Antioxidant vitamin therapy alters sepsis-related apoptotic myocardial activity and inflammatory responses

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Carlson, Deborah, David L. Maass, D. Jean White, Jing Tan, and Jureta W. Horton. Antioxidant vitamin therapy alters sepsis-related apoptotic myocardial activity and inflammatory responses. Am J Physiol Heart Circ Physiol 291: H2779–H2789, 2006. First published July 14, 2006; doi:10.1152/ajpheart.01258.2005.—This study examined the effects of antioxidant vitamins on several aspects of sepsis-related myocardial signaling cascades. Sprague-Dawley rats were divided into four groups: group 1, vehicle-treated shams; group 2, sham-operated rats given antioxidant vitamins (vitamin C, 24 mg/kg; vitamin E, 20 U/kg; vitamin A, 417 U/kg; and zinc, 3.7 ng/kg) by oral gavage in 0.5 ml water twice daily for 3 days and no septic challenge (vitamin-treated, sham-operated rats); group 3, intratracheal delivery of Streptococcus pneumoniae, 4 × 106 colony forming units in a volume of 0.3 ml phosphate buffer solution; group 4, S. pneumoniae challenge as described for group 3 plus antioxidant vitamins (as described for group 2). Hearts collected 24 h after septic challenge were used to examine several aspects of cell signaling and ventricular function. As a result, when compared with sham-operated rats, sepsis in the absence of antioxidant therapy promoted NF-κB activation, increased mitochondrial cytochrome c release, increased myocyte cytokine secretion, increased caspase activation, and impaired left ventricular function. Antioxidant vitamin therapy plus septic challenge prevented NF-κB activation, reduced mitochondrial cytochrome c release, decreased caspase activity, abrogated cardiomyocyte secretion of inflammatory cytokines, and improved myocardial contractile function. In conclusion, antioxidant vitamin therapy abrogated myocardial inflammatory cytokine signaling and attenuated sepsis-related contractile dysfunction, suggesting that antioxidant vitamin therapy may be a potential approach to treat injury and disease states characterized by myocardial dysfunction.

Aspiration-induced pneumonia; Streptococcus pneumoniae; cardiomyocytes; inflammatory cytokines; nuclear factor-κB activation; left ventricular performance

Sepsis, one of the main consequences of injury and disease, is a leading cause of morbidity and mortality in critically ill surgical patients (3). Mortality rates in septic patients have been demonstrated to range as high as 40–60% (1). Impaired myocardial contractile function is a well-documented feature contributing to the mortality associated with this pathological condition (28, 36). Despite the well-characterized physiological response, the precise mechanisms by which infection impairs myocardial contraction and relaxation remain unclear.

A large body of evidence suggests that the overproduction of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, and their secretion by cardiomyocytes act as early signaling events leading to sepsis-related cardiac dysfunction. (18, 19, 25, 26). The cellular pathways that have been demonstrated to modulate the cytokine cascade have been shown to include nuclear translocation of NF-κB, a known mediator of inflammatory and stress responses and apoptosis (7, 11, 24, 37). In unstimulated cells NF-κB exists as a latent cytoplasmic complex with the inhibitor protein IκB. IκB binds to the components of NF-κB, p50 and p65, inhibiting translocation to the nucleus (2, 7, 37). In response to stress such as sepsis, the cell responds by producing reactive oxygen species, nitrogen intermediates, and cytokines, all of which promote phosphorylation of IκB and nuclear translocation of NF-κB. NF-κB then triggers the transcription of proinflammatory genes as well as both anti- and proapoptotic genes. Both molecular and pharmacological strategies that prevent NF-κB activation have been shown to provide considerable myocardial protection in models of endotoxin challenge and in burn injury (7, 24), thus confirming the significant role of this transcription factor in mediating cardiac dysfunction.

