Adenosine Inhibits Lipopolysaccharide-Induced Cardiac Expression of Tumor Necrosis Factor-α

Daniel R. Wagner, Alain Combes, Charles McTiernan, Virginia J. Sanders, Bonnie Lemster, Arthur M. Feldman

Abstract—Tumor necrosis factor-α (TNF-α) is elevated in the failing heart. Very little is known about regulation of TNF-α in cardiomyocytes. TNF-α expression by macrophages is diminished by adenosine. Therefore, we hypothesized that a similar mechanism might occur in the heart. Neonatal rat myocytes were stimulated with lipopolysaccharide (LPS), and TNF-α was measured by ELISA. In the absence of LPS, myocytes did not release TNF-α in the medium. After exposure to LPS, TNF-α increased to 70.1±3.5 pg/mL at 6 hours. Immunofluorescent staining confirmed that TNF-α was expressed in myocytes. Adenosine decreased TNF-α in a dose-dependent manner (1 to 100 μmol/L, 37% to 65% decrease, $P<.01$). Adenosine also decreased TNF-α in cell homogenates by 78% ($P<.0001$). The effect of adenosine could be replicated by the A_2 agonist PD-125944 (DPMA), by cAMP agonists 8-bromo-cAMP, forskolin, and Ro 20–1724, but not by A_1 and A_3 agonists. Conversely, the effect of adenosine could be suppressed by the adenylate cyclase inhibitor MDL-12,330. Adenosine also inhibited TNF-α in adult rat ventricular myocytes (−60%, $P<.005$) and rat papillary muscles (−55%, $P<.05$). In neonatal myocytes, adenosine normalized LPS-induced calcium changes and improved LPS-induced negative inotropic ($P<.01$) and negative lusitropic ($P<.01$) effects. Our results demonstrate that adenosine can significantly diminish TNF-α levels in the heart. The effect appears to be mediated by the A_2 receptor and transduced through a G protein–adenylyl cyclase pathway. These results may explain some cardioprotective effects of adenosine and provide a novel pharmacological intervention in congestive heart failure. **(*Circ Res*. 1998;82:47-56.)**

Key Words: adenosine ■ tumor necrosis factor-α ■ cytokine ■ cardiomyocyte ■ myocardium

Tumor necrosis factor-α is a key mediator of cellular damage in immune and inflammatory responses.[1] Only recently has TNF-α been found to play a role in the myocardium. In 1990, it was first noted that patients with congestive heart failure have elevated levels of TNF-α.[2] Subsequently, investigators demonstrated that failing, but not normal, human myocardium expresses TNF-α.[3] Additionally, there is an inverse relationship between TNF-α levels and New York Heart Association classification of disease severity.[4] That elevated levels of TNF-α are not simply an epiphenomenon is demonstrated by the observation that when TNF-α is robustly overexpressed in the myocardium of transgenic animals, the mice develop a lethal cardiomyopathy.[5] In contrast, a more modest level of overexpression results in the development of a dilated cardiomyopathy in transgenic animals.[6] Similarly, continuous infusion of TNF-α results in the development of a dilated cardiomyopathy in rodents.[7] Although myocyte TNF-α expression is elevated with failure, little is known regarding the molecular and cellular mechanisms that regulate myocardial TNF-α expression.

Recently, investigators have demonstrated that the ability of macrophages to express TNF-α could be attenuated by adenosine.[8–10] Furthermore, a recent study suggests that patients harboring a single mutant allele of the AMP deaminase gene, a mutation with the potential to increase myocardial adenosine production, have a marked delay in the onset of symptoms of end-stage congestive heart failure.[11] Additionally, adenosine has been shown in numerous studies to have a cardioprotective effect in myocardial ischemia.[12,13] Therefore, we hypothesized that adenosine might regulate the expression of cardiac TNF-α. In the present study, we present data suggesting that physiological concentrations of adenosine can attenuate the expression of TNF-α by LPS-stimulated neonatal rat cardiomyocytes, adult rat ventricular myocytes, and rat papillary muscle.

Materials and Methods

Neonatal Rat Cardiomyocytes

Isolation

Cardiomyocytes were prepared from ventricles of 1-day-old Sprague-Dawley rats by the method of Toraason et al[14] as previously described[15] using a commercially available cardiomyocyte isolation kit (Worthington Biochemical). Cells recovered after trypsin and collagenase digestion were preplated on untreated plastic flasks for 1 hour to reduce nonmyocyte cell

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From the Division of Cardiology (D.R.W., A.C., C.M., B.L., A.M.F.) and the Division of Neuropathology (V.J.S.), University of Pittsburgh (Pa) Medical Center.

Correspondence to Arthur M. Feldman, MD, PhD, University of Pittsburgh Medical Center, Division of Cardiology, S572 Scaife Hall, 200 Lothrop St, Pittsburgh, PA 15213.

E-mail feldma@card2.cath.upmc.edu

Selected Abbreviations and Acronyms

CPA = N^6-cyclopentyladenosine
DMPX = 3,7-dimethyl-1-propargyl xanthine
DPMA = PD-125944
ITu = iodotubercidin
LPS = lipopolysaccharide
NECA = 5′-(*N*-ethylcarboxamido)-adenosine
PMA = phorbol 12-myristate 13-acetate
RGC = receptor–G protein–adenylate cyclase complex
TNF-α = tumor necrosis factor-α

numbers. Nonadherent cells enriched for cardiomyocytes were cultured in DMEM/F-12 containing 5% horse serum, 1 mmol/L glutamine, 10 mmol/L HEPES, 0.1 mmol/L bromodeoxyuridine, 5 μg/mL insulin, 5 ng/mL selenium, 5 μg/mL transferrin, and 10 μg/mL gentamicin. Horse serum was treated with AG1X-10 (Pharmacia) resin as previously described[16] to reduce serum triiodothyronine to undetectable levels as determined by radioimmunoassay. Cells were plated on pronectin (Promega)–coated tissue culture plates at a density of 1×10^5 cells/cm^2 and grown at 37°C in 95% O_2/5% CO_2. Cells were cultured for 48 hours before starting the experiments.

Immunohistochemistry

To prove that neonatal myocytes express TNF-α, cells were stained with antibody to rat TNF-α (R&D Systems). To determine the extent of contamination with nonmyocytes, cells were stained with antibody to myosin heavy chain as previously described.[17] Briefly, cell cultures were prepared as described above, and cells were grown on glass slides. Cells were washed twice in ice-cold PBS and fixed with a 1:1 mixture of methanol and acetone for 15 minutes at 4°C. After fixation, cells were incubated for 60 minutes in 1:10 diluted goat or rabbit serum (Sigma) to limit background staining. The rat TNF-α antibody was used in a 1:1000 dilution; the monoclonal anti-myosin antibody (MF-20), in a 1:2 dilution. Slides were washed 3 times with ice-cold PBS and incubated for 30 minutes. with secondary fluorescein (FITC, Sigma)–labeled rabbit anti-goat antibody (1:100 dilution) or secondary rhodamine (TRITC, Sigma)–labeled goat anti-mouse antibody (1:100 dilution). Slides were viewed with an inverted phase-immunofluorescence microscope (Nikon). Anti-myosin labeling showed that routine cell preparations contained ≈93% cardiomyocytes. The remaining cells consisted mainly of fibroblasts (≈7%) with traces of endothelial cells and smooth muscle cells.

