Nitric Oxide-Dependent and -Independent Mechanisms are Involved in

TNFα-Induced Depression of Cardiac Myocyte Contractility

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Abstract:

Previous studies have demonstrated the presence of myocardial depression in clinical and experimental septic shock. This response is mediated, in part, through circulating TNFα-induced, nitric oxide-dependent, early (< 1 hr) depression of basal myocyte contractility. Other mechanisms of early myocardial dysfunction involving decreased response to adrenergic stimulation may exist. This study evaluated the presence and nitric oxide-dependence of impaired adrenergic response to TNFα in in-vitro cardiac myocytes. The contraction of electrically-paced neonatal rat cardiac myocytes in tissue culture was quantified using a closed loop video tracking system. TNFα induced depression of baseline contractility over the first 20 minutes of cardiac myocyte exposure. This effect was blocked by N-methyl-arginine (NMA), a nitric oxide synthetase inhibitor, in all studies. Contractile and cyclic AMP response to increasing concentrations of isoproterenol was deficient in cardiac myocytes exposed to TNFα irrespective of the presence of NMA. In contrast, increasing concentrations of forskolin (a direct stimulant of adenylate cyclase) and dibutyryl cyclic AMP (a metabolically active membrane-soluble analogue of cyclic AMP) completely reversed TNFα-mediated depression, though only in the presence of NMA. Forskolin-stimulated cyclic AMP generation remained intact irrespective of NMA. Increasing concentrations of exogenous calcium chloride, unlike other inotropic agents, corrected TNFα-mediated defects of contractility independent of the presence of NMA. These data suggest that TNFα exposure is associated with a second nitric oxide-independent but calcium-dependent early depressant mechanism that is
manifested by reduced contractile and cAMP response to β-adrenergic stimulation.
The normal cardiac response to septic shock involves biventricular dilatation and decreased ejection fractions (14,24,25). Clinically, septic myocardial depression is also reflected by a suboptimal stroke volume response to fluid resuscitation (22) and infusion of exogenous catecholamines (15,20,32).

The causal role of circulating factors in myocardial depression in human septic shock is now well established (21,27-29,31). Measurements of isolated cardiac myocyte contraction in the presence of serum from patients with acute septic shock demonstrate a depression of maximum extent and peak velocity of myocyte shortening that correlates quantitatively and temporally with depression of the patient’s left ventricular ejection fraction as measured by radionuclide ventriculography (27,31). We have demonstrated that this depressant activity represents a synergistic combination of low circulating concentrations of the pro-inflammatory cytokines, TNFα and IL-1β (21). Each individual cytokine as well as combinations of the two reproduce the depressant effects of septic serum. Furthermore, elimination of TNFα and IL-1β eliminates the myocardial depressant activity of septic serum (21). This depressant effect appears to be mediated through a nitric oxide (NO)- and cyclic GMP-dependent mechanism (17). Co-incubation of TNFα, IL-1β, synergistic combinations of the two or human septic serum with competitive inhibitors of nitric oxide synthetase (NOS) attenuates or prevents depressant responses in isolated cardiac myocytes. TNFα also blunts generation of cyclic AMP and contractile response to catecholamine stimulation in these myocytes (18). This observation is congruent with evidence of sepsis-associated cardiovascular resistance to
adrenergic stimulation noted in a variety of clinical and experimental studies of sepsis and septic shock (15,16,20,26,33).

This study was designed to further examine the relationships between defects of adrenoreceptor signaling and NO generation in early (<1 hr) myocyte depression induced by TNFα. Specifically, we sought to determine the subcellular location of the defect in β-adrenoreceptor signaling induced by TNFα and whether or not the defect is related to NO synthesis.

**Materials and Methods:**
The methods utilized were a modification of those previously described (21,27). The basic myocyte assay is designed to measure the depression or enhancement of contractility of beating cardiac myocytes in cell culture in response to agents which are introduced into the growth medium.

