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Rapid Endotoxin-induced Alterations in Myocardial Calcium Handling

Obligatory Role of Cardiac TNF-α

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Background: **Bacterial endotoxin (lipopolysaccharide [LPS]) induces septic shock and depressed myocardial contractility. The mechanism of LPS-mediated cardiac dysfunction remains controversial. We hypothesized that LPS exerts significant effects on myocardial excitation-contraction coupling by rapid stimulation of tumor necrosis factor α (TNF-α) expression in the heart.**

Methods: **Isolated rat hearts were studied with and without recirculation of cell-free perfusate. The effects of LPS, exogenous TNF-α, anti-TNF-α antibody, and ceramidase inhibition were examined. Measurements included myocardial uptake of LPS, left ventricular contractility, myocardial oxygen consumption, intracellular calcium [Ca^{2+}] cycling, and TNF-α concentrations in coronary perfusate and myocardium.**

Results: **Lipopolysaccharide was rapidly taken up by the perfused heart. With non-recirculating perfusion, LPS had no effect on contractility, oxygen consumption, coronary vascular resistance, or intracellular free calcium concentration ($[Ca^{2+}]_i$). However, with recirculating perfusion contractility was significantly impaired after 30 min of LPS, associated with lower $[Ca^{2+}]_i$ levels and attenuated systolic rise in $[Ca^{2+}]_i$. Significant amounts of TNF-α accumulated in recirculating perfusate and myocardial tissue from LPS-perfused hearts. Ceramidase inhibition or neutralizing anti-TNF-α antibody inhibited the effects of LPS on contractility and $[Ca^{2+}]_i$. Recombinant rat TNF-α mimicked the LPS effects with faster onset.**

Conclusions: **Lipopolysaccharide exerts rapid, negative inotropic effects on the isolated whole rat heart. The reduction in contractility is associated with depressed intracellular calcium cycling. In response to LPS, TNF-α is rapidly released from the heart and mediates the effects of LPS *via* the sphingomyelinase pathway. The present study for the first time directly links LPS-stimulated TNF-α production, abnormal calcium cycling, and decreased contractility in intact hearts.**

DESPITE numerous advances in medical treatment, the mortality of sepsis continues to exceed 40%.[1] Bacterial endotoxin (lipopolysaccharide [LPS]) can trigger the systemic inflammatory response in Gram-negative sepsis, eventually resulting in dysfunction and failure of multiple organ systems. In addition to systemic vasodilation and arterial hypotension, decreased myocardial contractility with reduced contractile reserve is observed both clinically and experimentally and is an important factor in patient outcome.[2–5] Inflammatory cytokines such as tumor necrosis factor α (TNF-α) have been identified as mediators of cardiac depression during clinical sepsis[6] and in experimental models.[7–10] However, a direct effect of LPS on the heart has also been suggested.[11–14]

It is generally believed that the primary sources of the proinflammatory cytokines affecting the heart during sepsis are resident and circulating immune cells. However, it has recently been established that the cardiomyocyte is able to express TNF-α and other inflammatory mediators) in response to LPS and during myocardial ischemia/reperfusion.[7,8,15,16] TNF-α appears to depress myocardial contractility in a biphasic manner. Immediate negative inotropic effects have been observed in cultured cardiomyocytes, myofibrillar preparations, and isolated whole heart.[17–20] Later effects, observed with a latency of several hours in isolated myocytes and *in vivo*, are mediated in part by nitric oxide (NO).[12,21,22] In addition, early and late NO-independent effects on various cellular functions have also been reported.[23,24]

Much of the available information regarding LPS and TNF effects on myocardial excitation-contraction coupling is from experiments performed in isolated myocytes or myofibrillar preparations. Despite these reports, there is no information about the relation between LPS uptake, TNF-α expression, changes in contractility, and calcium homeostasis in intact hearts. These issues are important to understanding cardiac dysfunction during sepsis and cardiopulmonary bypass, two of the settings in which LPS and TNF are active. Therefore, we sought to examine (1) the immediate effects of LPS on contractility and intracellular calcium handling in the isolated perfused whole rat heart, (2) the role of myocardial TNF-α production in producing these changes, and (3) the effect of interfering with TNF signaling on LPS-in-

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duced myocardial dysfunction. We specifically tested the hypothesis that LPS is rapidly taken up by the perfused whole heart and exerts short-term effects on myocardial excitation-contraction coupling *via* rapid stimulation of TNF signaling.

Materials and Methods

Animal Care

All animals were given humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). The experimental protocol was reviewed and approved by the Animal Care Committee at Children's Hospital, Boston (Boston, MA).

Isolated Heart Preparation

Male Wistar rats (weight, 250–300 g) were euthanized by intravenous injection of 150 mg/kg ketamine, 2.5 mg/kg xylazine, and 500 U/kg heparin. Hearts were rapidly excised and placed in 4°C Krebs-Henseleit (K-H) buffer. After cannulation of the aorta, hearts were perfused retrograde in the Langendorff mode with modified K-H buffer (115 mM NaCl, 26 mM $NaHCO_3$, 11 mM glucose, 1.8 mM $MgSO_4$, 1.2 mM KH_2PO_4, 3.3 mM KCl, 1.25 mM $CaCl_2$, and 10 U/l insulin) that had been equilibrated with a 95% O_2–5% CO_2 gas mixture and passed through a 0.2-μm glass fiber filter. The final buffer pH was 7.35–7.45, partial pressure of oxygen (P_{O_2}) was 550–600 mmHg, and partial pressure of carbon dioxide (P_{CO_2}) was 30–40 mmHg. To minimize LPS contamination, the Langendorff system was sterilized and detoxified using Etoxaclean detergent (Sigma Chemical Company, St. Louis, MO) followed by sterile acidic and basic washes before every experiment. Solutions and perfusion apparatus were tested for endotoxin contamination using the E-toxate *Limulus* amebocyte lysate detection kit (Sigma). The temperature of perfused hearts was maintained at 37°C and monitored with a thermistor placed in the right-side ventricular cavity. The vena cavae and pulmonary veins were sutured closed, and a fluid-filled latex balloon connected to a micromanometry catheter (Millar Instruments, Houston, TX) was placed in the left ventricle *via* the left atrium. The balloon was filled to achieve a left ventricular end-diastolic pressure of 5 mmHg using a calibrated syringe. Hearts that developed a systolic pressure less than 70 mmHg after stabilization were discarded. The atrioventricular node was crushed, and hearts were paced at 250 beats/min *via* epicardial electrodes sutured to the right ventricle. Constant flow perfusion at a rate of 10 ml/min was chosen to minimize the effects of changes in coronary vascular resistance on myocardial function. Coronary perfusion pressure was monitored throughout the experiment with a pressure transducer connected *via* a side port to the aortic perfusion cannula. Myocardial oxygen consumption was determined by obtaining samples of aortic perfusate and coronary effluent and derived from the arteriovenous difference in oxygen content (Stat Profile Plus 9; Nova Biochemical, Waltham, MA), multiplied by coronary flow and normalized to dry heart weight.