In addition to a role as a transcription factor, NF-κB has also been identified as an oxidant-sensitive transcriptional regulatory protein (30, 33), and nuclear translocation of NF-κB has been implicated in the impairment of mitochondrial function, promoting a loss of high energy phosphates, enhanced mitochondrial cytochrome c release, mitochondrial free radical production, and oxidative injury to the cell (32, 35), all of which may negatively impact cardiac function. Several antioxidant therapies have been previously demonstrated to prevent NF-κB activation and to reverse the inflammatory cytokine response, which, in turn, may alleviate the associated cardiac dysfunction (16, 30).

As stated, NF-κB after translocation to the nucleus promotes the transcription of both pro- and antiapoptotic proteins. The relationship between cardiac NF-κB activity and apoptosis in injury remains unclear, and in some model systems, it has proven to be detrimental, and in others, protective (11). Although the role of myocardial apoptosis in cardiac dysfunction remains unclear during sepsis, it has been shown recently that activation of the apoptotic-associated caspasases plays a critical role in triggering myocardial dysfunction in the rat heart during sepsis (8, 9). Caspase-3 activation in the myocardium has been suggested to play an important role in endotoxin-induced cardiomyocyte dysfunction through changes in calcium myofilament response, contractile protein cleavage, and sarcomere disorganization (22). Other caspase activities that may influence cardiac function include caspase cleavage of gelsolin, which leads to gelsolin activation and subsequent gelsolin-mediated disruption of the actin filament network. In addition to gelsolin, a number of other intermediate filament proteins, as well as adherent junction proteins, are also cleaved by caspases (20).

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This present study was designed to examine the effects of antioxidant vitamin therapy on several aspects of sepsis-related myocardial inflammatory signaling and dysfunction. Antioxidant vitamin therapy was initiated 3 days before aspiration pneumonia challenge and continued throughout the sepsis period; the effects of antioxidant vitamin therapy on myocardial NF-κB activation and cellular integrity as measured by mitochondrial cytochrome c release and caspase activation were examined. In addition, cardiomyocyte secretion of inflammatory cytokines and cardiac contraction and relaxation were recorded as markers of myocardial function.

**MATERIALS AND METHODS**

**Experimental Model**

Adult Sprague-Dawley male rats (320–350 g) were used in the present study. Animals obtained from Harlan (Houston, TX) were conditioned in-house for 5–6 days after arrival with commercial rat chow and tap water available at will. All protocols performed in this study were reviewed and approved by The University of Texas Southwestern Medical Center’s Institutional Review Board for the care and handling of laboratory animals and conformed to all guidelines for animal care as outlined by the American Physiological Society and the National Institutes of Health.

**Antioxidant Vitamin Therapy**

To examine the potential beneficial effects of antioxidant vitamins on sepsis-related cardiomyocyte cytokine secretion and myocardial contractile dysfunction, adult rats were given a vitamin regimen twice each day for 3 days before septic challenge and for 24 h after intratracheal inoculation with *Streptococcus pneumoniae*. Rats received vitamin A (41 U/kg), vitamin C (38 mg/kg), vitamin E (27 U/kg), and zinc (3.7 ng) given in a total volume of 0.5 ml water by oral gavage twice each day (16). On the morning of the fourth day, vitamins were given; rats were anesthetized and randomized to receive either sham pneumonia or aspiration-induced pneumonia as described under Induction of Aspiration-Related Pneumonia.

**Catheter Placement**

Rats were anesthetized lightly with isoflurane, and body hair on the neck was closely clipped. The neck region was then treated with a surgical scrub, the left carotid artery was exposed, and a polyethylene catheter (PE-50) inserted and advanced retrogradely to the level of the aortic arch. In addition, a polyethylene catheter (PE-50) placed in the right jugular vein was used to administer fluids. All catheters were filled with heparinized saline, exteriorized, and secured at the nape of the neck.