**Myocyte Isolation and Culture:** Spontaneously beating newborn rat myocardial cells were established using a modification of the technique described by Harary and Farley(10). Using sterile technique, hearts from two day old Sprague Dawely rat pups were removed, pooled and minced into small blocks. The cells were disaggregated with 0.15% bovine pancreatic trypsin [Sigma Chemicals Co, St. Louis, MO] in a modified Hank's balanced salt solution (potassium, calcium and magnesium free) [Gibco Laboratories, Grand Island, NY]. Suspensions were centrifuged at 500 g for 15 minutes and plated into 35 X 10 mm petri dishes at a density of 300,000 cells/mL. Plating media (standard control media) consisted of 90% HEPES-buffered RPMI-1640 [Gibco Laboratories, Grand Island, NY] and
10% heat-inactivated newborn calf serum [Gibco Laboratories, Grand Island, NY] supplemented with glutamine [Sigma Chemical Co, St. Louis, MO], penicillin [Sigma Chemicals Co, St. Louis, MO] and streptomycin [Sigma Chemical Co, MO]. The solution pH was 7.35 in room air and approximately 7.30 in 5% CO₂. A second, custom-made modified version of this media was used for specified experiments involving calcium chloride as an inotropic agent. This media was calcium depleted (0.20 mM Ca²⁺) in comparison to normal media (1.57 mM Ca²⁺)[Gibco Laboratories, Grand Island, NY]. Previous experience has demonstrated that this modified media can be used in place of standard RPMI media and provides a comparable support for cardiac myocyte contractions.

After isolation, myocytes were incubated at 37°C in 5% CO₂. Media was changed every 48 hours. After plating, cells became confluent and began spontaneously beating within 4 days. Latex microbeads (Polysciences, Inc, Worthington PA) of 3 µM diameter were introduced into the culture after 4 days and affixed themselves to cell membranes. Beating cells were used for the assay between 5 and 10 days after plating. At this time, cardiac myocytes accounted for approximately 75-80% of the viable cells based on cell contraction in response to an electrical pulse.

All pipettes, plates and other equipment used for preparation, culture, or testing of cardiac myocytes were endotoxin-tested and disposable. All liquid media and other utilized solutions contained less than 1 pg/mL endotoxin with the exception of newborn calf serum which contained 0.48 ng/mL endotoxin. Human recombinant TNFα derived from yeast [Sigma Chemicals Co, St. Louis, MO] with
20,000 IU/µg L929 cytotoxicity assay activity was utilized for experiments. Recombinant TNFα contained less than 50 pg endotoxin/µg. Culture media and other test solutions were tested for endotoxin content using a quantitative, chromogenic Limulus amebocyte lysate assay [Whitaker M.A. Bioproducts, Walkersville, MD].

**Contractility Assessment:** Each petri dish containing beating 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 television monitor displayed the image of the target cells. A custom built electronic tracking system was used to quantify the rhythmic displacement of a latex bead selected from the many beads attached to the membranes of beating myocytes. The tracking system produced a signal proportional to the maximum extent of each contraction. In order to ensure a fixed contraction frequency, myocytes were paced using a custom built alternating current electrical pulse generator (12 V, maximum 40 mA, 0.7-7 millisecond pulse duration). The minimum current and pulse duration required to effectively pace cardiac myocytes was utilized for each experiment.

**Assay Protocol:** After plates were removed from the incubator, fresh growth media/standard control media was applied. Following 15 minutes equilibration to ambient pCO₂ and pO₂, plates were mounted on the microscope stage and myocytes were paced to 60 contractions/min. An appropriate bead was located and the maximum extent of myocyte shortening measured for 5 minutes.
(baseline contractility). If extent of cell contraction was stable (maximum 2.5% variation over 5 minutes), 1 mL of control media or control media containing either TNFα (50 ng/mL) or both TNFα (50 ng/mL) and N-methyl-arginine [NMA] (10 µM) was added. Subsequently, measurements of maximum extent of myocyte shortening were obtained every 5 minutes. After 20 minutes observation, 100 µL of control media containing one of the inotropic agents, isoproterenol, forskolin (a direct activator of adenylate cyclase), dibutyryl cyclic adenosine monophosphate [dBcAMP] (a membrane permeable, metabolically active analogue of natural cAMP), or calcium chloride was added and allowed to diffuse through the medium. Measurements of maximum extent of cardiac myocyte shortening were obtained every 5 minutes for an additional 15-20 minutes. The duration of observation for each test agent was based on preliminary experiments examining the time point of maximum response. Plates were not reused for additional experiments.