Fibroblasts

To determine whether contamination with fibroblasts might have influenced our results, experiments with fibroblast-rich preparations were performed. Fibroblast-rich preparations were obtained at the time of preplating. Cells were grown to confluence in DMEM/F-12 containing 5% horse serum, 1 mmol/L glutamine, 10 mmol/L HEPES, 5 μg/mL insulin, 5 ng/mL selenium, 5 μg/mL transferrin, and 10 μg/mL gentamicin. In fibroblast-rich preparations, <5% cardiomyocytes were present.

Chemiluminescence

Control studies were performed to determine whether contamination of the cardiomyocyte cultures with leukocytes (macrophages, monocytes, and neutrophils) may have contributed to TNF-α production. We used chemiluminescence to assess the extent of contamination with leukocytes as previously described.[18] The principle behind chemiluminescence is that it occurs naturally during phagocytosis, can be potentiated with a chemiluminescent agent, and correlates with the number of leukocytes. In brief, cardiomyocyte cultures were prepared as described above, and 10 μg/mL PMA or 100 ng/mL LPS *Escherichia coli* 0127 was added to induce phagocytosis. Chemiluminescence signal was measured in a luminometer (Lumat, Berthold Systems Inc) using 10 μmol/L luminol (Sigma) as a chemiluminescent agent.[18] Minimal chemiluminescence was induced by adding PMA or LPS to the cardiomyocyte cultures. We estimated that the number of contaminating leukocytes was <250/mL, or 0.1% of the cell population.

Adult Rat Ventricular Myocytes

Adult rat ventricular myocytes were isolated from female rats (≈250 g) as previously described.[19] Briefly, hearts were perfused by the Langendorff method with HEPES-buffered Krebs-Henseleit solution (pH 7.4, 37°C) containing (mmol/L) NaCl 118, KCl 5, KH_2PO_4 1, $MgSO_4$ 1, HEPES 25, $NaHCO_3$ 37.5, glucose 11, and pyruvate 5, along with 0.5% BSA and vitamins. Myocytes were isolated by the addition of collagenase (0.8 mg/mL, Worthington). The preparations were enriched with viable myocytes by sequential sedimentations through 4% BSA (Sigma). Cells were transferred to serum-free medium 199 (Sigma) containing 5 μg/mL insulin, 5 μg/mL transferrin, and 10 μg/mL gentamicin and attached to BSA-coated 60-mm dishes. The medium was changed after 1 hour. Cells were kept at 37°C in 95% O_2/5% CO_2 for 24 hours. This procedure typically resulted in preparations containing ≈95% rod-shaped cardiomyocytes after 24 hours in culture. The *Limulus* amebocyte lysate assay (E-Toxate, Sigma) was used to assess endotoxin presence during cell isolation.

Rat Papillary Muscle

Rat papillary muscles were isolated from female rats (≈250 g) and immediately cut into ≈2×1×1-mm strips. The muscle strips were incubated in DMEM/F-12 medium containing 5% horse serum, 1 mmol/L glutamine, 10 mmol/L HEPES, 5 μg/mL insulin, 5 ng/mL selenium, 5 μg/mL transferrin, and 10 μg/mL gentamicin. Muscle strips were equilibrated for 1 hour at 37°C in 95% O_2/5% CO_2 before starting the experiments. During the experiment, muscle strips were kept in commercially available 12-well plates at 37°C in 95% O_2/5% CO_2. The wet weight of muscle strips was determined at the end of the experiments.

Stimulation With LPS

Exposure to LPS was used to induce production of TNF-α in neonatal rat cardiomyocytes, in adult rat ventricular myocytes, and in rat papillary muscle preparations. We used LPS of *E coli* 0127 (Sigma) after preliminary experiments had shown a rank order of TNF-α response: LPS *E coli* 0127>*E coli* 055:B5>*Salmonella enteritidis*>*Salmonella typhimurium*.

Neonatal rat cardiomyocytes were exposed to LPS (10 ng/mL, 6-hour incubation) after 48 hours in culture. A higher dose of LPS (10 μg/mL) was necessary to induce TNF-α production in adult rat ventricular myocytes and rat papillary muscle. Adult rat ventricular myocytes were exposed to LPS (10 μg/mL, 6-hour incubation) after 24 hours in culture. Rat papillary muscle strips were exposed to LPS (10 μg/mL, 6-hour incubation) after a 1-hour equilibration period. Adenosine, adenosine receptor agonists and antagonists, and all other inhibitors were added with LPS unless stated otherwise.

Measurement of TNF-α

TNF-α Release

Release of TNF-α into the medium was determined in neonatal and adult cardiomyocytes and in preparations of papillary muscle strips. At 0, 3, and 6 hours after the addition of LPS *E coli* 0127, the supernatants were collected, immediately frozen in liquid nitrogen, and stored at −70°C until analysis. The levels of TNF-α in the supernatants were measured with a rat TNF-α ELISA kit (Factor-Test-X, Genzyme). This kit uses the multiple-antibody sandwich principle. The accuracy of this ELISA kit was verified by repeating measurements with a mouse TNF-α ELISA kit, which has the property of cross-reaction with rat TNF-α (Genzyme). Both kits provided comparable measurements for TNF-α in our rat cardiomyocyte cell cultures. The rat TNF-α ELISA kit has a lower limit of detection (10 pg/mL). In order to detect levels as low as 1 pg/mL, all samples were concentrated through Centricon 10 concentrators (Amicon) as previously described.[20] Recovery was equal for all measured samples, and the filtrate did not contain measurable TNF-α. For soluble TNF-α, data are reported as pg/mL of unconcentrated supernatant.

Intracellular TNF-α

Intracellular TNF-α was determined in preparations of neonatal and adult cardiomyocytes. Cells were suspended in ice-cold PBS (200 μL) with the protease inhibitor phenylmethylsulfonyl fluoride (2 mmol/L) and homogenized as previously described.[20] Cell homogenates were briefly centrifuged to remove excess particulate matter. Total protein levels were quantified using a commercially available assay (Bio-Rad) with BSA used as a standard (0 to 2 mg/mL); TNF-α was expressed as pg/mg protein.

Analysis of Cytosolic Calcium and Contraction/Relaxation in Neonatal Cardiomyocytes

Free cytosolic calcium and contractile parameters were measured in neonatal rat cardiomyocytes as previously described.[15,21] Neonatal cardiomyocytes were prepared as described above, plated onto glass coverslips, and cultured in the presence of LPS (*E coli* 0127, 100 ng/mL), LPS and adenosine (10 μmol/L), or diluent for 4 days. Treatments were performed after isolation and repeated on days 2 and 4. Preliminary analysis had shown that calcium and contraction in neonatal myocytes were not affected by short-term exposure to LPS.

