening in comparison to the control (P < 0.001). The combination of IL-1β and L-NMA exerted depression similar to control (P < 0.001 vs. IL-1β). L-Arg (but not D-Arg) in combination with IL-1β + L-NMA reestablished depression compared with either control (P < 0.001) or IL-1β + L-NMA (P < 0.001). Similar to L-NMA, MeB reversed IL-1β's depressant activity (P < 0.001 vs. IL-1β). Data for peak velocity of cardiac myocyte shortening was entirely parallel, with similar values for significance (not shown).

Cardiac myocyte cGMP concentrations from the experiment are shown in Fig. 3C. Similar to the TNF-α experiment, IL-1β resulted in a significant increase of intracellular cGMP concentration compared with control media (P < 0.001). The addition of L-NMA pre-
vented this increase (P < 0.001 vs. IL-1β). L-Arg (but not D-Arg) in combination with IL-1β + L-NMA reestablished the increased cGMP concentration (P < 0.005 vs. control, P < 0.01 vs. IL-1β + L-NMA). MeB with IL-1β also prevented the increase in cGMP seen with IL-1β (P < 0.005 vs. IL-1β). Again, intracellular cAMP levels were similar in all groups (not shown).

The effect of increasing concentrations of IL-1β on intracellular cGMP concentration of cardiac myocytes was assessed (Fig. 3D). In parallel to our previous finding of concentration-dependent IL-1β-mediated cardiac myocyte depression, IL-1β also induced a highly concentration-dependent increase in cGMP concentration (r² = 0.83, P < 0.001).

Figure 2 shows NO production by myocytes stimulated with IL-1β. Significantly more NO was detected in headspace gas above myocytes incubated with IL-1β for 30 min than myocytes exposed to standard test media without IL-1β (P < 0.01) or myocytes exposed to IL-1β in the presence of L-NMA (P < 0.01).

Contractility, NO production, and cyclic nucleotide content of cardiac myocytes exposed to TNF-α + IL-1β. Figure 4A shows the slopes of the regression lines representing change in myocyte shortening as a function of time for myocytes exposed to TNF-α (0.05 ng/ml) + IL-1β (2.0 ng/ml) in combinations as specified with L-NMA (10, 5, and 1 µM), L-Arg (25 mM), D-Arg (25 mM), and MeB (2.5 µM). The combination of TNF-α and IL-1β resulted in substantial myocyte depression compared with myocytes exposed to control media (P < 0.001). The addition of increasing concentrations of L-NMA serially reduced the depressant effect of the cytokine combination, with 10 µM abrogating the effect (P < 0.001 vs. TNF-α + IL-1β). A highly significant concentration-response relationship was demonstrated to exist (P < 0.0001). L-Arg (but not D-Arg) in combination with TNF-α/IL-1β + L-NMA reestablished depression in comparison to both control (P < 0.001) and TNF-α/IL-1β + L-NMA (P < 0.001). MeB also reversed the depressant activity of the cytokine combination (P < 0.001 vs. TNF-α/IL-1β). The findings for peak velocity of cardiac myocyte shortening data were similar (not shown) and exhibited similar values for significance.

Cardiac myocyte cGMP concentrations from the experiment are shown in Fig. 4B. Thirty minutes of cardiac myocyte exposure to TNF-α/IL-1β generated a significant increase of intracellular cGMP concentration (P < 0.001 compared with control). L-NMA at 10 µM prevented this increase (P < 0.001 vs. TNF-α/IL-1β). L-Arg (but not D-Arg) with TNF-α/IL-1β + L-NMA restored the increased cGMP concentration (P < 0.005 vs. control, P < 0.001 vs. TNF-α/IL-1β + L-NMA). MeB also prevented the increase in cGMP...
levels seen with the cytokine combination (P < 0.005 vs. TNF-α/IL-1β). Myocyte intracellular cAMP levels were not significantly different (not shown).

The effect of increasing concentrations of TNF-α and IL-1β on intracellular cGMP concentration of cardiac myocytes is demonstrated in Fig. 4C. A highly concentration-dependent increase in cGMP concentration is shown to exist in association with increasing concentrations of a combination of TNF-α and IL-1β (r² = 0.62, P < 0.001). This finding supports data in a previous manuscript (39) in which these same increasing cytokine concentrations resulted in increasing amounts of cardiac myocyte contractility depression.

As with TNF-α and IL-1β individually, significantly more NO was detected in headspace gas above myocytes incubated with the combination of TNF-α and IL-1β than control myocytes (P < 0.01) or myocytes exposed to the cytokine combination in the presence of L-NMA (P < 0.01) (Fig. 2).