Isoproterenol [Abbott Laboratories, Chicago, IL] in 50 µL media was added to final (plate) concentrations of 0, 0.005, 0.025, 0.125, 0.5, and 2.5 µM (n=10 each group). Similarly, final concentrations for other inotropic agents were as follows: forskolin [Sigma Chemical Co, MO] 0, 10^{-5}, 10^{-3}, 10^{-1}, 10 µg/L (n=10 each group); dBcAMP [Sigma Chemical Co, MO] 0, 0.001, 0.1, 1 and 10 mM (n=10 each group); CaCl₂ [Sigma Chemical Co, MO] 0, 0.35, 0.7, 1.4, 2.8 mM (n=8 each group).

In separate experiments using an identical protocol, the cyclic adenosine monophosphate [cyclic AMP or cAMP] content of cardiac myocytes was
determined. These additional experiments were performed without observations of myocyte contractility (n=4 each group). At the time point at which maximum contractile response to isoproterenol or forskolin was observed, myocytes were washed with calcium and magnesium-free phosphate buffered saline, frozen by direct exposure to liquid nitrogen and then stored at -70°C.

Cyclic AMP concentrations were measured in duplicate using the Biotrak cAMP enzyme-immunoassay (EIA) system (dual range) according to the protocol of the manufacturer [Amersham International, UK]. 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 immersed in boiling water for 10 minutes to denature and precipitate protein (7). Following 3000g centrifugation (4°C) for 10 minutes, 100 μL of supernatant was utilized according to the non-acetylation protocol instructions of the manufacturer. Results were normalized to cell count.
Statistical Analysis

By comparing the maximum extent of myocyte shortening at each 5 minute interval to the baseline value, changes in contractility were referenced to initial contractility. In order to confirm the existence of TNF-induced cardiac myocyte depression, the change in maximum extent of cardiac myocyte shortening over the first 20 minutes (immediately before the specified inotropic agent was introduced) was examined. Percentage change from baseline was used as the dependent variable and was analyzed as a function of time for control and test solutions using analysis of variance (ANOVA). Paired comparisons at specific time points were performed using least square means.

To examine the inotropic response to each agent (isoproterenol, forskolin, dBcAMP and calcium chloride), the concentration-response relationship at the point of maximum response (for each inotrope) was assessed. Response curves (generated from the point of maximum response) for cells exposed to TNF ± NMA were compared to curves for myocytes exposed to control medium by analysis of covariance (ANCOVA). Second degree polynomial regression analysis was used to assess the existence of a concentration-dependent cAMP response to isoproterenol and forskolin and to compare the concentration-response curves for TNFα, TNFα with NMA and control media.
Results:
In each experimental set, TNFα alone generated depression of extent of shortening of cardiac myocytes over the first 20 minutes of exposure (i.e. prior to the introduction of any inotropic agent) (each group p<0.05, ANOVA). The presence of NMA, in each experimental group, abrogated this response.

1) Effects of Isoproterenol on Contractility and cAMP Generation Response of TNFα ± NMA-Exposed Cardiac Myocytes: Figure 1a shows cardiac myocyte contractility (expressed as maximum extent of myocyte shortening) for cells exposed to control or TNFα (50 ng/mL) ± NMA (10 µM)-containing media stimulated with 0.005 uM isoproterenol. Figure 1b shows the similar cells stimulated with 2.5 µM isoproterenol. The inotropic effect of isoproterenol peaked at approximately 5 minutes following introduction.

Figure 1c shows the response (change in maximum extent of cardiac myocyte shortening) of TNFα ± NMA-exposed cardiac myocytes to increasing concentrations of isoproterenol (at 5 minutes post-introduction). A significant isoproterenol concentration-cardiac myocyte contractility relationship was demonstrated for control and TNFα-exposed cardiac myocytes (p<.0001 and p=.021 respectively, ANCOVA). The response curve for TNFα + NMA-exposed myocytes did not reach statistical significance. Despite maximally tolerated concentrations of isoproterenol, the response curve for TNFα-exposed cardiac myocytes remained significantly offset from the response curve for control media alone (p<.0001, ANCOVA). TNFα-exposed cardiac myocytes demonstrated less
of an inotropic response to any given concentration of isoproterenol (p<.05 at any time point). Even at the highest concentration of isoproterenol tested (2.5 µM), TNFα-exposed cardiac myocytes fail to demonstrate maximum extent of cardiac myocyte shortening equal to myocytes not exposed to TNFα (p=.0002). Higher concentrations of isoproterenol resulted in significant toxicity (rhythmic perturbations and impaired myocyte contractility).