Cytosolic Calcium

Myocytes were loaded with fura 2-AM (Molecular Probes) by incubating the coverslips for 20 minutes in 2 mL of Tyrode's solution containing (mmol/L) NaCl 137, KCl 5, glucose 15, $MgSO_4$ 1.3, NaH_2PO_4 1.2, HEPES 20, and $CaCl_2$ 1, as well as fura 2-AM (3 μmol/L) and D-Pluronic (Molecular Probes) (3 μL of 25% [wt/wt] in dimethyl sulfoxide). Myocytes were then rinsed with Tyrode's solution and maintained for 15 minutes at room temperature to allow for deesterification of the dye. Coverslips were transferred to a temperature-regulated chamber (33°C) mounted on a Nikon Diaphot 300 inverted microscope stage, and cells were perfused with pre-warmed modified Tyrode's solution. Cells were paced by electrical field stimulation at 1 Hz (15 V, 4-millisecond pulse duration) (model S11 stimulator, Grass Instruments) using platinum electrodes. Fluorescence of intracellular fura 2 was determined by alternatively illuminating cells with 340- and 380-nm light and measuring emission at 520 nm (Ionoptix Corp). The sampling rate for collection of ratio values was 100 Hz. Theoretically, free cytosolic calcium ion concentrations can be calculated from the fura 2 fluorescence ratios at two wavelengths. However, improper calibration of fura 2 is difficult to exclude because of compartmentalization in loaded cells and differences in spectral properties between cells and buffer solutions. The fura 2 fluorescence ratio was used as an indicator of free cellular calcium as previously reported.[21]

Contraction and Relaxation Parameters

To provide high contrast spots for tracking contractile activity, glass beads (2.1±0.5 μm, Duke Scientific Corp) were added to the neonatal myocytes. The preparation was illuminated with red light through a dichroic mirror, and a video edge-detection system (VED 104, Crescent Electronics) was used to record the motion of glass beads attached to the surface of contracting myocytes. Data from 10 consecutive beats from 8 to 10 cells were recorded from each coverslip; at least four coverslips were prepared for each condition.

Data Analysis

A data analysis program (IonWizard 4.3, Ionoptix Corp) was used to measure fura 2 parameters (baseline and peak systolic calcium) and calculate maximum speed of contraction, maximum speed of relaxation, and peak amplitude of contraction. Calibration of contractile distance was determined by using Cell-VU grid coverslips (Erie Scientific Corp).

Immunohistochemistry of Rat Papillary Muscle Sections

Immunohistochemical staining of rat papillary muscles was performed as previously described.[22] Tissue was surrounded with OCT medium and snap-frozen. Blocks were cut on a cryostat at 10 μm, and sections were mounted on Superfrost Plus slides (Fisher). Tissue sections were immersion-fixed in 95% ethanol, rinsed in PBS, and treated for 30 minutes with 5% goat serum. Sections were treated with rabbit anti-human TNF-α (Genzyme) in a 1:100 dilution for 24 hours at 4°C. This antibody binds human TNF-α and rat TNF-α.[23] Sections were rinsed briefly with PBS and then treated with a 1:200 dilution of biotinylated goat anti-rabbit secondary antibody (Caltag). After rinsing with PBS, sections were treated with avidin-biotin complex (Vector Laboratories) for 1 hour. Visualization of the reaction was achieved by adding 0.01% of 3,3′-diaminobenzidine, 0.6% nickel ammonium sulfate, 0.05% imidazole, and 0.0003% H_2O_2 in 0.05 mmol/L Tris buffer. Sections were weakly counterstained with 1% neutral red.

Materials

Adenosine, 2-chloroadenosine, NECA, and CPA were obtained from Sigma. Dipyridamole, DPMA, N^6-benzyl-NECA, DMPX, Ro 20–1724, forskolin, 8-bromo-cAMP sodium, MDL-12,330, and ITu were obtained from Research Biochemicals Intl. The anti-myosin antibody MF-20 was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Md, and the Department of Biological Sciences, University of Iowa, Iowa City, under contract N01-HD-2–3144 from the National Institute of Child Health and Human Development.

Statistical Analysis

Results are expressed as mean±SEM of duplicate determinations of at least three different experiments. Data were subjected to ANOVA (one-way ANOVA, Fisher's test), and a value of $P<.05$ was considered to be statistically significant.

Results

Immunostaining of Neonatal Cardiomyocytes

The extent of contamination of neonatal myocyte preparations with nonmyocytes was determined by staining cells with antibody to myosin heavy chain. In Fig 1, panels A and B show a routine preparation consisting of 93% cardiomyocytes. To demonstrate that neonatal myocytes express TNF-α, cells were stained with antibody to rat TNF-α. Panels C and D show that neonatal cardiomyocytes stain positively for TNF-α after exposure to LPS (10 ng/mL) for 6 hours; in the absence of LPS, TNF-α could not be demonstrated with this technique.

Release of TNF-α by Neonatal Cardiomyocytes

In accordance with our immunostaining findings, neonatal rat cardiomyocytes did not release detectable amounts of TNF-α in the supernatant in the absence of LPS. The limit of detection for TNF-α was 1 pg/mL. However, in the presence of LPS (*E coli* 0127), TNF-α increased rapidly, reaching a peak at 6 hours. The maximum effect was seen at 10 ng/mL of LPS (46.0±2.1 pg/mL at 3 hours and 70.1±3.5 pg/mL at 6 hours, n=15). The lowest dose of LPS to elicit a significant release of TNF-α was 1 ng/mL (9.7±0.5 pg/mL at 3 hours and 22.2±2.3 pg/mL at 6 hours, n=3). To verify whether the presence of serum might influence the effect of LPS, some experiments were also performed in serum-free medium. The absence of serum did not significantly alter the response of neonatal myocytes to LPS. However, cell beating appeared diminished and less synchronous in serum-free medium. Therefore, all further experiments with neonatal myocytes were performed in serum-rich medium.