Contractility, NO production, and cyclic nucleotide content of HSS-stimulated cardiac myocytes. The effects of 10% HSS alone and in combination with 10 µM L-NMA, 10 µM L-NMA + 25 mM L-Arg, and 25 µM MeB on cardiac myocyte contractility are shown in Fig. 5, A–E. In contrast to the TNF-α and IL-1β experiments, only the regression-derived slopes derived from the plots of maximum extent and peak velocity of cardiac myocyte shortening as a function of time are shown. Similar results were found with each of the five HSS samples. In each case, 10% HSS caused significant depression of both extent and velocity of shortening compared with controls (minimum P < 0.001). L-NMA prevented this depressant effect (minimum P < 0.01 vs. HSS, but P = NS vs. control), whereas L-Arg reestablished it (minimum P < 0.001 vs. control, P < 0.01 vs. TNF-α + L-NMA). Similar to L-NMA, MeB reversed the depressant effects of HSS in each of the 5 sera tested (minimum P < 0.01 vs. HSS).

Three of the five patient sera (HSS) were assayed for cGMP (Fig. 6, A–C) and cAMP (not shown). Results were again similar in each case. HSS-exposed cardiac myocytes demonstrated a significant increase in intracellular cGMP content compared with myocytes exposed to the cytokine combination in the presence of L-NMA (P < 0.01) (Fig. 2).

NO production by HSS is demonstrated in Fig. 2. Overall, more NO was detected in headspace gas above HSS-stimulated cardiac myocytes than above myocytes...
exposed to control nonseptic serum (P < 0.01) or myocytes exposed to HSS with L-NMA (P < 0.01).

Effect of L-NMA, L-Arg, D-Arg, and MeB on contractility and cyclic nucleotide content of cardiac myocytes. The individual effects of L-NMA, L-Arg, L-NMA + L-Arg, D-Arg, and MeB on cardiac myocyte contractility are shown in Fig. 7A. None of these agents alone had a significant effect on either maximum extent or peak velocity (not shown) of cardiac myocyte shortening. No substance or combination of substances exerted a depressant or inotropic effect relative to control media. Similarly, Fig. 7B demonstrates that none significantly altered intracellular cGMP concentrations compared with control media. cAMP concentrations were similar in all groups also (not shown).

NOS activity in myocardial tissue. Constitutive NOS (cNOS) activity was measured in myocytes from 7- to 10-day-old rat cardiac myocytes cultures. Samples were obtained from three separate harvests. The mean calcium-dependent (cNOS) activity before incubation with control or test media was 4.23 ± 0.11 (SE) pmol L-citrulline·mg protein⁻¹·min⁻¹. Calcium-dependent (cNOS) activity following 30-min incubation with control media (10% NCS) or test media containing either 10% NCS with specified concentrations of TNF-α and/or IL-1β or 10% HSS in place of 10% NCS was not significantly different from baseline. Concurrent mean calcium-independent [inducible NOS (iNOS)] activity was 0.63 ± 0.09 pmol·mg protein⁻¹·min⁻¹ in the preincubation (baseline) sample. Values for postincubation test and control samples were not significantly different.

Endotoxin and cytokine levels in test media. The mean ± SE concentrations of TNF-α, IL-1β, and endotoxin in HSS samples were 72 ± 6, 167 ± 18, and 440 ± 120 pg/ml, respectively. Media containing 10% NCS, including those with cytokines, consistently demonstrated endotoxin concentrations between 40 and 65 pg/ml (comparable to 10% HSS). Growth media with 10% FCS demonstrated endotoxin concentrations below the limit of detection.
The major finding of this study is that the in vitro myocyte depressant activity of the circulating MDS of human septic shock [which we have previously identified as a synergistic combination of TNF-α and IL-1β (39)] is mediated, at least in part, through the sequential generation of NO by cNOS and cGMP by soluble guanylate cyclase. In addition, we have shown that the depressant activity of TNF-α, IL-1β, and a combination of TNF-α and IL-1β together (at concentrations comparable to those found in serum during human septic shock) is also mediated by the sequential generation of NO and cGMP. Although others have previously demonstrated that early TNF-α-induced cardiac myocyte depression may be NO dependent (22), early (as opposed to late) IL-1β-mediated depression has not been linked to NO and cGMP. Perhaps most importantly, this study provides the first direct evidence of a pathogenetic link between human septic serum-induced depression of myocardial tissue and the generation of NO and cGMP.