The isoproterenol concentration–contractile response for TNF + NMA-exposed myocytes was relatively flat across increasing concentrations compared to control myocytes (p=.0310, ANCOVA) (Figure 1c). The inclusion of 10 µM NMA with TNFα primarily affected contractility at absent or low concentrations of isoproterenol (basal contractility) (Figure 1a,c). Extent of contraction (shortening) in myocytes incubated with TNFα + NMA 5 minutes after introduction of 0 and 0.005 µM isoproterenol was unchanged compared to control-media exposed myocytes. However, increasing isoproterenol concentrations failed to elicit increases in extent of contraction shortening comparable to those seen in control media-exposed myocytes (Figure 1c). Correction of TNFα-induced depression by NMA was deficient at even maximally tolerated concentrations of isoproterenol (p=.0008) and the contractile response was quantitatively similar to myocytes exposed to TNFα alone (Figure 1b,c).

Intracellular cAMP concentrations of cardiac myocytes from the preceding experiment are shown in Figure 1d. Cells were harvested at 5 minutes following the introduction of isoproterenol. All groups (control media, TNF alone, TNF ± NMA) exhibited a significant positive isoproterenol concentration-dependent
increases in cAMP content (each p<.001, 2nd order polynomial regression). However, the cAMP response of TNFα- and TNFα + NMA-exposed cardiac myocytes to increasing isoproterenol concentrations was significantly blunted compared to controls (both p<.02, polynomial regression).

2) Effects of Forskolin on Contractility and cAMP Generation Response of TNFα-Exposed Cardiac Myocytes: Maximum response to forskolin occurred approximately 10 minutes after introduction. Increasing concentrations of forskolin generated a distinct contractile and cAMP response relative to isoproterenol (Figure 2). Figure 2a shows the response (change in maximum extent of cardiac myocyte shortening) of cardiac myocytes 10 min following the introduction of increasing concentrations of forskolin. Forskolin caused a concentration-dependent increase in myocyte contraction shortening across all groups (control, TNFα alone, TNFα + NMA; each p<0.02 ANCOVA). Similar to the isoproterenol experiments, TNFα alone caused significant depression of cardiac myocyte contractile response to forskolin throughout all tested forskolin concentrations (p<.0001, ANCOVA)(Figure 2a). In contrast to the results with isoproterenol, the presence of NMA with TNFα completely corrected the TNFα-induced defect of myocyte contractility. With the addition of NMA to TNFα, increasing concentrations of forskolin resulted in a response curve similar to control media alone.

Intracellular cAMP concentrations of cardiac myocytes from the forskolin studies are shown in Figure 2b. Cells were harvested at 10 minutes following the
introduction of forskolin. Similar to the results with isoproterenol, all groups demonstrated a highly forskolin concentration-dependent increase in intracellular cAMP concentration (p<0.001, 2nd order polynomial regression). However, unlike isoproterenol, there was no attenuation of the response in the presence of TNFα. All three response curves (control, TNFα alone, TNFα + NMA) were similar to each other.

3) Effects of Dibutyryl Cyclic AMP on Contractility Response of TNFα-Exposed Cardiac Myocytes: Peak contractile response to dBcAMP occurred 20 minutes after introduction. dBcAMP produced a maximal response pattern that was similar to forskolin (Figure 3). A concentration-dependent increase in extent of myocyte contraction was seen across all three groups (each p<0.005, ANCOVA). TNFα again caused a highly significant depression of depression of cardiac myocyte contraction response to dBcAMP across all concentrations (p<0.001, ANCOVA). In contrast to the response to isoproterenol and similar to forskolin, the presence of NMA with TNFα resulted in complete correction of the TNFα-induced defect of cardiac myocyte contraction. The contraction response curve to increasing concentrations of dBcAMP in the presence of TNFα + NMA was similar to the response curve in control media.