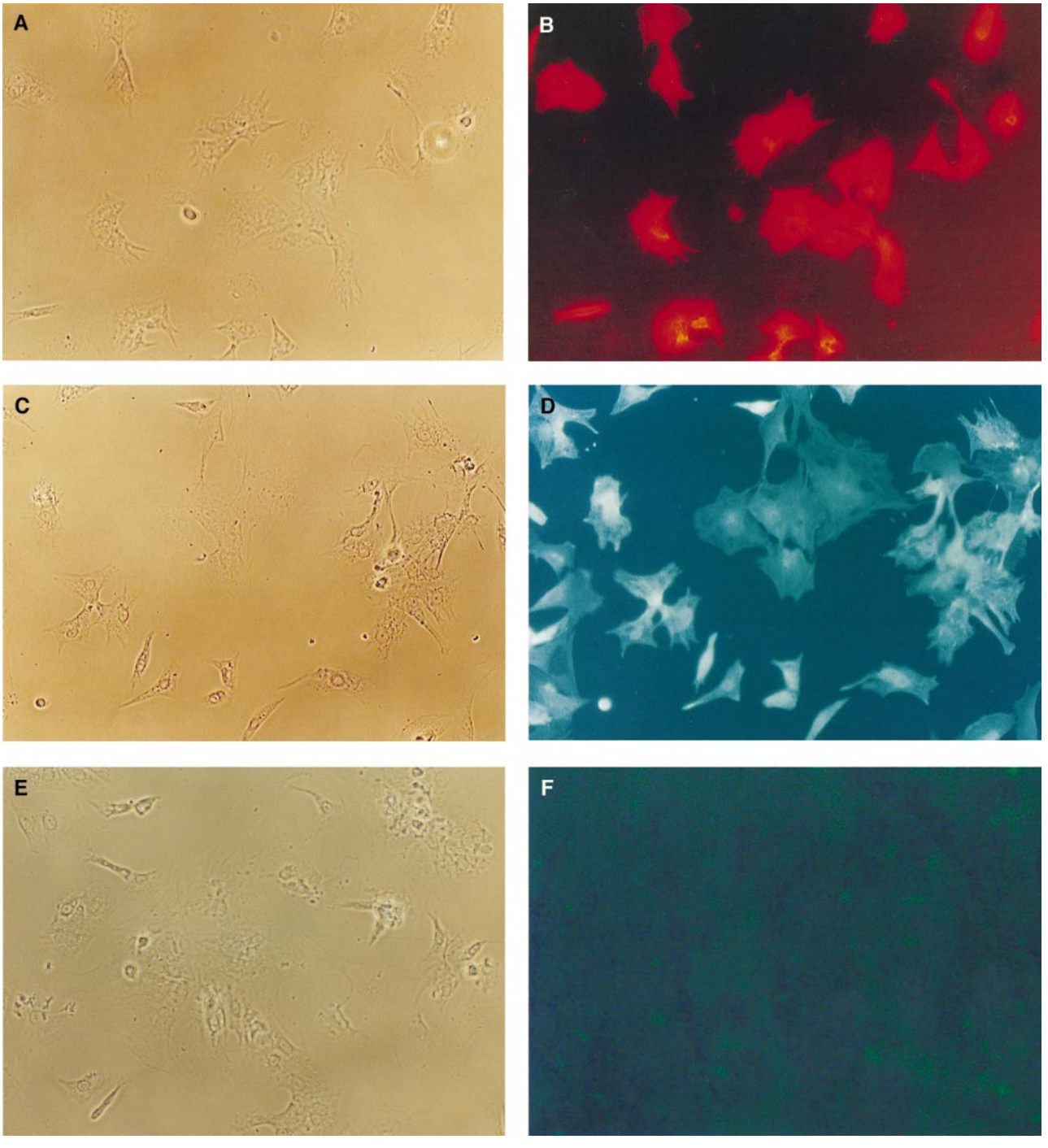

Figure 1. Light microscopy (A, C, and E) and immunohistochemical staining (B, D, and F) of cultured neonatal rat cardiomyocytes with antibodies to myosin heavy chain (B) and rat TNF-α (D and F). Panel F shows non–LPS-stimulated myocytes stained for TNF-α. The primary antibodies to myosin heavy chain and TNF-α were stained with secondary antibodies conjugated with rhodamine (red fluorescence) and fluorescein (green fluorescence), respectively. Myocytes were exposed to LPS (10 ng/mL) for 6 hours. Anti-myosin labeling shows that ≈93% of the cells are cardiomyocytes. Note that cells stain positive for TNF-α in the presence of LPS (D). Magnification ×400.

Effect of Adenosine on TNF-α Release by Neonatal Cardiomyocytes

As seen in Fig 2A, adenosine decreased TNF-α release in a concentration-dependent manner. Adenosine was equally effective in the presence of either 100 ng/mL or 1 ng/mL LPS (data not shown). The inhibitory effects of adenosine were not temporally related to activation by LPS, as there was no significant difference between levels of inhibition when myocytes were exposed to adenosine 1 hour before LPS, at the time of LPS exposure, or 1 hour after LPS treatment (Fig 2B). However, when cells were exposed to adenosine 3 hours after LPS challenge, adenosine was not effective in attenuating myocyte TNF-α expression.

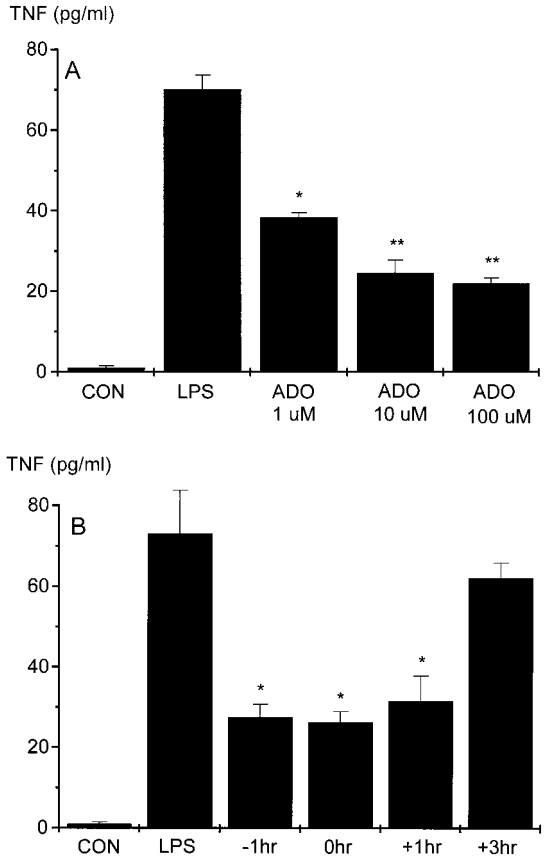

Figure 2. Neonatal rat cardiomyocytes were stimulated with 10 ng/mL LPS (*E coli* 0127) at time 0. After 6 hours, supernatant was collected, and TNF-α was measured with ELISA. In control (CON, in the absence of LPS), TNF-α was below the limit of detection (1 pg/mL). A, Dose effect of adenosine. Adenosine (ADO, 1 to 100 μmol/L) was added at time 0. B, Timing of ADO addition. ADO (10 μmol/L) was added 1 hour before LPS (−1 hour), at the same time as LPS (0 hour), 1 hour after LPS (+1 hour), and 3 hours after LPS (+3 hours). *P<.001 and **P<.0001 vs LPS (n=3, ANOVA).

Effect of Adenosine on Intracellular TNF-α Production

To assess the effects of adenosine on myocyte TNF-α levels, we measured the concentration of immunogenic TNF-α. Using our measurement system, we were unable to detect intracellular TNF-α at baseline. However, after addition of LPS, TNF-α levels were detectable in cell homogenates (18.2±4.3 pg TNF-α/mg protein). In addition, 10 μmol/L adenosine decreased TNF-α to 4.0±0.4 pg/mg protein (78% decrease, P<.0001) (n=5).

Effect of Adenosine-Regulating Agents on TNF-α

The adenosine-regulating agents dipyridamole and ITu increase endogenous adenosine in isolated cardiomyocytes.[19,24] Dipyridamole is an inhibitor of adenosine transport, and ITu is an inhibitor of adenosine kinase. In the presence of 10 μmol/L dipyridamole, TNF-α was decreased by 30% at 6 hours (P<.05) (n=3) (Fig 3). The effect of dipyridamole was less prominent than the effect of adenosine. Adenosine had a significant additive effect in experiments in which both dipyridamole and adenosine were added (P<.01). As seen in Fig 3, addition of 10 μmol/L ITu completely suppressed the release of TNF-α (P<.0001). These changes were not due to cell death, as myocytes maintained regular beating at the end of incubations with ITu.

