and for controls, HLA-DQ primers GH26 and GH27 (*22*). A modified quantitative PCR (*23*) protocol was used. One of each of the HIV primer pairs was end-labeled with $^{32}P\gamma ATP$; 10^6 cpm (5 to 10 pM) of labeled and 10 to 20 pM of the unlabeled complementary primer were included in each reaction. DNA for PCR was prepared (*5*) by washing the cells in phosphate-buffered saline (PBS) and suspending in hypotonic lysis buffer with proteinase K (600 μg/ml at 56°C for 1 hour followed by 95°C for 10 min). Samples were stored at −20°C until amplified in a Perkin-Elmer thermal cycler (5 min at 94°C, 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, 5 min at 72°C extension after 28 cycles). As a control, 25 pM of each of the unlabeled DQ primers were included in the gag reactions and the reaction product resolved and visualized on a 2% agarose stained with ethidium bromide.

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24. HIV-1 was grown in blood mononuclear cells, except for $HTLV\text{-}III_B$, which was also grown in the CEM T cell line. Patient isolates were obtained by coculturing patient cells with phytohemagglutinin (PHA)-induced blasts and passaged twice in PHA blasts. Virus was harvested at the peak of RT activity, passed through a 0.2 μM filter, and stored at −70°C. HIV with T and macrophage tropism was titered on primary T blasts, whereas $HTLV\text{-}III_B$ was titered on CEM. Viral supernatants used in PCR experiments were first incubated for 0.5 hours with deoxyribonuclease I (30 to 50 U/ml) (Boehringer Mannheim, Indianapolis, IN) and filtered. We prepared inactivated HIV-1 by heating virus supernatant to 56°C for 30 min. To infect target cells, virus supernatant at multiplicities of 0.05 to 0.5 was added for 1.5 hours at 37°C. Cells were washed three times in medium and resuspended.
25. RT was assayed by a microtiter method as modified for HIV-1 (*26*). Supernatant (10 μl) was harvested into microtitre plates and stored at −70°C. The reaction mix contained 50 mM tris (pH 7.8), 7.5 mM KCl, 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 0.05% Nonidet NP-40, 0.1 Å units (about 6 μg/ml) $pA_n{\cdot}dT_{12-18}$ template primer (Pharmacia, Piscataway, NJ) and 20 μCi/ml ^{32}P-dTTP (Amersham, Arlington Heights, IL). Reaction mix (50 μl) was added to each well and the plate incubated at 37°C for 1.5 hours. The 6 μl of solution was spotted onto DEAE-cellulose paper, air-dried, washed five times in 2× standard saline citrate (SSC) twice in 95% ethanol, dried, and counted with scintillant.
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Negative Inotropic Effects of Cytokines on the Heart Mediated by Nitric Oxide

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The direct effects of pro-inflammatory cytokines on the contractility of mammalian heart were studied. Tumor necrosis factor α, interleukin-6, and interleukin-2 inhibited contractility of isolated hamster papillary muscles in a concentration-dependent, reversible manner. The nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) blocked these negative inotropic effects. L-Arginine reversed the inhibition by L-NMMA. Removal of the endocardial endothelium did not alter these responses. These findings demonstrate that the direct negative inotropic effect of cytokines is mediated through a myocardial nitric oxide synthase. The regulation of pro-inflammatory cytokines and myocardial nitric oxide synthase may provide new therapeutic strategies for the treatment of cardiac disease.

Pro-inflammatory cytokines are a class of secretory polypeptides that are synthesized and released locally by macrophages, leukocytes, and endothelial cells in response to injury (*1–4*). Reperfusion of ischemic myocardium is associated with the infiltration by leukocytes and macrophages that may be responsible for a transient depression of myocardial contractility ("stunned myocardium") (*5–9*). This report attempts to determine if pro-inflammatory cytokines produce direct reversible inotropic effects in isolated papillary muscle preparations.