Effects of LPS

To determine the contribution of substances released from the myocardium in response to LPS, experiments were performed both with and without perfusate recirculation. After 20-min stabilization in the Langendorff perfusion mode, baseline measurements were obtained. Five micrograms per milliliter LPS from *Salmonella typhosa* (Sigma) was added to the perfusate, and measurements performed every 5 min. In a separate set of experiments, perfusate recirculation was started after LPS was added with a total volume of 100 ml K-H buffer under continued filtration and oxygenation. This recirculation volume was chosen based on pilot experiments (approximately 8 recirculations occurred in 60 min) and did not compromise contractile function, calcium cycling, or oxygen consumption in control hearts.

Myocardial LPS Uptake

To estimate the time-course of myocardial LPS uptake in the isolated whole heart, a separate set of hearts was perfused with LPS from *S. typhosa* that had been fluorescently labeled with Texas Red (Molecular Probes, Eugene, OR).[25] Briefly, the succinimidyl ester of Texas Red X was conjugated to LPS using the FluoReporter Amine Labeling system (Molecular Probes) as described by the manufacturer. Labeled LPS was separated from unreacted fluorophore by gel filtration using 10 mM monobasic sodium phosphate buffer (pH 6.8) on a NAP 5 column (Pharmacia, Piscataway, NJ). The activity of Texas Red–labeled LPS was confirmed by the *Limulus* amebocyte lysate assay and its ability to stimulate TNF production in a macrophage cell line.

In these experiments, isolated hearts were perfused with K-H buffer containing 5 μg/ml Texas Red–labeled LPS. Myocardial uptake of labeled LPS was determined in a modified spectrofluorometer (SLM-Aminco, Ithaca, NY) by using 524 nm excitation light and monitoring real-time emission light between 560 and 650 nm; LPS uptake was visualized as the increase in fluorescence over time measured at 589 nm (isosbestic point for myocardium).[26] To account for both background fluorescence and fluorescence from intravascular LPS, the fluorescence level after 2-min perfusion with Texas Red-labeled LPS was considered baseline and the data expressed as increase in fluorescence as compared with that baseline.

In separate experiments, 5-μm sections of left ventricular myocardium from hearts that had been perfused with Texas Red X-LPS for 30 min, followed by a 30-min washout period, were fixed in 2% paraformaldehyde, paraffin embedded, and mounted for visualization on a BioRad MRC1024 confocal microscope (BioRad, Hercules, CA) fitted with a 60× oil immersion objective. Half-micron-thick optical sections were merged and projected using the BioRad software; the laser excitation wavelength was 568 nm, and fluorescence emission was detected between 589 and 621 nm.

Inhibition of LPS Effects

To test the hypothesis that LPS exerts its effects on the heart *via* TNF-α secreted from the myocardium, a specific antibody to TNF-α (goat anti-rat TNF-α immunoglobulin G; R&D Systems, Minneapolis, MN) was added to the perfusate to achieve a final concentration of 0.3 μg/ml. This antibody was selected for its ability to neutralize the biologic activity of rat TNF-α. The neutralizing dose (ND_{50}) for this lot was estimated to be 0.3 μg/ml in the presence of 0.025 ng/ml recombinant rat TNF-α. Perfusion with anti-rat TNF-α alone was shown to have no effect on cardiac function (data not shown). The antibody was added to the perfusate simultaneously with LPS.

In other experiments, the contribution of TNF signaling to LPS effects was investigated using the cell-permeable ceramidase inhibitor N-oleoylethanolamine (NOE; Sigma). Dissolved in ethanol, NOE was added at a concentration of 1 μM to the perfusate 20 min before administration of LPS and given continuously throughout the experiment. At this concentration, NOE alone had no effect on function of the isolated rat heart for the duration of the experiment (data not shown).

Effects of TNF-α

To determine whether exogenous TNF-α mimics LPS effects, 10 ng/ml recombinant rat TNF-α (PharMingen, San Diego, CA) was added to the perfusate. In preliminary experiments a significant degree of species specificity was noted: neither human nor murine recombinant TNF-α had a significant impact on contractility of rat hearts, nor did rat TNF-α affect function of isolated rabbit hearts (data not shown).

Myocardial Calcium Transients

Measurement of beat-to-beat intracellular calcium transients was performed as previously described and validated in detail.[26,27] In brief, after 15 min of stabilization, hearts were loaded with the Ca^{2+}-sensitive dye Rhod-2 (Molecular Probes) by perfusion with the cell-permeable acetoxymethyl ester (Rhod-2-AM, 0.2 mg/0.1 ml ethanol infused over a period of 2 min at 37°C without recirculation). Dye loading was followed by a 10-min washout period to remove extracellular or unhydrolyzed dye. A modified spectrofluorometer (SLM-Aminco, Springfield, IL) provided excitation light at 524 nm and recorded emission light at 589 nm. Recordings were performed with a time-resolution of 4 ms for analysis of single free intracellular calcium concentration $[Ca^{2+}]_i$ transients and 40 ms for observation of changes in $[Ca^{2+}]_i$ over longer time periods. Because Rhod-2 has no spectral shift after Ca^{2+} binding, it is necessary to measure differences in dye loading or changes in tissue dye concentration over time (*e.g.*, leakage or photobleaching). To accomplish this, tissue Rhod-2 absorbance was quantified using the ratio of scattered excitation light at 524 nm (peak Rhod-2 absorbance in myocardial tissue) and 589 nm (isosbestic point for myocardium). The change in absorbance over time was used to normalize the calculated intracellular calcium concentration.