Our conclusions are supported by a number of our observations. Human septic serum, TNF-α, IL-1β, and low concentrations of TNF-α + IL-1β together each result in a depression of maximum extent and peak...
velocity of cardiac myocyte shortening. Along with contractility depression, each also causes a parallel increase in measurable intracellular cGMP. L-NMA, a competitive inhibitor of both cNOS and iNOS, prevents both depression of contractility and accumulation of intracellular cGMP. L-Arg, the natural substrate of NOS, reestablishes both. d-Arg (the NOS-insensitive dextro-isomer) fails to reestablish cardiac myocyte depression and cGMP accumulation in cardiac myocytes exposed to either TNF-α or IL-1β (alone or together) with L-NMA. MeB, an irreversible inhibitor of both NOS and guanylate cyclase, prevents depression of myocyte contractility and intracellular accumulation of cGMP induced by TNF-α, IL-1β, TNF-α + IL-1β, or HSS. These findings occur in the absence of any significant changes in intracellular cAMP concentrations.

The potential role of cGMP in cytokine-induced cardiac myocyte depression is reinforced by the demonstration that concentration-dependent increases of intracellular cGMP induced by TNF-α, IL-1β, or both parallel the concentration-dependent decreases of cardiac myocyte contractility that we have demonstrated previously (39). The role of NO is supported by the demonstration that cultured cardiac myocytes exhibit substantial NOS activity. It is further buttressed by observations that show that cardiac myocytes stimulated with TNF-α, IL-1β, both together, or HSS produce detectable NO gas in the culture flask headspace and that this NO production is inhibited by the presence of L-NMA.

We have previously demonstrated the finding of early (<10 min) depression of HSS-exposed cardiac myocytes (46, 48). More recently, we have shown that TNF-α, IL-1β, both TNF-α and IL-1β together (at extremely low concentrations consistent with human septic shock), and supernatants of activated macrophages individually depress cardiac myocyte contractility within the same period (38, 39). This rapid timeframe for depressant response has been supported by other in vitro studies of myocardial tissue, which also demonstrate the existence of early depression following cytokine exposure (22, 37, 58, 68). This would appear to be too short a period for de novo synthesis of iNOS. This finding of early NO-sensitive myocyte depression along with early NO production argues strongly that cNOS (as opposed to the inducible form) is involved in NO generation in this model of septic myocardial depression. This position is reinforced by our demonstration that cultured cardiac myocytes exhibit significant calcium-dependent but not calcium-independent NOS activity. Calcium-dependent NOS activity is characteristic of cNOS isoforms, whereas calcium-independent activity is typical of inducible NOS isoforms. The demonstration that measurable cardiac myocyte cNOS and iNOS activity is unchanged following incubation with TNF-α, IL-1β, TNF-α and IL-1β together, and HSS is also consistent with cNOS, rather than iNOS, mediated NO generation. cNOS activity, which is measured under ideal conditions in the L-citrulline assay, is expected to be unchanged despite increased NO generation because cellular cNOS activity is substantially regulated by substrate and cofactor conditions rather than fixed structural modifications or increases in cellular enzyme content. In contrast, assayed iNOS activity would be expected to increase with enhanced iNOS cellular activity because cellular iNOS activity is primarily a function of the amount of enzyme present (53, 54).

The probable clinical relevance of early septic serum-induced cNOS-dependent cardiac myocyte depression is supported by the significant correlation Parrillo and colleagues (46, 48) have demonstrated between the in vivo depression of left ventricular ejection fraction among patients with acute septic shock and the in vitro myocardial depressant activity of the same patients’ sera. Although it cannot be concluded that cNOS-dependent MDS activity (which appears to be responsible for early depressant effects in vitro) is solely responsible for relatively prolonged (up to 7–10 days) myocardial depression seen during human septic shock, an association between in vitro septic serum-induced cardiac myocyte depression and clinical septic myocardial dysfunction is clear. Supporting the potential clinical relevance of MDS-induced early cardiac myocyte depression are in vivo canine studies that confirm the ability of TNF-α to induce early myocardial depression (within 1 h following initiation of infusion) (20, 66). This myocardial depression occurs too early to be caused by de novo iNOS generation. It is, however, consistent with cytokine-stimulated cNOS activity.