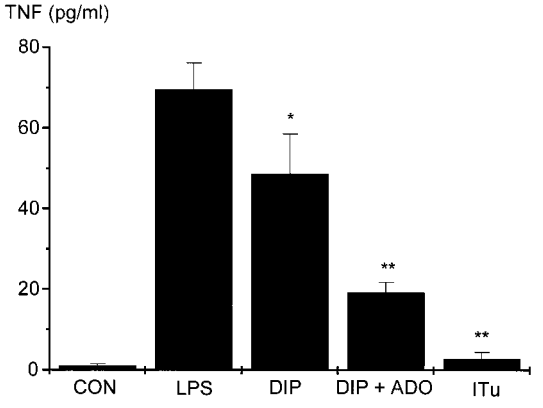

Figure 3. Effect of adenosine (ADO)-regulating agents. Dipyridamole (DIP), an inhibitor of ADO transport, and ITu, an inhibitor of ADO kinase, are known to increase endogenous ADO in isolated cardiomyocytes. Neonatal rat cardiomyocytes were stimulated with 10 ng/mL LPS (*E coli* 0127) at time 0. Where indicated, ADO (10 μmol/L), DIP (10 μmol/L), and ITu (10 μmol/L) were added at time 0. After 6 hours, supernatant was collected, and TNF-α was measured with ELISA. In control (CON, in the absence of LPS), TNF-α was below the limit of detection (1 pg/mL). *P<.05 and **P<.0001 vs LPS (n=3, ANOVA).

Effect of Selective Adenosine Agonists and Antagonists

Addition of 10 nmol/L DPMA, a selective adenosine A_2 receptor agonist, decreased TNF-α by 48% (P<.001) and 52% (P<.0001) at 3 and 6 hours, respectively (n=3) (Fig 4). The selective adenosine A_1 receptor agonist CPA (5 nmol/L) and the selective adenosine A_3 receptor agonist N^6-benzyl-NECA (10 nmol/L) had no effect on the release of TNF-α (Fig 4). However, at high concentrations (100 μmol/L), these selective

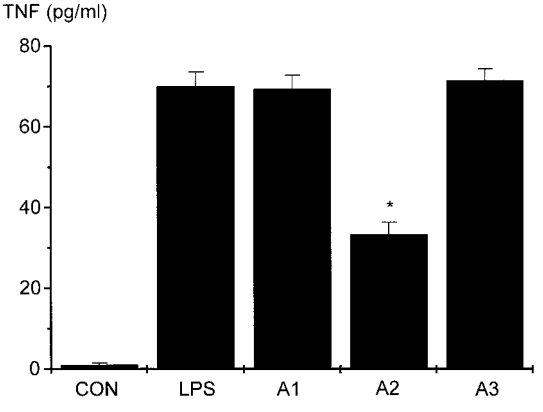

Figure 4. Effect of adenosine receptor agonists. Neonatal rat cardiomyocytes were stimulated with 10 ng/mL LPS (*E coli* 0127) at time 0. Selective adenosine receptor agonists CPA (A_1 receptor) (5 nmol/L), DPMA (A_2 receptor) (10 nmol/L), and N^6-benzyl-NECA (A_3 receptor) (10 nmol/L) were added at time 0. After 6 hours, supernatant was collected, and TNF-α was measured with ELISA. In control (CON, in the absence of LPS), TNF-α was below the limit of detection (1 pg/mL). *P<.0001 vs LPS (n=3, ANOVA).

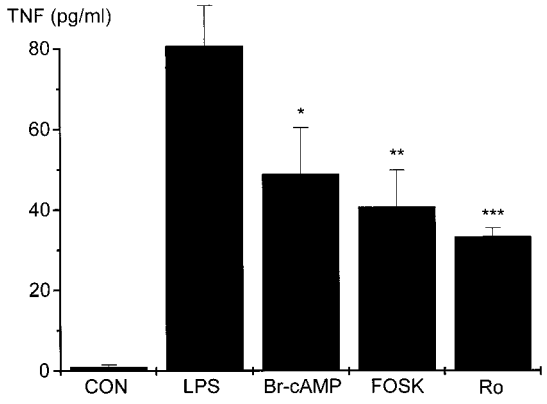

Figure 5. Effect of cAMP agonists. Neonatal rat cardiomyocytes were stimulated with 10 ng/mL LPS (*E coli* 0127) at time 0. Cells were treated with various agents that raise cAMP: 8-bromo-cAMP (Br-cAMP, 10 μmol/L), forskolin (FOSK, 100 μmol/L), or Ro 20–1724 (Ro, 100 μmol/L) was added at time 0. After 6 hours, supernatant was collected, and TNF-α was measured with ELISA. In control (CON, in the absence of LPS), TNF-α was below the limit of detection (1 pg/mL). *$P<.01$, **$P<.005$, and ***$P<.001$ vs LPS (n=3, ANOVA).

agonists and the nonselective agonists 2-chloroadenosine (A_2/A_1 receptors) and NECA (A_2/A_1 receptors) all decreased TNF-α by ≈50%; this reduction was probably due to nonselective stimulation of A_2 receptors. At 100 μmol/L, DPMA completely suppressed the release of TNF-α. Consistent with the effects of adenosine A_2 agonists, the selective A_2 receptor antagonist DMPX (10 μmol/L) abrogated the adenosine (1 μmol/L) response by 50%. This change appeared to be concentration dependent, as DMPX (10 μmol/L) attenuated the response of 10 μmol/L adenosine by only ≈30% (data not shown).

Effect of Stimulators and Inhibitors of Signaling Pathways on TNF-α

The effects of the RGC signaling pathway in mediating the inhibition of adenosine on myocardial TNF-α expression was assessed because this pathway mediates the A_2 response in most tissues. Using pharmacological agents known to stimulate and inhibit selective sites in the RGC pathway, we found that Ro 20–1724 (100 μmol/L), a phosphodiesterase inhibitor, decreased TNF-α by 60% ($P<.001$), forskolin (100 μmol/L) by 50% ($P<.005$), and 8-bromo-cAMP (10 μmol/L) by 40% ($P<.01$) (n=3) (Fig 5). Conversely, MDL-12,330 (20 μmol/L), a specific inhibitor of adenylate cyclase, was able to completely suppress the effect of adenosine and the A_2 agonist DPMA on TNF-α (data not shown).

Release of TNF-α by Fibroblasts

To determine whether contamination with fibroblasts (≈7%) might have influenced our results, experiments with fibroblast-rich preparations were performed. Fibroblast-rich preparations contained ≈5% cardiomyocytes. Table 1 shows a direct comparison between cardiomyocyte and fibroblast-rich preparations, each containing 100 000 cells (n=3). It can be seen that, per cell, fibroblasts release ≈3 to 4 times less TNF-α than do cardiomyocytes in response to LPS. Interestingly, neither adenosine at 10 μmol/L nor the selective A_2 receptor agonist DPMA at 10 nmol/L had an effect on TNF-α release by fibroblasts. Therefore, it is unlikely that contamination with fibroblasts influenced the results obtained with neonatal cardiomyocytes.