To quantify $[Ca^{2+}]_i$ at the end of each experiment, 100 μM 2,2'dithiodipyridine (Sigma) was infused over a period of 2 min to induce calcium release from the sarcoplasmic reticulum. This was immediately followed by bolus injection of the calcium ionophore A23187 (10 μM calcimycin; Sigma) in 1 ml calcium solution, 10%, to maximize calcium entry from the extracellular space. These procedures established maximum fluorescence (F_{max}), which was used to calculate systolic and diastolic calcium concentration using the following equation:

$$[Ca^{2+}]_i = K_d \times (F_t - F_o)/(F_{max} - F_t)/A_t$$

where $[Ca^{2+}]_i$ is the free intracellular calcium concentration, K_d is the dissociation constant for Rhod-2 with calcium (710 mM in the presence of 0.5 mM myoglobin), F_t is fluorescence at a specific time point, F_o is autofluorescence measured before dye loading, and A_t is tissue light absorbance at a given time point. Interference with Rhod-2 emission and absorption spectra by LPS or any of the inhibitors used was excluded in preliminary experiments. The slope of the systolic calcium upstroke was assessed by calculating the first derivative of the $[Ca^{2+}]_i$ transient recording ($d[Ca^{2+}]_i/dt$). The rate of diastolic Ca^{2+} removal ($\tau[Ca^{2+}]_I$) from the cytosol was assessed by fitting calcium transient data points to a monoexponential function that describes the descending slope of the $Ca^{2+}{}_i$ transient, using the following equation:

$$[Ca^{2+}]_i\,(t) = [(Ca^{2+}{}_i)_{max} - (Ca^{2+}{}_i)_{infin}] \times \exp\,(-\tau\, Ca^{2+}{}_i \times t)$$

where $(Ca^{2+}{}_i)_{max}$ is $Ca^{2+}{}_i$ at maximum $d[Ca^{2+}]_i/dt$, and $(Ca^{2+}{}_i)_{infin}$ is $[Ca^{2+}]_i$ extrapolated to infinite time. The $\tau[Ca^{2+}]_i$ was calculated using this equation as the calcium transient declined from 90% to 10% of its maximum value.

Myofibrillar Calcium Response

The calcium responsiveness of the contractile apparatus was assessed by plotting left ventricular developed

pressure as a function of systolic $[Ca^{2+}]_i$. To manipulate $[Ca^{2+}]_i$, the calcium concentration in the perfusate was sequentially varied between 0.5 and 2.5 mM in 0.5-mM increments; $[Ca^{2+}]_i$ and developed left ventricular pressure were measured at each extracellular calcium concentration. These experiments were performed immediately after the measurement of baseline $[Ca^{2+}]_i$ after 60 min perfusion with or without LPS.

Transmembrane Calcium Flux

To estimate changes in transsarcolemmal Ca^{2+} influx, the rate of fluorescence quenching by Mn^{2+} was studied in separate groups of LPS-treated (recirculating perfusion) and control hearts. $MnCl_2$ was added to phosphate-free perfusion buffer to achieve a final Mn^{2+} concentration of 0.3 mM. After 60-min perfusion of Rhod-2–loaded hearts, the perfusate was switched to one containing Mn^{2+} with constant perfusate flow. Rhod-2 calcium transients were completely quenched in all hearts, and the rate of fluorescence decline was determined by fitting the systolic $[Ca^{2+}]_i$ values to a monoexponential decay function. Mn^{2+} enters the cardiomyocyte through L-type Ca^{2+} channels with identical kinetics to calcium; thus, the rate of Mn^{2+}-induced quenching of Rhod-2 fluorescence can be used to estimate transmembrane calcium influx (given similar perfusate flow, heart rate, and heart size, as were present in these experiments).[28]

TNF-α Determination

In another set of experiments, using both recirculating and non-recirculating perfusion with and without LPS, 3 ml coronary effluent was collected every 10 min and the samples snap frozen in liquid nitrogen. Undiluted 50-μl samples were assayed for TNF by enzyme-linked sandwich immunoassay (ELISA) (Quantikine M TNF-α kit; R&D Systems). After incubation and preparation according to the manufacturer's instructions, optical density was measured with an automated microplate reader, and the TNF-α concentration was derived from a standard calibration curve using rat TNF-α. To determine the TNF-α content of myocardial tissue, hearts were snap frozen in liquid nitrogen and stored at −80°C. Left ventricular tissue was homogenized in ice-cold buffer containing (all from Sigma) 20 mM Tris HCL (pH 7.4), 2 mM EDTA (disodium salt), 0.5 mM EGTA, 1 mM phenylmethylsufonyl fluoride, 25 μg/ml leupeptin, and 0.3 mM sucrose, and centrifuged at 1,000*g* for 15 min; protein content was determined in the supernatant (diluted 1:5 vol/vol in lysis buffer). Using the Quantikine M TNF-α ELISA kit, 50-μl samples of the diluted supernatant were incubated as described above. TNF-α concentration was normalized for protein content, and the TNF-α concentration was expressed as picograms of TNF per gram myocardial wet weight.