4) Effects of Calcium Chloride on Contractility Response of TNFα-Exposed Cardiac Myocytes: The contractile response to calcium chloride was maximal at 10 minutes post-introduction. Calcium chloride generated a significant
concentration-dependent increase in cardiac myocyte shortening in all groups (control, TNFα, TNFα + NMA; each p<0.01, ANCOVA) (Figure 4). TNFα alone was associated with depression of myocyte contraction. This effect existed only at absent or the lowest concentrations of exogenously introduced calcium (p<0.05)(Figure 4). Higher concentrations of exogenous calcium chloride completely reversed TNFα-mediated myocyte depression irregardless of the presence of NMA. The response curve to exogenous calcium for TNF-exposed cardiac myocytes was significantly different than that for myocytes exposed to control media (p=.0466, ANCOVA). The inclusion of NMA in TNFα-containing media resulted in correction of depressed basal contractility so that the calcium chloride contractility response curve was similar to that generated by myocytes in control media alone (Figure 4).

Discussion:

TNFα contributes to myocardial depression in a wide variety of pathological conditions including sepsis/septic shock. Studies of human septic shock have suggested the existence of a circulating myocardial depressant substance(s) whose early (<1 hour) in-vitro depressant activity quantitatively correlates to measures of cardiac contractility (i.e. ejection fraction) documented in-vivo (27). We have previously demonstrated that TNFα and IL-1β are responsible for the in-vitro myocardial depressant activity of serum from patients with septic shock(21). This depressant activity (inclusive of TNFα) has been shown to be mediated, in part, by NO (generated by a calcium-dependent NOS) and cyclic
GMP(17). An adrenoreceptor signaling defect in response to catecholamine stimulation has also been implicated in early TNFα-mediated dysfunction of isolated cardiac myocyte contraction(3,18). In this study, we sought to determine whether TNFα may act through multiple mechanisms to generate depression of cardiac myocyte contraction and whether these mechanisms are NO-dependent or independent.

Our data suggest that at least two discrete mechanisms of TNFα-induced myocyte depression exist. As we have previously documented, exposure of cardiac myocytes to TNFα generates an early (<1 hour) NO-dependent depression of basal contractility (Figure 1c) (17,21). This depressant response appears to be abrogated in the presence of NMA (17). However, in this study increasing concentrations of isoproterenol added to TNFα-exposed cardiac myocytes failed to correct the depressant effect even at the highest tolerated concentrations. Although addition of NMA to TNFα corrected depression at absent or low concentrations of isoproterenol (basal contractility), there was no impact at higher concentrations (Figure 1c). This experiment suggests that a second, early NO-independent defect of β-adrenoreceptor signal transduction is induced by exposure of cardiac myocytes to TNFα. This possibility is supported by the demonstration that cAMP generation in response to isoproterenol is also substantially blunted by TNFα and is not improved in the presence of NMA (Figure 1d).

The location of this second early mechanism of TNFα-induced myocardial depression is addressed by the experiments with forskolin and dBcAMP. β-
adrenoreceptor signaling is known to involve a coupled G-protein complex that activates adenylate cyclase which generates cyclic AMP(2). Defects of β-adrenoreceptor signal transduction could involve the receptor itself (e.g. receptor internalization), G-protein subunits, or adenylate cyclase.

In myocytes exposed to NMA and TNFα, dBcAMP and forskolin cyclase generated a contractile response distinct from that seen with isoproterenol (Figure 1c, 2a and 3). At absent or low concentrations of isoproterenol, TNFα-induced decreases in basal myocyte contractility were prevented by NMA (Figure 1c). However, maximally tolerated concentrations of isoproterenol failed to fully correct TNFα-induced myocyte depression even in the presence of NMA i.e. at maximally tolerated concentrations of isoproterenol, myocyte contraction was still deficient relative to control myocytes. The isoproterenol-stimulated increase in extent of contraction of these TNFα- and NMA-exposed myocytes was similar to myocytes exposed to TNFα without NMA.