TABLE 1. Comparison Between Neonatal Myocytes and Fibroblasts

TNF, pg/mL	Cardiomyocytes	Fibroblasts
Control	0.4±0.3	0.5±0.5
LPS	23.4±1.2	6.5±0.7
Adenosine	8.2±1.1	5.8±0.2
A_2 agonist	11.1±1.0	4.1±0.6

A cardiomyocyte-rich preparation containing 100 000 cells (93% cardiomyocytes) and a fibroblast-rich preparation containing 100 000 cells (95% fibroblasts) were exposed to 10 ng/mL LPS (*E coli* 0127) for 6 hours (n=3). Where indicated, adenosine (10 μmol/L) or the A_2 agonist DPMA (10 nmol/L) was added at time 0. After 6 hours, supernatants were collected, and TNF-α was measured with ELISA. Values are mean±SEM.

TNF-α Production by Adult Ventricular Myocytes

After isolation through collagenase perfusion, adult ventricular myocytes stained positive for TNF-α in the absence of LPS. This was the case whether myocytes were stained immediately on isolation or after 24 to 96 hours in culture. As seen in Fig 6, adult myocytes increased their production of TNF-α in response to LPS, albeit ≈10 times less than seen in neonatal cardiomyocytes. However, as seen in neonatal myocytes, adenosine (10 μmol/L) decreased TNF-α by 60% ($P<.005$) (n=5). Similarly, the A_2 agonist DPMA (10 nmol/L) and the adenosine-regulating agent ITu (10 μmol/L) decreased TNF-α by 75% ($P<.005$) and 72% ($P<.01$), respectively (Fig 6). The cause of the baseline activation of TNF-α after collagenase extraction in the adult myocytes may be explained by the contamination of the collagenase solution with endotoxin (>0.125 EU/mL by *Limulus* amebocyte lysate assay). We were unable to detect concentrations of TNF-α in the medium of adult cardiomyocytes in contrast to neonatal

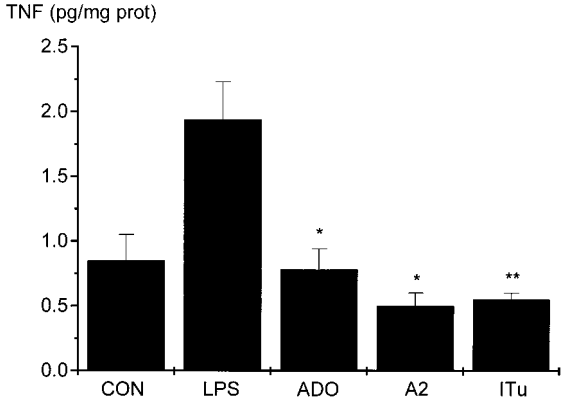

Figure 6. Effect of adenosine on adult cardiomyocytes. Adult rat ventricular myocytes were stimulated with 10 μg/mL LPS (*E coli* 0127) at time 0. Where indicated, adenosine (ADO, 10 μmol/L), the A_2 agonist DPMA (10 nmol/L), or the ADO kinase inhibitor ITu (10 μmol/L) was added at time 0. After 6 hours, cell pellets were collected, and intracellular TNF-α was measured with ELISA. *$P<.005$ and **$P<.001$ vs LPS (n=5, ANOVA).

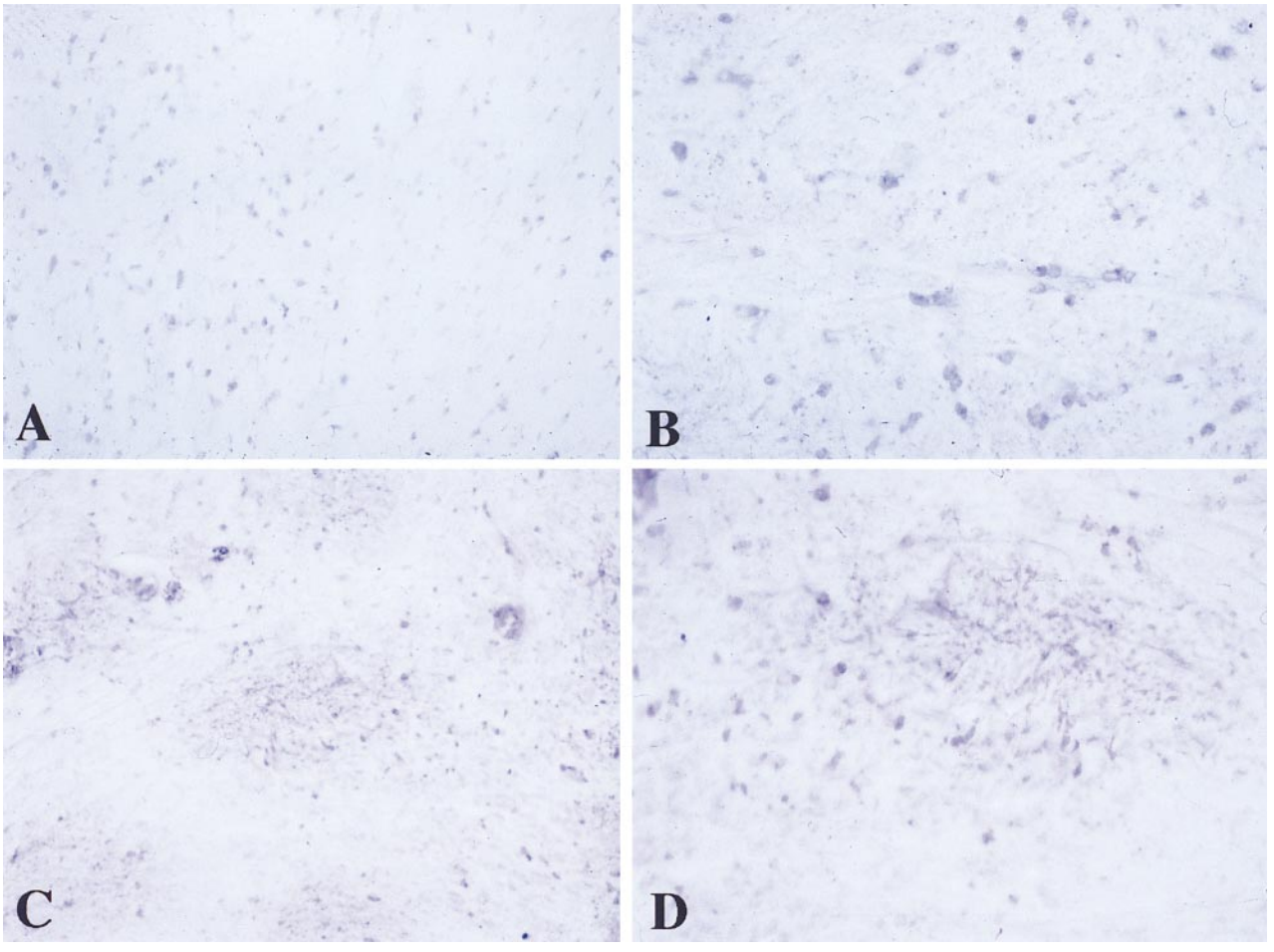

Figure 7. Immunohistochemical analysis of TNF-α in sections of rat papillary muscles. A through D, Control (A) and after treatment with LPS (*E coli*, 10 μg/mL) for 6 hours (C) (magnification $\times$200). B and D, Control (B) and LPS treatment (D) at higher magnification ($\times$400). Primary antibody was stained with secondary biotinylated antibody. There is no evidence of immunostaining in the absence of LPS. LPS induces expression of TNF-α in cardiomyocytes. Sections were weakly counterstained with 1% neutral red.

myocytes. However, in separate experiments, we demonstrated that collagenase totally inhibits the TNF-α ELISA, thus making it likely that traces of collagenase present in the culture medium of adult myocytes were interfering with the ELISA.