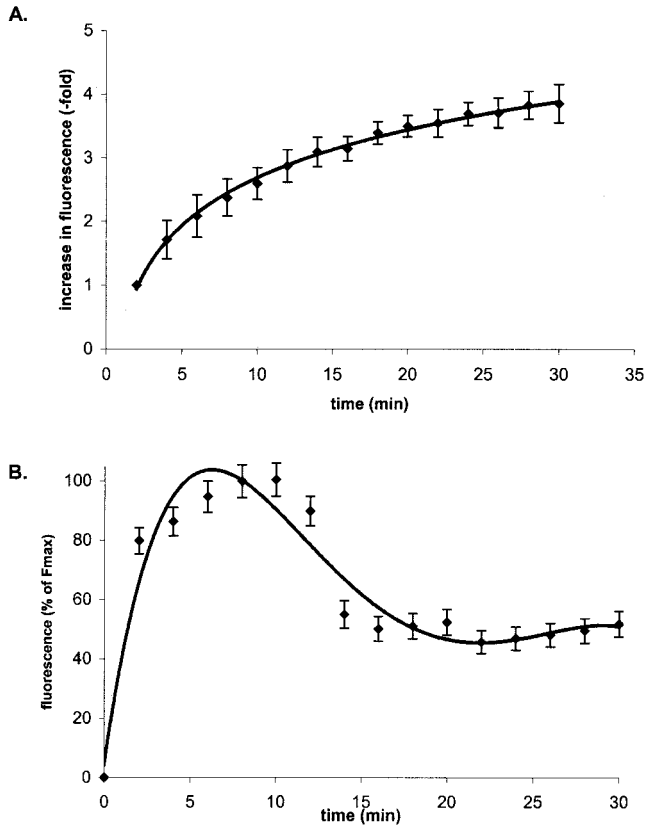

Fig. 1. (*A*) Time course of uptake of fluorescence-labeled lipopolysaccharide (LPS) in beating rat hearts. Surface fluorescence is recorded at 589 nm using excitation light of 524 nm. Data are expressed as increase in fluorescence light ± SD as compared with fluorescence light intensity after 2-min perfusion with Texas Red LPS. Logarithmic regression, $R^2 = 0.99$. (*B*) Intracellular uptake of fluorescent LPS assessed in intact perfused rat hearts. Hearts were perfused for 8.5 min with fluorescent LPS followed by 21.5-min washout with LPS-free perfusate. Values were expressed a percentage of the peak fluorescence, and the mean values of four experiments were fitted to a polynomial regression ($R^2 = 0.87$). All measurements have been adjusted for background autofluorescence.

Statistical Analysis

Analysis of calcium recordings was performed using Sigma Plot software (version 4.0; SPSS Inc., Chicago IL). Data are expressed as mean ± SD, and statistical analysis was performed using the SPSS software package (version 9.0; SPSS Inc.). Multiple group comparisons were tested for significance by analysis of variance using the Bonferroni correction for multiple comparisons. If no significant departure from normal distribution was confirmed using the Kolmogorov-Smirnov test ($P > 0.05$), Student *t* test was used to compare individual data sets.

Results

LPS Uptake

As depicted in figure 1A, LPS accumulated rapidly in the myocardium. This measurement does not distinguish between LPS localized inside and outside of cells. To

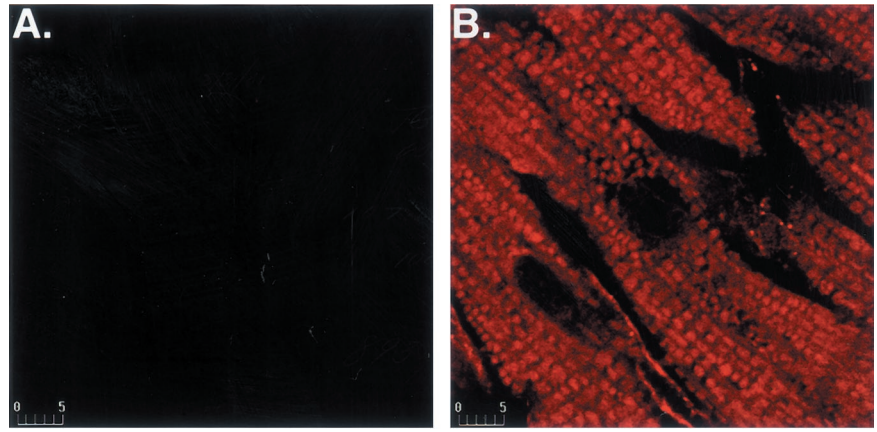

Fig. 2. Representative confocal photomicrographs of the left ventricular free wall from a heart perfused with (*A*) unreacted Texas Red X alone or (*B*) 5 μg/mL Texas Red X–labeled lipopolysaccharide (LPS). Each panel shows a 0.5-μm optical section of myocardium perfused with either unreacted fluorophore or Texas Red X–labeled LPS for 30 min. After a 30-min washout period with Krebs-Henseleit buffer, hearts were fixed, paraffin-embedded, sectioned, and used for microscopic analysis. The specific intracellular accumulation of LPS in cardiomyocytes is shown. Scale bars represent 5 μm.

account for fluorescence arising from intravascular LPS, the data are expressed as the increase in fluorescence beyond that present after 2-min perfusion with labeled LPS. To further assess the degree of intracellular LPS uptake, hearts were perfused for 8.5 min with labeled LPS followed by 30-min washout period using LPS-free perfusate (fig. 1B). An initial drop in fluorescent emission is consistent with labeled LPS being washed out from vascular lumen and interstitial space. Then fluorescence intensity remained stable until the end of the perfusion protocol, suggesting that approximately 50% of the Texas Red X-LPS present in the heart is localized within myocardial cells. This notion is supported by results depicted in figure 2, in a tissue section representative of myocardial LPS fluorescence at the end of the washout period. Although our method does not allow for quantification of the absolute tissue concentration of LPS, it is apparent that a large amount of LPS accumulates in the whole heart within several minutes and that significant intracellular concentrations persist after LPS removal.

Effects of LPS on Contractility and Calcium Handling

Using non-recirculating perfusion of isolated rat hearts perfused with up to 5 μg/ml LPS, no significant change in myocardial contractility (fig. 3A), oxygen consumption, or intracellular calcium handing was noted. However, with recirculation left ventricular developed pressure began to decline after approximately 20 min, reaching statistical significance after approximately 35 min. After 60 min of recirculating perfusion with LPS, developed pressure was 20–30% lower than in control hearts (fig. 2A). The changes in +dp/dt (the first derivative of the left ventricular developed pressure recording) followed the changes in developed pressure; +dp/dt at 60 min was 1,358 ± 314 mmHg/s in LPS-treated hearts *versus* 1,718 ± 295 mmHg/s in control hearts ($P < 0.02$, analysis of variance). Coronary perfusion pressure increased late during the perfusion period (74 ± 11 mmHg *vs.* 55 ± 8 mmHg in control hearts at 60 min; $P < 0.05$, Student *t* test). Myocardial oxygen consumption declined in parallel with the change in developed pressure (0.68 ± 0.11 ml · min^{-1} · g^{-1} dry weight in LPS-treated hearts *vs.* 0.82 ± 0.09 ml · min^{-1} · g^{-1} dry weight in control hearts at 60 min; $P < 0.05$, Student *t* test). Perfusion of the heart with 10 ng/ml TNF-α caused changes in developed left ventricular pressure similar to changes with LPS with recirculation, but with faster onset of effect (fig. 3B) (see also Effects of TNF-α on Contractility and Calcium Handling).