Until recently, few data have been available regarding the intracellular mechanisms underlying septic myocardial depression. Our observations, like those of several others (4, 8, 9, 22, 54), generally support the existence of direct NO-mediated depression of myocardial tissue by a myocardial NOS stimulated by endotoxin or inflammatory cytokines. Our work, however, also addresses the specific role of myocardial cNOS in cytokine and HSS-induced depression of myocyte contractility. Like ourselves, several investigators have also noted early-onset (<30 min) depression of myocardial tissue exposed to cytokines in vitro, consistent with NOS involvement (22, 26, 68). In particular, Finkel and colleagues (22) have shown early NO-dependent depression of guinea pig papillary muscle exposed to cytokines, including TNF-α, IL-2, and IL-6. Goldhaber and colleagues (26) have similarly shown that high concentrations of TNF-α caused an early NO-dependent depression of cardiac myocyte contractility. The rapidity of the effect in each study suggests cNOS involvement. Yokoyama and colleagues (68), however, have suggested that early TNF-α-induced depression of adult cardiac myocytes is not dependent on NO. That same group (43) has recently implicated sphingosine in the immediate myocardial depressant effects of TNF-α in adult feline myocardial cells, a finding recently supported by Cain et al. (11) using the combination of TNF-α and IL-1β with human myocardial tissue. The reason for the divergent results are not clear. No evidence yet links the NO and sphingosine signaling pathways. Potential differences that might account for the divergent results include the model used (differing species and ages of animals from which cardiac tissue
was obtained) and differing NOS inhibitors and concentrations of those inhibitors. It is also possible that myocyte pacing frequency may play a role in cNOS activity in some studies. However, the pacing rate of 1 Hz used in this study appears to be well below the 3-Hz threshold that Kaye et al. (36) have suggested is required to demonstrate pacing-induced cNOS activity.

In contrast to early, presumably cNOS-dependent myocardial depression, a series of other studies have tended to support only later-onset (hours to days) cytokine-driven depression of myocardial contractility during inflammatory myocardial dysfunction (4, 14, 19, 21, 27, 33, 63). Several investigators have shown that exposure of myocardial tissue in vitro to TNF-α, IL-1β, and supernatants from activated macrophages (which contain cytokines including TNF-α and IL-1β) results in depression of contractility following a delay of several hours or days (4, 14, 19, 21, 27, 33, 63). Disruption of β-adrenergic signal transduction due to an alteration in G protein interactions has been implicated as a cause of this phenomenon by one group of investigators (14, 27). The existence of increased inhibitory G protein subunits resulting in potential adrenoceptor dysfunction has been confirmed in catecholamine-resistant human septic shock (7, 49). The generation of iNOS, NO, and cGMP in response to IL-1β, TNF-α with IL-1β, and supernatants of activated macrophages after a period of several hours has also been implicated in later-onset depression of both baseline and isoproterenol-stimulated contractility of in vitro myocardial preparations (4, 5, 21, 33, 50, 53, 56, 57, 62, 63). Similarly, in vivo endotoxia results in the production of an iNOS and NO in the myocardium, which is associated with a decrease in contractility of excised myocardial tissue (8, 16, 53). Recent data suggest that iNOS-generated NO in these circumstances may act via cholinergic modulation of the inotropic response to β-adrenergic stimulation (3, 28, 30, 31, 65). Both mechanisms of late depression (G protein alteration and iNOS induction), although not mutually exclusive of each other, appear to be distinct from those causing early depression of cytokine-stimulated myocardial tissue in vitro.

A study by Kinugawa and colleagues (37) may help to reconcile these divergent findings of early versus late NOS-dependent myocardial depressant activity in models of sepsis. In their avian cardiac myocyte model, they were able to demonstrate both early (<30 min) and late (24 h) cardiac myocyte depression following IL-6 exposure. Myocyte depression appeared to be related to sequential cNOS activation followed by later iNOS generation. Such a possibility is also indirectly supported by evidence that suggests cNOS may potentially contribute to early septic or cytokine-mediated vascular dysfunction (23, 51, 59) even though iNOS is thought to be responsible for sustained septic vascular dysfunction (6, 32, 35, 47). The precise mechanism by which cytokines, supernatants of activated macrophages, and septic serum might stimulate myocardial cNOS remains undefined at this time. Potential mechanisms include those postulated for endothelial cNOS stimulation by shear stress, histamine, and vascular endothelial growth factor [heat shock protein 90 (24)] or by endotoxin (51) [endothelium-derived kinins (23) or platelet-activating factor (60)].

Clinical septic myocardial depression may represent a biphasic process involving cytokine/MDS-stimulated cNOS production of NO in the early phase, followed by cytokine/MDS-driven induction of iNOS in the later phase. Both processes may culminate in myocardial depression through the stimulation of guanylate cyclase and the production of cGMP, a nucleotide with known myocardial depressant functions. The association of MDS-produced early-onset cardiac myocyte depression in vitro (decreased maximum extent and peak velocity of shortening) with relatively prolonged clinical myocardial depression in vivo in septic patients (as measured by ventricular ejection fraction) (46, 48) can be explained by a dual action of the circulating myocardial depressant substance of sepsis (i.e., TNF-α/IL-1β) in both activating myocardial cNOS (early myocardial depressant effects) and stimulating myocardial iNOS production (prolonged myocardial depressant effects).

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