TNF-α Release by Rat Papillary Muscle

To ensure that the effects of adenosine were not limited to isolated myocytes, we also exposed rat papillary muscle sections to LPS. Although normal muscle demonstrated no TNF-α, LPS initiated the expression of TNF-α (Fig 7). By use of immunohistochemistry (Fig 7), myocytes were identified as a primary source of TNF-α in the papillary muscle. Adenosine (10 μmol/L) also inhibited TNF-α expression in papillary muscles (Fig 8) (a 55$\pm$15% reduction, $P<.05$, n=5). The A_2 agonist DPMA (10 μmol/L) and the adenosine-regulating agent ITu (10 μmol/L) decreased TNF-α release by papillary muscles by 96% ($P<.005$) and 99% ($P<.005$), respectively (Fig 8).

Effect of Adenosine on Cytosolic Calcium and Contraction in Neonatal Myocytes

Fig 9 shows a representative calcium transient of a group of normal, LPS-treated, and adenosine-treated neonatal myocytes. Cumulative data are summarized in Table 2. The baseline diastolic fura 2 ratio was similar in the three groups. LPS *E coli* (100 ng/mL) significantly reduced the peak systolic calcium by 17% ($P<.01$, n=38). This was associated with a 50% decrease ($P<.01$) in the shortening fraction, as well as a significant decrease in contraction velocity ($P<.01$) and relaxation velocity ($P<.01$). As demonstrated, adenosine (10 μmol/L) totally abolished the LPS effect on cytosolic calcium. Adenosine also significantly improved the shortening fraction ($P<.01$), contraction velocity ($P<.01$), and relaxation velocity ($P<.01$). However, although contraction and relaxation parameters improved, they did not return to normal in the presence of adenosine. Measurements of TNF-α in the medium of electrically driven cells confirmed that changes in calcium and contraction due to LPS were paralleled by changes in TNF-α.

Discussion

The present study demonstrates that adenosine inhibits the expression of TNF-α in neonatal cardiomyocytes, in adult cardiomyocytes, and in rat papillary muscles. In control conditions, neonatal myocytes do not synthesize or release measurable amounts of TNF-α. However, the addition of LPS induces substantial production and release of TNF-α. This is

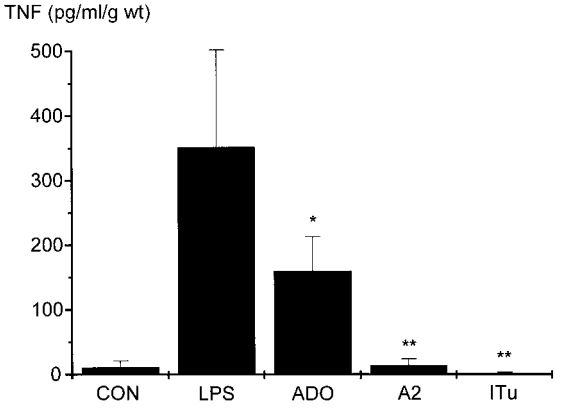

Figure 8. Effect of adenosine (ADO) on rat papillary muscles. Rat papillary muscle sections were stimulated with 10 μg/mL LPS (*E coli* 0127) at time 0. Where indicated, ADO (10 μmol/L), the A_2 agonist DPMA (10 nmol/L), or the ADO kinase inhibitor ITu (10 μmol/L) was added at time 0. After 6 hours, supernatants were collected, and TNF-α was measured with ELISA. In control (CON, in the absence of LPS), TNF-α was below the limit of detection (1 pg/mL). *$P<.05$ and **$P<.005$ vs LPS (n=5, ANOVA).

consistent with an earlier report showing TNF-α release by neonatal mouse myocytes after the administration of LPS.[25] In the present study, release of TNF-α was associated with a 17% decrease in peak systolic calcium ($P<.01$), a 50% decrease in contraction ($P<.01$), and abnormal relaxation ($P<.01$) in neonatal myocytes after 4 days. That TNF-α is expressed by myocytes was confirmed by immunofluorescent staining of myocytes with anti-myosin and anti–TNF-α. This finding was supported by chemiluminescence analysis of white blood cell contamination and experiments with fibroblast-rich preparations. In contrast to neonatal myocytes, ELISA and immunostaining were able to detect TNF-α synthesis in adult myocytes in the absence of LPS. However, this was probably due to the presence of trace amounts of endotoxin in the perfusion system. Nevertheless, adult myocytes responded to LPS with enhanced TNF-α production, albeit that response was significantly less than that of neonatal myocytes. These findings were consistent with earlier studies in which production of TNF-α protein occurred in feline cardiomyocytes isolated from LPS-perfused hearts.[26] In contrast to cultured adult rat myocytes, rat papillary muscles did not show evidence of baseline TNF-α stimulation by either ELISA or immunohistochemistry. However, consistent with the cultured cells, papillary muscles responded to LPS with marked TNF-α release.

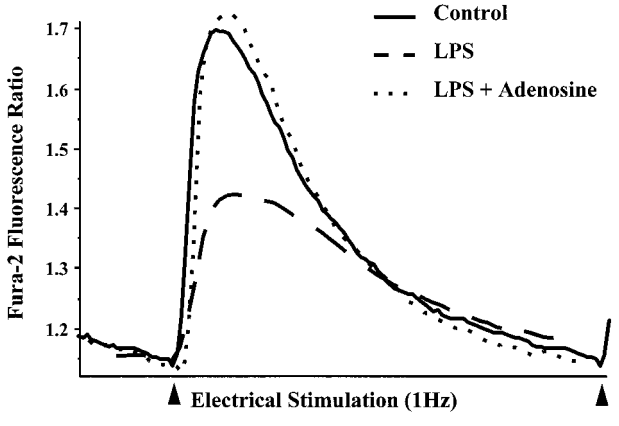

Figure 9. Representative superimposed tracings of calcium transients in electrically driven (1 Hz) cultured rat neonatal cardiomyocytes. Myocytes were treated with diluent (control), LPS *E coli* (100 ng/mL), and LPS+adenosine (10 μmol/L) for 4 days; treatments were performed after isolation and repeated on days 2 and 4. Myocytes were plated on coverslips, and intracellular calcium was measured as a fura 2 fluorescence ratio.