Representative calcium transients recorded during recirculating perfusion with LPS are shown in figure 4. Systolic $[Ca^{2+}]_i$ decreased in response to LPS with a similar time course as contractility (fig. 5A). In control hearts, systolic $[Ca^{2+}]_i$ was 553 ± 33 nM immediately after dye loading and 537 ± 43 nM after 60-min recirculating perfusion. In LPS-treated hearts, systolic $[Ca^{2+}]_i$ decreased from 546 ± 23 to 393 ± 28 nM throughout the 60-min recirculation period ($P < 0.01$, analysis of variance) (fig. 5A), resulting in a smaller amplitude of the calcium transient. Diastolic $[Ca^{2+}]_i$ also declined during the perfusion period in LPS-treated hearts (194 ± 13 nM at 0 min *vs.* 135 ± 31 nM at 60-min recirculating LPS perfusion; $P < 0.05$, analysis of variance) (fig. 5B). The rate of systolic rise in $[Ca^{2+}]_i$ ($d[Ca^{2+}]_i/dt$) was significantly slower in LPS-treated hearts (fig. 6A), but the $\tau[Ca^{2+}]_i$ did not change (fig. 6B). The rate of fluorescence quenching by Mn^{2+} was not different between LPS-treated and control hearts (173 ± 51 *vs.* 191 ± 48 ms^{-1}; $P = 0.8$, Student *t* test), indicating that the kinetics of transsarcolemmal Ca^{2+} influx *via* L-type Ca^{2+} channels was not substantially altered by LPS exposure.

Myofibrillar Calcium Response

The calcium responsiveness of the contractile apparatus is characterized by the force generated for a given $[Ca^{2+}]_i$. Hence, we plotted left ventricular developed pressure as a function of the measured $[Ca^{2+}]_i$ at varying perfusate Ca^{2+} concentrations in control hearts and hearts treated with recirculating LPS (fig. 7). Neither the

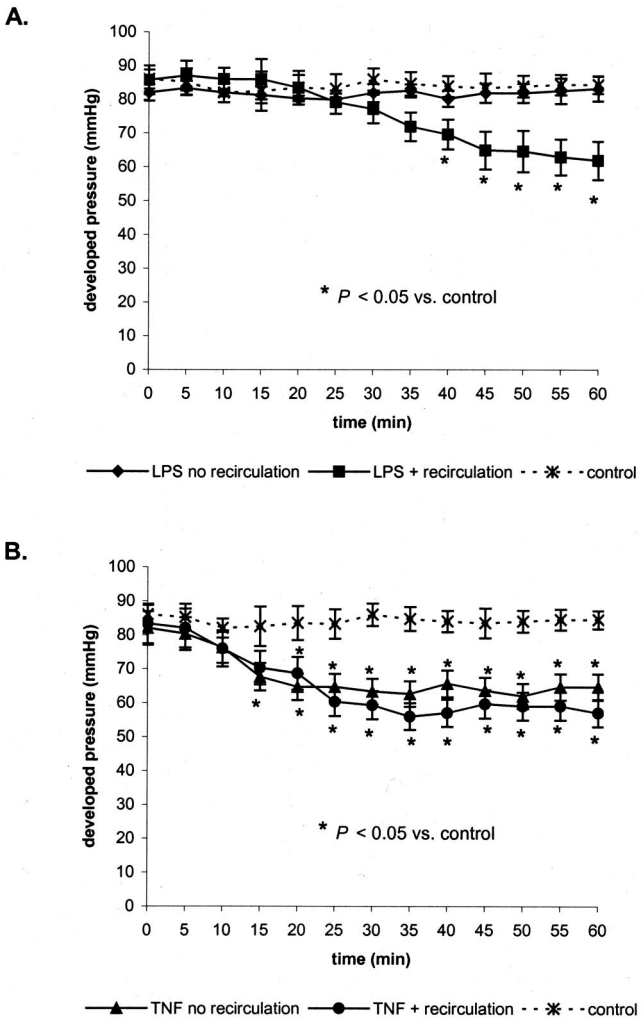

Fig. 3. Effects of (*A*) 5 μg/ml lipopolysaccharide (LPS) and (*B*) 10 ng/ml recombinant rat tumor necrosis factor (TNF)α on left ventricular developed pressure in isolated rat hearts subjected to non-recirculating and recirculating perfusion. Each data point represents the mean ± SD of six experiments. Groups were compared with one-way analysis of variance using the Bonferroni correction.

position nor the slope of the calcium-force relation was altered in LPS-treated hearts, indicating that the calcium responsiveness of the contractile apparatus is not changed during short-term LPS exposure in the *in vitro* rat heart.

TNF-α Expression in LPS-treated Hearts

In recirculating perfusate, TNF-α was first detected after 20-min perfusion with LPS (fig. 8). The concentration in the perfusate increased progressively to reach 165 ± 48 pg/ml at 60 min. In left ventricular tissue, the TNF-α concentration after 60-min perfusion with LPS was 511 ± 304 pg/g wet weight, as compared with 5.7 ± 2.3 pg/g wet weight in control hearts ($P < 0.01$, Student *t* test). For comparison, the myocardial tissue concentration of TNF-α in hearts perfused with exogenous TNF-α was 702 ± 114 pg/g wet weight after 60-min perfusion and 2-min washout.

Effects of TNF-α on Contractility and Calcium Handling

In hearts perfused with buffer containing 10 ng/ml recombinant rat TNF-α, left ventricular developed pressure started to decrease after 10 min with both recirculating and non-recirculating perfusion, reaching a statistically significant difference after 20 min (fig. 3B). The effects on developed pressure and dp/dt were slightly more pronounced with recirculating perfusion, with a developed pressure at 60 min of 64 ± 11 mmHg with non-recirculating TNF-α compared with 57 ± 7 mmHg with recirculating TNF-α ($P = 0.04$, analysis of variance). Myocardial oxygen consumption decreased proportionately to the depression of left ventricular developed pressure (data not shown). The