The addition of recombinant human tumor necrosis factor α (TNF-α) and interleukin-6 and interleukin-2 (IL-6 and IL-2) to the medium bathing isolated papillary muscles resulted in a concentration-dependent, reversible negative inotropic effect (Fig. 1). The negative inotropic effects were observed within 2 to 3 min, were maximal after 5 min, remained constant for at least 20 min, and were completely reversed within 40 min after the cytokines were removed from the bath. Recombinant human IL-1α had little inotropic effect.

Concentration response curves were generated for each of the cytokines (Fig. 2). TNF-α demonstrated a negative inotropic effect in a concentration range from 150 to 3200 U/ml. The majority of the effect was observed at concentrations below 900 U/ml (59 ± 7% and 44 ± 3% of baseline tension

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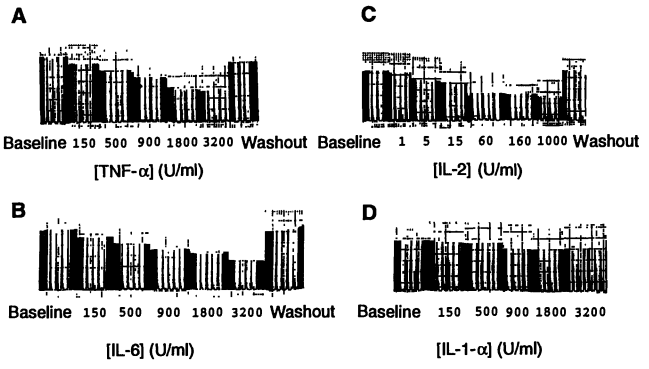

Fig. 1. Photographs of representative chart recordings illustrating the effects of adding increasing concentrations of cytokines on tension generated at 1 Hz by isolated papillary muscles prepared from 4-month-old F_1B control Syrian hamsters (Biobreeders Inc., Fitchburg, Massachusetts) (*19*). Vigorously beating hearts were removed and placed in oxygenated Tyrode's solution containing 130 mM NaCl, 4.7 mM KCl, 20 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 5.6 mM glucose oxygenated with 95% O_2 and 5% CO_2 (pH 7.4 at 25°C). Left ventricular papillary muscles were excised and attached at one end to a pressure transducer in a bath (5 ml) containing the same Tyrode's solution with bovine serum albumin (1%) oxygenated with 95% O_2 and 5% CO_2 warmed to 37°C (pH 7.4). Papillary muscles were stimulated at twice the threshold voltage with bipolar platinum electrodes and the tension generated was recorded with a Gould model 3400 recorder with built-in dc bridge preamplifier and transducer. We administered recombinant human cytokines (Genzyme, Cambridge, Masschusetts) by pipetting portions (50 μl) directly into the tissue bath, and then the medium was replaced with continuous perfusions of Tyrode's solution alone at 7.5 ml/min for 30 min; 150 to 3200 U/ml of TNF-α (**A**), 150 to 3200 U/ml of IL-6 (**B**), 1 to 1000 U/ml of IL-2 (**C**), 150 to 3200 U/ml of IL-1-α (**D**).

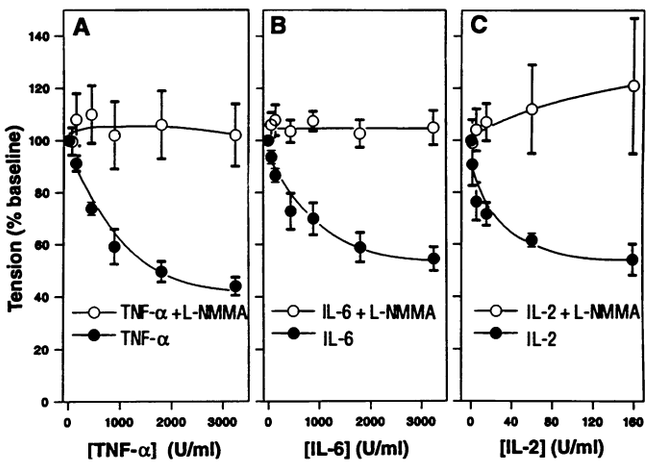

Fig. 2. Graphs depicting the negative inotropic effects of increasing concentrations of TNF-α (**A**), IL-6 (**B**), and IL-2 (**C**) alone (closed circles) or in the presence of L-NMMA (10 μM) (open circles). L-NMMA alone (10^{-7} to 10^{-3} M) had no significant inotropic effect (n = 6). Values represent the means ± SEM of six different determinations in six different papillary muscle preparations.