**Preparation of Inoculum**

*S. pneumoniae* type 3 was obtained from the American Type Culture Collection (ATCC 6303, Rockville, MD) in lyophilized form. Bacteria were reconstituted and then passed through the cerebrospinal fluid of a rabbit to increase virulence; aliquots were prepared and stored at –80°C. Before each experiment, individual aliquots were thawed, inoculated into Muller Hinton broth with supplement C (Difco, Kansas City, MO), and incubated overnight at 37°C in the presence of 95% O2–5% CO2. The broth was then centrifuged, and the resultant pellet was washed twice with sterile endotoxin-free PBS to remove any impurities adherent to the bacteria. The bacteria were then resuspended in sterile endotoxin-free PBS, agitated, and then drawn up into sterile tuberculin syringes in 0.3 ml aliquots. Bacterial colony forming units (CFU) were determined by plating 100 μl of the bacterial suspension onto blood agar plates in serial dilutions and incubating the plates overnight at 37°C. The number of viable bacteria inoculated into animals in either the pneumonia alone group or in the burn plus pneumonia group was ~4 × 10⁶ CFU (17, 39).  

**Induction of Aspiration-Related Pneumonia**

On the fourth day after initiating oral vitamin therapy, animals were anesthetized with isoflurane, placed in a supine position, intravenous catheters were placed, and the area over the trachea was prepped with a surgical scrub (povidone-iodine, Betadine). A midline incision was made over the trachea; the trachea was identified and isolated via blunt dissection. An aliquot of either bacterial suspension (4 × 10⁶ CFU/0.3 ml PBS) or sterile endotoxin-free PBS was injected directly into the trachea using a 27-gauge needle; the wound was then closed with surgical staples. Animals were placed on a 30° incline with the head up to ensure that the injected fluid entered the lungs. Immediately after intratracheal administration of either buffer or *S. pneumoniae*, the external jugular catheter was connected to a swivel device (BSP99 syringe pump, Braintree Scientific, Braintree, MA) for fluid administration (lactated Ringer solution), and 25 ml were given over the first 8 h after intratracheal *S. pneumoniae* challenge. Additional lactated Ringer solution (10 ml/rat) was given over the next 16 h after *S. pneumoniae* challenge. Buprenorphine (0.5 mg/kg) was given every 12 h after septic challenge. Rats did not display discomfort or pain, moved freely about the cage, and consumed food and water within 20 min after recovering from anesthesia. Rats given intratracheal buffer (no bacterial challenge) received identical regimens of analgesics (buprenorphine) and received lactated Ringer solution (12 ml/24 h) to ensure adequate hydration states. Body temperature was measured with a rectal temperature probe (YS-TEL Thermometer, model 44TA), and respiratory rate was monitored by counting respiratory movement. Systemic blood pressure was measured by using a Gould-Statham pressure transducer (model IDP23, Gould Instruments, Oxford, CA) connected to a medical recorder (model 7D Polygraph, Grass Instruments, Quincy, MA). A tachycardiograph (model 7P4F, Grass Instruments) was used to monitor heart rate. Data from the Grass recorder were input to a Dell Pentium computer, and a Grass Poly VIEW Data Acquisition System was used to convert acquired data into digital form.

**Experimental Groups**

Rats received vitamin therapy twice each day for 3 days. On the morning of the fourth day, vitamins (or vehicle) were given and rats were anesthetized and randomized to receive either sham pneumonia or aspiration-induced pneumonia as described under Induction of Aspiration-Related Pneumonia.
tion of myocyte cytokine secretion (n = 4 heart/experimental group), to examine NF-kB activation, and to examine mitochondrial cytochrome c release and caspase activation (n = 6 hearts/groups). Hearts collected for these in vitro studies were cleared of fat and vessels, freeze-clamped in liquid nitrogen at the time of harvest, and stored at –80°C.

Cardiac NF-kB Activation

Nuclear protein extraction. A modified procedure based on the method of Grabellus et al. (14) was used. All steps were performed on ice.