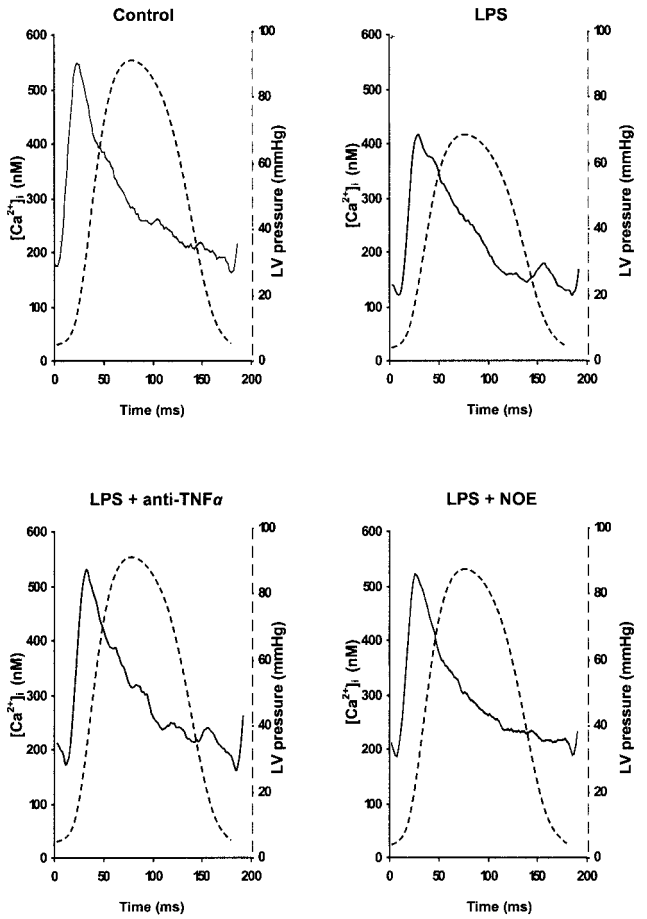

Fig. 4. Representative calcium transients and left ventricular pressure recordings from a rat heart perfused with 5 μg/ml lipopolysaccharide (LPS) in recirculating perfusion mode at the onset of LPS perfusion (control) and after 60-min LPS perfusion (LPS). LPS + anti–tumor necrosis factor α (anti-TNF-α) = LPS-perfused heart with 0.3 μg/ml neutralizing anti-TNF-α added to the recirculating perfusate. LPS + N-oleoylethanolamine (NOE) = LPS-perfused heart with 1 μM of the ceramidase inhibitor NOE added to the recirculating perfusate. Solid line and left-side y-axis = intracellular calcium; interrupted line and right-side y-axis = left ventricular pressure.

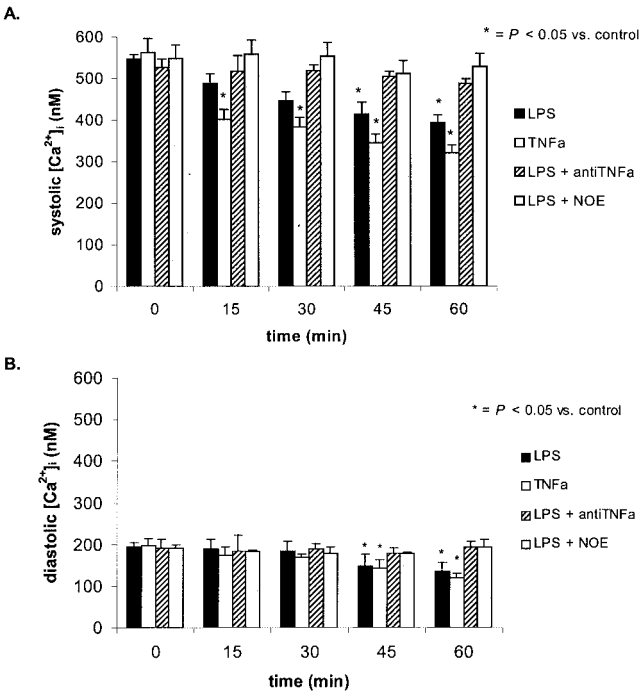

Fig. 5. Effects of lipopolysaccharide (LPS) and recombinant rat tumor necrosis factor (TNF)α on (*A*) systolic and (*B*) diastolic intracellular calcium levels of isolated rat hearts subjected to recirculating perfusion. Concomitant administration of 0.3 μg/ml anti-rat TNF-α and 1 μM N-oleoylethanolamine inhibited the LPS-induced decline of intracellular free calcium concentration ($[Ca^{2+}]_i$). Each data point represents the mean ± SD of six experiments. Groups were compared with one-way analysis of variance using the Bonferroni correction.

changes induced by exogenous TNF in intracellular calcium handling paralleled the time course of the decline in myocardial contractility, with an early decrease of systolic $[Ca^{2+}]_i$ (fig. 5A), together with a reduced rate of rise of the calcium transient (fig. 6A), followed by a decrease of diastolic $[Ca^{2+}]_i$ (fig. 5B). In summary, exogenous TNF-α mimicked the functional effects of LPS but occurred more quickly.

Role of TNF Signaling in LPS Effects

Administration of anti-rat 0.3 μg/ml TNF-α antibody significantly attenuated the effects of recirculating LPS on contractility and intracellular calcium handling. In the presence of anti-rat TNF-α antibody, we noted only a slight decline in developed pressure at the end of the perfusion period (fig. 9); systolic $[Ca^{2+}]_i$, as well as $d[Ca^{2+}]_i/dt$, remained unchanged throughout (figs. 5 and 6). Furthermore, coronary perfusion pressure was not different from control (59 ± 11 *vs.* 55 ± 9 mmHg; $P = 0.9$, Student *t* test). Pretreatment with the ceramidase (a key intermediate in the sphingomyelinase signaling pathway) inhibitor NOE also effectively inhibited the effects of LPS on contractility and calcium handling. After NOE administration, developed pressure, systolic $[Ca^{2+}]_i$, diastolic $[Ca^{2+}]_i$, and $d[Ca^{2+}]_i/dt$ did not significantly change throughout the experiment (figs. 4–6). Coronary perfusion pressure was not different from control at 60 min in the presence of NOE.

Discussion

The mechanism of septic cardiodepression remains controversial. In the present study, LPS-induced dysfunction occurred only with recirculation of the perfusate, suggesting that the accumulation of one or more substances in response to LPS was necessary. A prominent causative role for the stimulation of myocardial TNF production by LPS was indicated by the findings that (1) LPS induced rapid TNF production within the myocardium and release into the coronary circulation, (2) eliminating TNF-α signaling directly by using neutralizing anti-TNF-α effectively inhibited the effects of LPS on contractility and calcium cycling, (3) administration of exogenous rrTNF-α mimicked LPS effects on calcium handling and contractility but with faster onset, and (4) inhibition of sphingosine production with the ceramidase inhibitor NOE also prevented the development of short-term LPS effects in the heart. The current study is the first to link LPS-stimulated myocardial TNF-α production and impaired calcium cycling with contractile dysfunction in intact hearts.