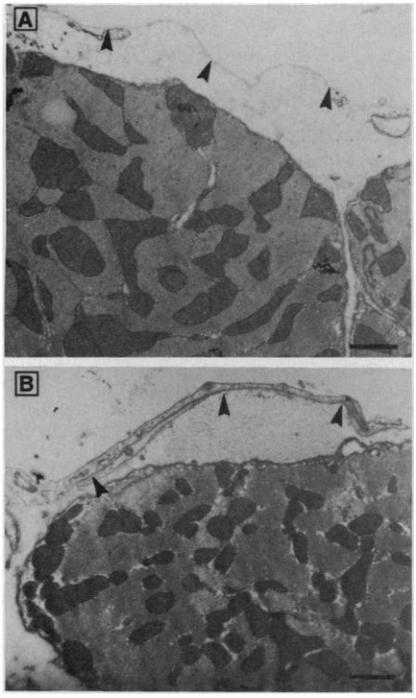

Fig. 3. Representative electron micrographs of hamster papillary muscles demonstrating the effect of chemically denuding the endocardial endothelium. Papillary muscles were immersed in Triton X-100 (0.5%) for 2 s and immediately placed into glutaraldehyde (2.5%) in phosphate-buffered saline to fix for 1 hour. Samples were subsequently processed with standard methods for plastic embedment (*20*). Thin (70 nm) sections were cut with a Reichert Ultracut S, mounted on copper grids, double-stained with uranyl acetate (2%) and citrate, and observed with a JEOL 100CX or 100B electron microscope. (**A**) Papillary muscle treated with Triton X-100. The endothelium surrounding the papillary muscle was lost, but the subendothelial basement membrane remained (arrowheads), and the subcellular architecture of the muscle was normal (×9000) (bar = 1 μm). (**B**) Similarly prepared papillary muscles not treated with Triton X-100 retained a thin endothelial layer surrounding the papillary muscle (×9000) (bar = 1 μm).

at 900 and 3200 U/ml, respectively; n = 6) [Baseline tension (100%) = 195 ± 9 mg/mm^2; n = 6]. The effects of IL-6 were similar to those of TNF-α (70 ± 6% and 55 ± 5% of baseline tension at 900 and 3200 U/ml, respectively; n = 6). The negative inotropic effects of IL-2 were observed at lower concentrations than TNF or IL-6 (62 ± 2% and 50 ± 2% of baseline tension at 66 and 1000 U/ml, respectively; n = 6). TNF and IL-6 had no inotropic effects at 66 U/ml and required 3200 U/ml to achieve maximal effects (P < 0.01, n = 6 by analysis of variance). IL-1α had little effect at the concentrations studied (87 ± 6% of baseline tension at 3200 U/ml; n = 6). No cytokines reduced tension below 44% of baseline.

Endothelium-derived relaxing factor (EDRF) is identical to nitric oxide (NO) (*10–12*). NO is formed from L-Arg by NO synthase (*10–12*). N^G-monomethyl-L-arginine (L-NMMA) is a specific inhibitor of NO synthase that can be reversed with L-Arg. Physiologic effects of cytokines are blocked by L-NMMA and reversed with L-Arg (*13–16*). These observations provide evidence of NO-mediated effects of cytokines in noncardiac tissues (*13–16*). Incubation of papillary muscles with L-NMMA (10 μM) had no effect alone but completely blocked the negative inotropic effects of TNF-α, IL-6, and IL-2 (Fig. 2).