In the present study, we demonstrate that the addition of adenosine to the culture medium effects a significant attenuation of the release of TNF-α by neonatal cardiomyocytes. The adenosine effect was concentration dependent and occurred with a physiological concentration of adenosine (1 μmol/L).[27] In addition, adenosine inhibits TNF-α release when cells were exposed to adenosine 1 hour before LPS treatment, at the time of LPS treatment, or up to 1 hour after LPS challenge. By contrast, adenosine was not effective when given 3 hours after LPS challenge. Furthermore, the anti-cytokine effects of adenosine could not be attributed to an inhibition of TNF-α release, since adenosine also inhibited the cellular production of TNF-α by 78% ($P<.0001$). The effect of adenosine was not limited to neonatal myocytes, as it inhibited TNF-α production in adult myocytes by 60% ($P<.005$) and TNF-α release in papillary muscles by 55% ($P<.05$).

The physiological relevance of our observation is illustrated by the fact that adenosine normalized LPS-induced calcium changes in neonatal myocytes (Fig 9). This was associated with significant improvement in cardiac contraction ($P<.01$) and relaxation ($P<.01$). The LPS-induced calcium changes concur with known effects of TNF-α on calcium transients.[28,29] However, it is unlikely that LPS-induced changes were solely related to TNF-α. Indeed, it is possible that the effects of LPS on calcium and contraction were due to the activation of other cytokines in the inflammatory cascade. The direct negative inotropic effects of LPS and TNF-α have been shown previously in cardiomyocytes.[26,30] Our results indicate that LPS also has a significant ($P<.01$) negative lusitropic effect. Adenosine significantly improved contraction and relaxation in cells exposed to LPS. That adenosine did not completely normalize contraction and relaxation may be explained by the fact that adenosine also activates the adenosine A_1 receptor, which mediates negative inotropic effects in cardiomyocytes.

There are four known subclasses of adenosine receptors (A_1, A_{2a}, A_{2b}, and A_3).[31,32] The cardiac A_1 receptor and its antiadrenergic actions are well characterized; the A_1 receptor appears to be coupled to the activation of an inhibitory guanine nucleotide regulatory protein (G_i) and therefore decreases intracellular production of cAMP. More recently,[33–35] the presence and function of A_2 receptors on ventricular myocytes have been described. It appears that cardiac A_2 receptors are responsible for increases in inotropy via cAMP-dependent as well as cAMP-independent mechanisms. The presence of A_3 receptors on cardiomyocytes has also been proposed in recent studies.[31,32] However, the physiological role of cardiac A_3 receptors remains to be determined. Our finding that only the A_2 receptor agonist DPMA inhibited TNF-α expression in the nanomolar range led us to postulate that the cytokine inhibitory effects of adenosine are mediated through an A_2-dependent pathway. This hypothesis was supported by the fact that

TABLE 2. Effect of LPS and Adenosine on Cellular Calcium and Contraction/Relaxation in Neonatal Cardiomyocytes

	Control (n=38)	LPS (n=38)	LPS+Adenosine (n=38)
Diastolic fluorescence ratio	1.15±0.01	1.14±0.01	1.15±0.01
Peak systolic fluorescence ratio	1.70±0.02	1.42±0.01*	1.73±0.02†
Maximum amplitude of contraction, μm	3.02±0.15	1.51±0.10*	2.45±0.15*†
Maximum speed of contraction, μm/s	36.94±1.80	20.15±1.16*	30.92±2.07*†
Maximum speed of relaxation, μm/s	25.63±1.37	15.39±0.86*	21.92±1.17*†

Cultured neonatal myocytes were treated with diluent (control), LPS *E coli* (100 ng/mL), and LPS+adenosine (10 μmol/L) for 4 days; treatments were performed after isolation and repeated on days 2 and 4 when medium was changed. Myocytes were plated on coverslips and electrically driven (1 Hz). Intracellular calcium was measured as fura 2 fluorescence ratio, and contraction/relaxation parameters were determined using video-edge detection. Values are mean±SEM.

*$P \leq .01$ vs control; †$P \leq .01$ vs LPS (n=38, ANOVA).

the A_2-selective antagonist DPMX suppressed the ability of adenosine to inhibit TNF-α release. It should be noted that inhibition of TNF-α expression occurred at higher concentrations (micromolar) of many of these inhibitors; however, this was likely due to nonselective occupancy of the A_2 receptor at high agonist concentrations.

To further support the hypothesis that adenosine effects a robust inhibition of myocardial cytokine release, we studied the response to LPS in the presence of dipyridamole, an inhibitor of adenosine transport, and ITu, an inhibitor of adenosine kinase. Both inhibitors have been shown to increase endogenous adenosine levels in isolated cardiac myocytes.[19,24] Dipyridamole decreased TNF-α by 30%, with exogenous adenosine having a significant additive effect. These results were consistent with results by Bouma et al[9] that were obtained in activated human monocytes exposed to draflazine, an inhibitor of adenosine transport. In contrast, the addition of ITu (10 μmol/L) totally suppressed LPS-stimulated TNF-α release. It remains unclear why ITu was more effective than exogenous adenosine. However, it is possible that in the presence of ITu, endogenous adenosine was compartmentalized with the A_2 receptor. The results with adenosine-regulating agents were confirmed in adult cardiomyocytes and papillary muscles, where ITu (10 μmol/L) suppressed TNF-α by 72% ($P<.01$) and 99% ($P<.005$), respectively. Our results obtained with adenosine-regulating agents are consistent with a previous report in which draflazine, another adenosine-regulating agent, attenuated TNF-α release by activated monocytes.[9]

Previous studies have suggested that TNF-α is decreased by cAMP-dependent mechanisms in macrophages.[36,37] The results of the present study suggest that cAMP-dependent mechanisms are also responsible at least in part for the inhibitory effects of adenosine on TNF-α production in myocytes. Activation of the A_2 receptor, an RGC-coupled receptor, significantly decreased TNF-α release. In addition, when intracellular cAMP was increased with forskolin, 8-bromo-cAMP, or Ro 20–1724, TNF-α was decreased by ≈50%. Conversely, the adenylate cyclase inhibitor MDL 12,330 was able to inhibit completely the effect of adenosine and the A_2 agonist DPMA.

The effects of adenosine on the immune system and, in particular, its inhibitory effects on phagocytic function and adherence of neutrophils are well recognized.[38,39] Recently, Parmely et al[8] have shown that the production of TNF-α by LPS-treated macrophages can also be modulated by adenosine. They demonstrated that 1 to 10 μmol/L adenosine inhibited the TNF-α production of activated mouse peritoneal macrophages by at least 50%. Similar results were obtained by Bouma et al[9] using activated human monocytes and Reinstein et al[40] using activated rat Kupffer cells. Therefore, the results of the present study suggest that the inhibitory effects of adenosine on cytokine production may be a ubiquitous phenomenon. However, whether the effects of adenosine are at the pretranslational or posttranslational level remains to be determined.

An increasing amount of data suggest a pathophysiological role for cytokines in the development of the end-stage heart failure phenotype. In the present study, we demonstrate that adenosine can substantially inhibit myocyte TNF-α expression in neonatal cardiomyocytes, adult cardiomyocytes, and papillary muscles. If adenosine effects a similar inhibition of TNF-α expression in vivo, regulation of myocardial TNF-α expression by modulation of adenosine levels may provide a new and novel therapeutic target for the pharmacological or molecular therapy of end-stage congestive heart failure. However, adenosine only attenuated TNF-α expression when cells were exposed before or shortly after LPS activation. Therefore, the therapeutic window for adenosine may limit its clinical usefulness.

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