As with isoproterenol, TNFα-induced decreases in basal myocyte contractility were prevented by NMA at absent or low concentrations of forskolin or dBcAMP, (Figure 1c, 2a and 3). However, in the presence of NMA, maximally tolerated concentrations of forskolin or dBcAMP completely corrected TNFα-induced myocyte depression (Figure 2a and 3). This contrasts the findings seen with isoproterenol.

These data suggest that the location of the defect in β-adrenoreceptor signaling induced by TNFα is proximal to adenylate cyclase. This proposition is further supported by the demonstration that concentration-dependent forskolin-
mediated cAMP generation was not adversely affected by TNFα exposure whether or not NMA was present (unlike the response seen with isoproterenol) (Figure 1d and 2b). The cAMP response curves for control, TNFα and TNFα + NMA-containing media in response to increasing concentrations of forskolin were all similar. This would only be expected if the TNFα-induced β-adrenoreceptor signal transduction defect was proximal to adenylate cyclase. Although G-protein uncoupling is likely, these observations cannot rule out the possibility of direct β-adrenoreceptor responses (e.g. rapid receptor internalization).

Finally, experiments using exogenous calcium chloride as an inotropic agent suggest common distal mechanistic elements in defects of both basal and catecholamine-stimulated contractility. In this set of experiments, NMA again prevented TNFα-induced depression of basal cardiac myocyte contraction (Figure 4). However, in contrast to forskolin (Figure 2a) and dBcAMP (Figure 3), NMA was not required to eliminate TNFα-induced depressant effects at higher concentrations of exogenous calcium chloride (Figure 4). Exogenous calcium completely corrected TNFα-induced myocyte depression irregardless of the presence of NMA. This suggests that reduced calcium availability or sensitivity represents a final common pathway through which both mechanisms of TNFα-associated depressant activity are mediated.

Most of the work on TNFα-induced myocyte responses has focused on β-adrenoreceptor dysfunction associated with late (> 1 hour) depression (3,4,6,9,36). Early depressant effects (< 1hour) of TNFα on isolated myocyte contractility have been ascribed to various mechanisms. These include
sequential NO and cGMP generation (via calcium-dependent NOS activation rather than calcium-independent NOS induction consistent with the short time frame to onset of myocardial depression)(5,8,17) and sphingomyelinase-dependent signaling (5,23,35). There is some suggestion that these mechanisms do not necessarily operate exclusively of each other(5,19,30).

Although significant data links calcium-dependent NOS to physiologic modulation of adrenergic responsiveness(1,11-13,37), there has been little work on the question of β-adrenoreceptor signaling alterations in immediate/early myocyte depression induced by TNFα. We have previously demonstrated that TNFα exposure results in an early effect of attenuation of β-adrenoreceptor-mediated increases in myocyte contractility and cAMP generation(18). Bick and colleagues have also shown that short-term neonatal rat cardiac myocyte exposure to TNFα results in blunted contractility and cyclic AMP response to isoproterenol but that responses to forskolin are preserved (3). Similarly, Sugishita and colleagues have shown that TNFα acutely suppresses β-adrenergic-induced increases in contractility and I_{Ca} transients(35).

There exist two major limitations to this model. First, the neonatal rat cardiac myocytes utilized have the disadvantage of not being terminally differentiated as are adult myocytes. Their characteristics change as they age. They exhibit metabolic differences from adult cells including significant differences in membrane receptors (34). It can be argued that phenomena observed in neonatal rat myocytes may have limited relevance to human adult cardiac pathophysiology. However, this study was performed in order to better
understand septic myocardial depression. In this context, we have already shown a significant relationship between septic serum-induced in-vitro cardiac depression in this model and in-vivo cardiac depression (decreased ejection fraction) in the septic shock patients from whom the serum is obtained (21,27,31). To the extent that TNFα is a central component of septic serum-induced myocyte depression(21), our current findings provide insight into the pathophysiologic mechanisms which may underlie septic myocardial depression. In addition, the use of serum based media, although a necessary component of this study, also introduces additional variables. Bovine serum contains a variety of poorly understood, unidentified factors which can affect myocyte metabolism. However, the use of controls using the same 10% bovine serum for comparison purposes compensates for this variable.