The negative inotropic effects of the cytokines could be mediated through the endocardial endothelium or myocardium. Treatment of papillary muscles with Triton X-100 removed the endocardial endothelium (*17*) (Fig. 3), and further mechanistic studies were conducted comparing papillary muscles with and without an intact endothelium (Fig. 4). Inhibition of the inotropic effects of the cytokines by L-NMMA was reversed by the subsequent addition of L-Arg (Fig. 4). The addition of increasing

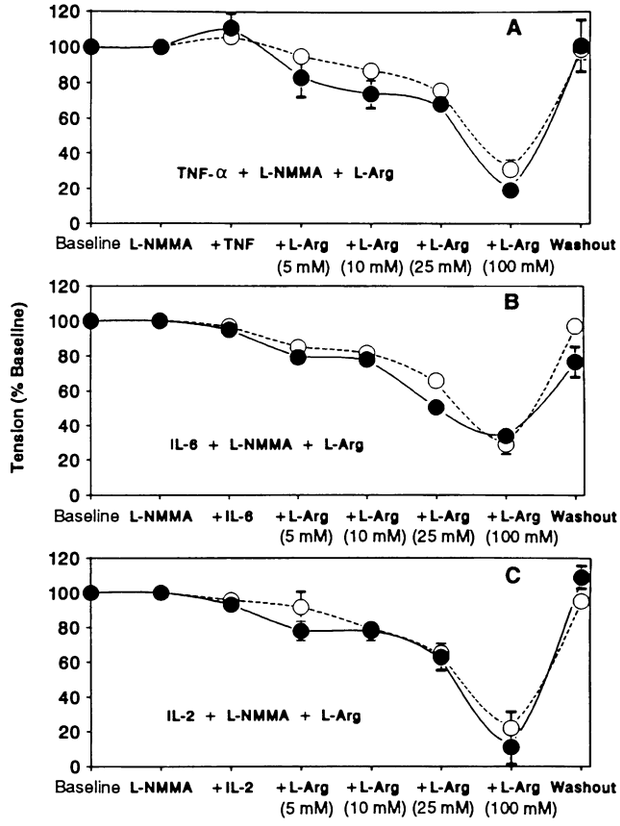

Fig. 4. Inhibition of inotropic effects of cytokines by treatment of muscle with L-NMMA (10 μM) and reversal of that inhibition by L-Arg. Intact papillary muscles (closed circles) or muscles lacking endothelium (open circles) were treated with L-NMMA (10 μM) for 10 min. TNF-α (1000 U/ml) (**A**), IL-6 (1000 U/ml) (**B**), or IL-2 (160 U/ml) (**C**) was added for 10 min. Subsequently, L-Arg was added in increasing concentrations for 10 min. Tension returned to baseline within 30 min after the medium containing inotropic agents was removed and replaced with unsupplemented medium. L-Arg alone (100 mM) reduced tension to 31 ± 6% of baseline (n = 6). No inotropic effect of L-Arg was observed at concentrations from 10^{-7} to 10^{-3} M (n = 6). Values represent the means ± SEM of six different determinations in six different papillary muscles. Chemically denuding the endothelium did not alter the inotropic responses of the papillary muscles to the cytokines.

concentrations of L-Arg (5 to 100 mM) to muscles treated with L-NMMA and TNF-α resulted in a more pronounced negative inotropic effect than that seen with TNF-α alone [19 ± 4% of baseline tension with TNF-α and L-Arg as compared to 59 ± 7% of baseline tension with TNF-α alone ($P < 0.01$, $n = 6$; Student's two-tailed t test)] (Fig. 4A). This suggests that L-Arg enhanced the negative inotropic effect of TNF-α by providing additional substrate for NO production. This effect was also greater than that seen with L-Arg (100 mM) alone (31 ± 6% of baseline tension; $P < 0.01$, $n = 6$; Student's two-tailed t test). The addition of L-Arg (100 mM) to muscles treated with L-NMMA and IL-6 reduced tension to 35 ± 3% (Fig. 4B). The addition of L-Arg (100 mM) to muscles treated with L-NMMA and IL-2 reduced tension to 11 ± 10% of baseline (Fig. 4C). All of these inotropic effects were completely reversed within 30 min after the cytokines or other agents were washed away (Fig. 4, A through C). Removal of the endothelium did not alter the negative inotropic responses of the papillary muscles to cytokines (Fig. 4, A through C).