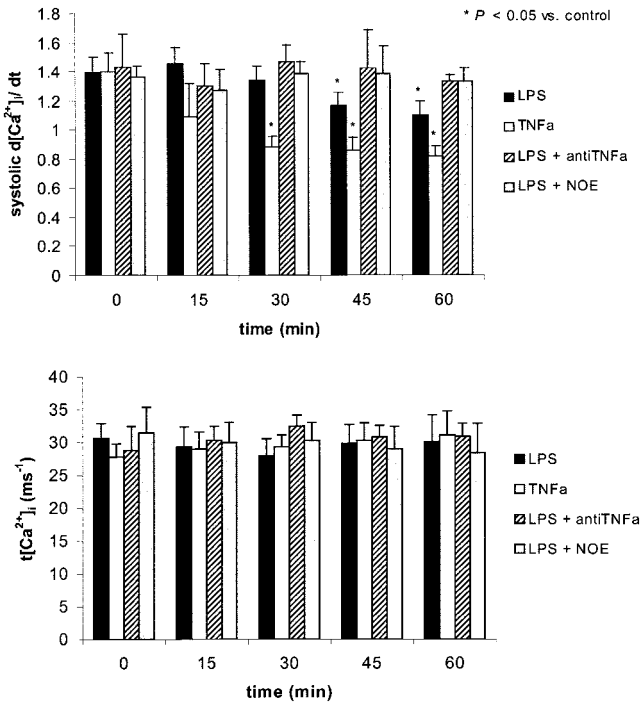

Fig. 6. Effects of lipopolysaccharide (LPS) and recombinant rat tumor necrosis factor (TNF)α on (*A*) systolic first derivative of the $[Ca^{2+}]_i$ transient recording ($d[Ca^{2+}]_i/dt$) and (*B*) rate of diastolic calcium decline ($\tau[Ca^{2+}]_i$) in isolated rat hearts subjected to recirculating perfusion. Concomitant administration of 0.3 μg/ml anti-rat TNF-α and 1 μM N-oleoylethanolamine inhibited the LPS-induced reduction of the $d[Ca^{2+}]_i/dt$. Each data point represents the mean ± SD of six experiments. Groups were compared with one-way analysis of variance using the Bonferroni correction.

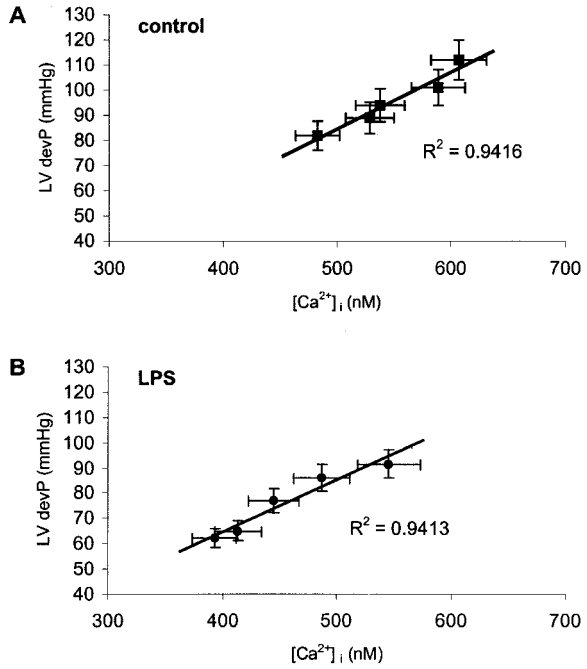

Fig. 7. Left ventricular developed pressure as a function of free intracellular calcium concentration ($[Ca^{2+}]_i$). The slope of the calcium-force relation is not different (control = 0.225 x; LPS = 0.201 x), indicating that the calcium responsiveness of the contractile apparatus is unchanged. LV devP = left ventricular developed pressure.

LPS and Cardiac TNF-α Expression

Myocardial TNF-α expression has been identified in endothelial cells, smooth muscle cells, and cardiomyocytes.[8] Production of TNF by the cardiomyocyte, as well as other myocardial cell types, has been demonstrated recently in response to injuries such as ischemia-reperfusion and pressure overload.[29,30] Intramyocardial production of TNF-α in response to LPS has also been demonstrated previously, but the functional consequences and mechanisms of TNF effect on function were unclear.[7,8,31] Production of TNF-α by isolated neonatal and adult cardiac myocytes was associated with depressed contractility and apoptosis.[7] The sarcolemmal TNF-α receptor, TNFR-1, has been identified in rat cardiomyocytes and linked with changes in intracellular calcium handling.[32] Our data clearly demonstrate that LPS is efficiently taken up by the isolated rat heart, leading to rapid stimulation of myocardial TNF-α production. The demonstration of myocellular LPS uptake is important, for we have recently shown that the physical process of LPS internalization in the cardiomyocyte may be critical for initiating subsequent LPS-stimulated signaling events.[25]

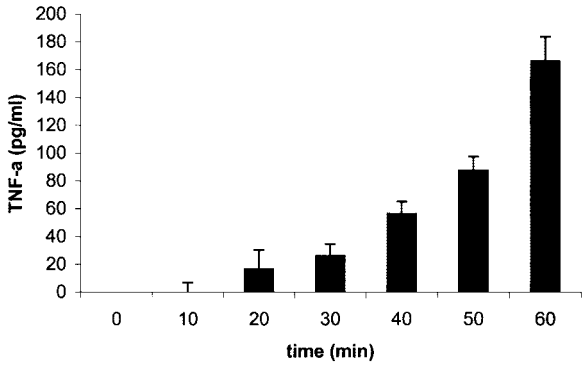

Fig. 8. Tumor necrosis factor α (TNF-α) concentration in recirculating coronary effluent of rat hearts perfused with crystalloid solution containing 5 μg/ml lipopolysaccharide (LPS). The TNF-α concentration in myocardial tissue after 60-min perfusion was 511 ± 304 pg/g wet weight in LPS-treated hearts and 5.7 ± 2.3 pg/g wet weight in control hearts.