Several conclusions can be drawn from our current data. First, early generation of NO (presumably by a calcium -dependent NOS) is clearly implicated in immediate/early depression of depression of basal cardiac myocyte contractility induced by TNFα. In addition, TNFα exposure is associated with a second independent early depressant mechanism which is manifested by reduced contractile and cAMP response to β-adrenergic stimulation. This second mechanism appears to be NO-independent but calcium-dependent. Further, the signaling defect is located proximal to adenylate cyclase. Alterations in the β-adrenoreceptor itself or in the coupled G-protein complex are likely.

These mechanisms, although similar is in some respects to those described with more prolonged exposure of cardiac myocytes to TNFα, represent
distinct responses that may represent specific targets for intervention in pathological states of inflammatory myocardial dysfunction. Further clarification of the nature of these disturbances will be required in order to develop specific therapies. Future studies should examine β-adrenoreceptor density/localization on the myocyte cell membrane and integrity of coupled G-protein subunit signaling activity following exposure to TNFα.
Figure Legends:

Figure 1) Effect of isoproterenol on contractility and cAMP generation response of TNFα ± NMA-exposed cardiac myocytes a) contractility response to 0.005 µM isoproterenol over time b) contractility response to 2.5 µM isoproterenol over time c) myocyte contractility response at 5 minutes post-introduction of increasing concentrations of isoproterenol d) intracellular cyclic AMP levels across increasing isoproterenol concentrations (5 minutes post-introduction). R² values for control, TNFα and TNFα+NMA responses were 0.84, 0.45 and 0.52 respectively.  iso = isoproterenol Error bars = standard error of the mean.

Figure 2) Effect of forskolin on contractility and cAMP generation response of TNFα ± NMA-exposed cardiac myocytes a) myocyte contractility response at 10 minutes post-introduction of increasing concentrations of forskolin b) intracellular cyclic AMP levels across increasing forskolin concentrations (10 minutes post-introduction). R² values for control, TNFα and TNFα+NMA responses were 0.78, 0.63 and 0.67 respectively.  Error bars = standard error of the mean.

Figure 3) Effect of dBcAMP on contractility response of TNFα ± NMA-exposed cardiac myocytes. Myocyte contractility was assessed 20 minutes after introduction of increasing concentrations of dBcAMP.  Error bars = standard error of the mean.
Figure 4) Effect of exogenous calcium chloride on contractility response of TNFα ± NMA-exposed cardiac myocytes. Myocyte contractility was assessed 10 minutes after introduction of increasing concentrations of calcium chloride (Ca^{2+}). Error bars = standard error of the mean.
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-% change in extent of myocyte shortening at 5 min post-isoproterenol introduction

- control
- TNF
- TNF α + NMA

* = p<.05 vs control

TNF α vs control, p<.0001
TNF α + NMA vs control, p=.031

isoproterenol concentration (μM)
The graph shows the cyclic AMP concentration (fmol/10^6 cells) at 5 min post-isoproterenol introduction as a function of isoproterenol concentration (μM). The lines represent different conditions:

- **control**
- **TNF α**
- **TNF α + NMA**

Statistical comparisons are indicated as follows:

- **TNF α vs control**, p = 0.0188
- **TNF α + NMA vs control**, p = 0.0102
TNFα vs control, p < 0.0001
TNFα + NMA vs control, p = NS
* = p < 0.01 vs control

% change in extent of myocyte shortening
[at 10 min post-forskolin introduction]

forskolin concentration (µg/L)
TNF α vs control, p=NS
TNF α + NMA vs control, p=NS

Cyclic AMP concentration (fmol/10^6 cells) at 10 min post-forskolin introduction

Forskolin concentration (µg/L)


**Graph Legend:**
- **control**
- **TNF**
- **TNF α + NMA**

**Key Points:**
- TNF α vs control, p < .0001
- TNF α + NMA vs control, p = NS
- * = p < .05 vs control

**Axes:**
- X-axis: dBcAMP concentration (mM)
- Y-axis: % change in extent of myocyte shortening at 20 min post-dBcAMP introduction
TNFα vs control, p = .0466
TNFα + NMA vs control, p = NS

- control
- TNF
- TNFα + NMA

* = p < .05 vs control