Cytokines increase the amount of NO in noncardiac tissues by inducing the transcription of an inducible NO synthase (*13–16*). The rapid onset and reversibility of the effects seen in this report argue against an effect requiring gene transcription. The negative inotropic effects of these cytokines in the papillary muscle preparation appear to result from enhanced activity of a constitutive NO synthase enzyme in the myocardium.

The observed inotropic effects of pro-inflammatory cytokines raise the possibility that they participate in reversible, postischemic myocardial depression ("stunning"). Myocardial stunning frequently occurs after cardiopulmonary bypass and may complicate successful recovery from cardiac surgery (*5–9*). We found elevated concentrations of IL-6 (1800 to 4000 U/ml) in bronchoalveolar fluid from patients after cardiopulmonary bypass (*18*). IL-6 also reversibly decreased tension generated by pectinate muscles removed from patients at the time of surgery (*18*). These preliminary observations in patients support the clinical relevance of our findings with the Syrian hamster papillary muscle preparation. Thus, the regulation of pro-inflammatory cytokines and myocardial NO synthase may provide new therapeutic strategies for the management of cardiac patients.

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Structure and Functional Expression of an ω-Conotoxin–Sensitive Human N-Type Calcium Channel

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N-type calcium channels are ω-conotoxin (ω-CgTx)–sensitive, voltage-dependent ion channels involved in the control of neurotransmitter release from neurons. Multiple subtypes of voltage-dependent calcium channel complexes exist, and it is the α_1 subunit of the complex that forms the pore through which calcium enters the cell. The primary structures of human neuronal calcium channel α_{1B} subunits were deduced by the characterization of overlapping complementary DNAs. Two forms ($\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$) were identified in human neuroblastoma (IMR32) cells and in the central nervous system, but not in skeletal muscle or aorta tissues. The $\alpha_{1B\text{-}1}$ subunit directs the recombinant expression of N-type calcium channel activity when it is transiently co-expressed with human neuronal β_2 and α_{2b} subunits in mammalian HEK293 cells. The recombinant channel was irreversibly blocked by ω-CgTx but was insensitive to dihydropyridines. The $\alpha_{1B\text{-}1}\alpha_{2b}\beta_2$-transfected cells displayed a single class of saturable, high-affinity (dissociation constant = 55 pM) ω-CgTx binding sites. Co-expression of the β_2 subunit was necessary for N-type channel activity, whereas the α_{2b} subunit appeared to modulate the expression of the channel. The heterogeneity of α_{1B} subunits, along with the heterogeneity of α_2 and β subunits, is consistent with multiple, biophysically distinct N-type calcium channels.

Voltage-dependent Ca^{2+} channels are multisubunit complexes through which extracellular Ca^{2+} enters excitable cells. In rabbit skeletal muscle, four tightly coupled subunits, α_1, α_2, β, and γ, make up the channel complex (*1*). The primary structure of each subunit has been determined and α_1, α_2, and β cDNAs have been used to characterize transcripts expressed in other tissues (*2*). The α_1 and β subunits are each encoded by a gene family, including at least five distinct genes for α_1 subunits and three genes for β subunits (*3–6*). Primary transcripts of each of the α_1 genes, the α_2 gene, and two of the β genes have been shown to yield multiple, structurally distinct, subunits by means of differential processing (*6–9*). Expression studies have shown that the α_1 subunit forms the pore through which Ca^{2+} enters the cell (*10, 11*).

On the basis of biophysical and pharmacological characteristics, three subtypes of neuronal, high-voltage–activated Ca^{2+} channels (L-, N-, and P-type) have been proposed (*2*). These high-voltage–activated

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