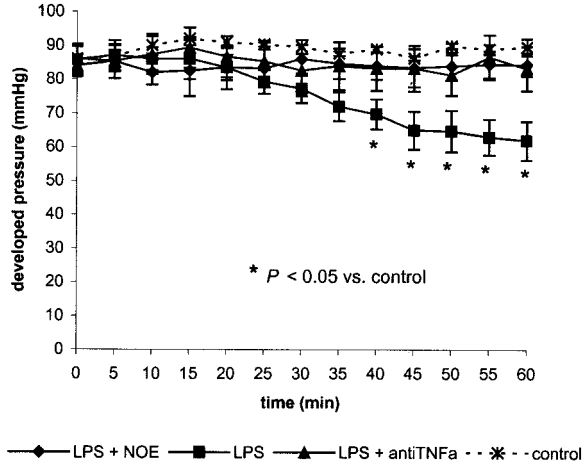

Fig. 9. Inhibition of LPS-induced decrease in left ventricular developed pressure in isolated rat hearts subjected to recirculating perfusion. Lipopolysaccharide (LPS) concentration was 5 μg/ml; 0.3 μg/ml anti-rat tumor necrosis factor α was added to the perfusate together with LPS; 1 μM N-oleoylethanolamine was administered 20 min before LPS perfusion. Each data point represents the mean ± SD of six experiments. Groups were compared with one-way analysis of variance using the Bonferroni correction.

LPS, TNF, and Excitation-contraction Coupling

The effects of LPS on myocardial contractility and calcium handling are controversial. Several groups, including our own, have recently shown that LPS leads to increased calcium cycling and/or reduced myofilament calcium responsiveness,[11,13,27,33,34] whereas other authors have found no change in myofilament Ca^{2+} sensitivity[35] and either no change or reduced calcium transients and transmembrane calcium flux.[11,34–36] There are numerous possible explanations for these differences, including species, experimental preparation, and duration of LPS exposure. Changes in intracellular calcium handling and myocardial contractility that become prominent several hours after LPS or TNF administration *in vivo* or *in vitro* are probably due, at least in part, to a NO-mediated increase in cyclic guanosine monophosphate and appear to be fundamentally different from early, NO-independent changes.[9,23,27]

Similar conflict can be found regarding the effects of TNF, although the bulk of reports support a significant

role for TNF in altering myocardial contractility, calcium handling, and energetics.[6,9,10,14,23,37] In the present study, we noted rapid LPS-induced changes in myocardial calcium handling that included a decline in systolic $[Ca^{2+}]_i$, reduced amplitude calcium transients, and slower rate of rise of systolic $[Ca^{2+}]_i$; the rate of diastolic $[Ca^{2+}]_i$ removal was unchanged. In contrast to later effects,[27] myofibrillar calcium responsiveness was unchanged. These short-term changes in calcium handling were associated with a similarly rapid depression of myocardial contractility, were completely prevented by neutralization of TNF-α protein or signal transduction, and were mimicked by exogenous recombinant rat TNF-α. Negative inotropic effects of TNF-α have recently been characterized in other experimental models, including transgenic mice overexpressing TNF-α[38] and exogenous TNF-α given to isolated perfused hearts.[17] In isolated cardiac myocytes, TNF caused a rapid decrease in peak $[Ca^{2+}]_i$ without a change in inward calcium current.[19,20]

In adult cardiac myocytes, the amplitude of the calcium transient and, hence, the systolic intracellular calcium level are primarily determined by the amount of calcium released from the sarcoplasmic reticulum *via* the calcium release channel (ryanodine receptor). The explanation for reduced contractility resulting from LPS-induced myocardial TNF production is suggested by the observation that LPS-induced changes in calcium handling and contractility were prevented by inhibiting sphingosine production. TNF-α binding to membrane TNF receptors activates sphingomyelinase, which catalyzes the production of ceramide from membrane sphingomyelin. Sphingosine is then cleaved from sphingomyelin by the enzyme ceramidase.[39] There is currently substantial evidence that sphingosine directly inhibits calcium release by the sarcoplasmic reticulum.[40–43] Other evidence in support of this notion includes work showing reduced calcium release and altered ryanodine receptor function in sarcoplasmic reticulum isolated from endotoxemic dogs.[44,45] Sarcoplasmic reticulum calcium release is closely coupled to sarcolemmal calcium influx during the action potential *via* L-type calcium channels (calcium-induced calcium release), so inhibition of the L-type calcium channel can also result in a reduced amplitude of the calcium transients. TNF-α and sphingosine can reduce L-type channel calcium current in isolated cells.[20,32,42] We attempted to assess the role of reduced sarcolemmal calcium influx to reduced systolic calcium levels by measuring the manganese quenching rate of calcium-induced fluorescence of Rhod-2. In this model, we could not demonstrate reduced calcium influx through cell membrane calcium channels in response to LPS or TNF. These findings point to the ryanodine receptor as the primary cause for reduced Ca^{2+} transient amplitude and, consequently, reduced contractile function. However, it is important to note that the Mn^{2+} quenching rate is a somewhat insensitive measure that may underestimate actual changes in sarcolemmal calcium influx during the cardiac action potential.

LPS stimulation can result in the production of numerous mediators in addition to TNF that have been shown to affect cardiac function and cardiomyocyte survival, including interleukin-1, platelet activating factor, nitric oxide, and various prostanoids.[9,10,12,20,46,47] In contrast to the present study, LPS has been shown to cause rapid coronary vasodilation (perhaps in conjunction with the production of an unidentified protein) in a pharmacologically preconstricted coronary circulation.[47] It is also important to point out that sphingolipid metabolites are central to a number of signal transduction pathways. For example, they can mediate mitogenic or apoptotic effects depending on cell type, initiating stimulus, and the context in which they are produced. Ceramide, in particular, has been viewed as a central regulator of cell cycle status, apoptosis, and cell senescence, whereas a ceramide metabolite, sphingosine-1-phosphate, may be mitogenic and protect from ceramide accumulation and apoptosis.[48] Thus, promoting ceramide accumulation, as would be expected to occur from ceramidase inhibition by NOE, might have significant long-term effects that were not assessed by the present experiments.

These experiments demonstrate that LPS causes acute depression of left ventricular contractility and intracellular calcium handling in intact heart. These effects require rapid myocardial TNF-α production and are mediated *via* the sphingomyelin-sphingosine signaling cascade. The pathophysiologic role of innate and adaptive immune responses triggered in myocardial cells in response to injuries such as ischemia-reperfusion, abnormal hemodynamic loads, and heart failure is receiving increasing attention. Strategies to neutralize the biologic activity of TNF-α and other inflammatory mediators are currently in development[49,50] and may prove useful to limit cardiovascular dysfunction, both in the short term for sepsis and in more chronic disorders.

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