Whole hearts were thawed in the presence of a lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.1% Nonidet P-40, 0.5 mM DTT, and protease inhibitors (Complete Mini EDTA-free, Roche, Mannheim, Germany). The hearts were allowed to thaw on ice for 20 min and were suspended in 500 µl of lysis buffer. Tissue was then homogenized by hand using a dounce homogenizer. The cells were then allowed to incubate for 20 min on ice and were then centrifuged at 1,200 g for 5 min at 4°C. The supernatants (cytosolic fraction) were removed and frozen at –80°C.

The remaining pellet was resuspended in 200 µl of ice-cold resuspension buffer (20 mM HEPES, pH 7.9; 420 mM NaCl; 1.8 mM MgCl₂; 0.2 mM EDTA, pH 8; and 25% glycerol) and protease inhibitors as described above plus 0.5 mM EDTA. The pellets were completely resuspended by pipetting and were incubated for 20 min on ice with gentle resuspension of the pellets every couple of minutes. After incubation, the pellets were centrifuged for 5 min at full speed at 4°C. The supernatant was collected (nuclear fraction) and frozen at –80°C. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Nuclear and cytosolic extracts were stored at 80°C.

EMSA. Double-stranded oligonucleotides corresponding to the consensus NF-kB binding site of the κ light chain enhancer (5’-AGTTGAGGGGACTTTCCAGGC-3’) were purchased from Promega Biotech (Madison, WI). In a total volume of 10 µl, 3.5 pmol of oligonucleotide, 10 U of T4 polynucleotide kinase in 1× forward buffer (GIBCO-BRL, Gaithersburg, MD), and 30 µCi of [γ-32P]ATP (DuPont NEN, Boston, MA) were incubated at 37°C for 60 min. The reaction was stopped by adding 1 µl of 0.5 mM EDTA. Volume was brought up to 50 µl with the addition of STE buffer (10 mM Tris·HCl, pH 7.5; 10 mM NaCl; and 1 mM EDTA). Labeled probe was separated from unbound ATP using ProbeQuant G-50 Micro Columns from Pharmacia Biotech (Piscataway, NJ). The activity of labeled probe was determined, and the probe was stored at –20°C.

Nuclear proteins (20 µg) were incubated in 1× gel shift buffer (100 mM HEPES, pH 7.6; 250 mM KCl; 5 mM MgCl₂; and 25% glycerol). In addition, these were incubated with poly(deoxyxyno-sine-deoxythymidyl)ic acid and 2 µl of probe between 400,000 and 500,000 counts/min. The final reaction volume was made up to 20 µl with water. The reactions were incubated at room temperature for 20 min, and then 2 µl of loading buffer were added. Loading buffer (10×) consisted of 30% glycerol, 0.25% xylene cyanol, and 0.25% bromophenol blue. The samples were then separated on an 8% polyacrylamide gel in 0.5× TBE (25 mM Tris·HCl, 25 mM boric acid, and 0.5 mM EDTA). Finally, the gels were dried and exposed to X-ray film. Competition analyses were performed by including a 50 M excess of unlabeled double stranded DNA oligonucleotide in the binding reaction. Nonspecific competitor DNA contained an activator protein-1 binding element (7).

Western Blot Analysis of p50 and p65

Protein extracts (20 µg), cytoplasmic and nuclear, harvested as stated in the EMSA assay were combined with 2 µl of 2× sample-loading buffer (0.063 M Tris·HCl, pH 6.8; 2% SDS; 5% mercapto-ethanol; and 10% bromophenol blue). The samples were boiled for 5 min and resolved on a 12.5% SDS-polyacrylamide gel. The gels were then transferred to polyvinylidene difluoride (PVDF) membrane (Per-kin Elmer Life Sciences, Boston, MA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline with 0.05% Tween 20 (TBS-T) (120 mM Tris, pH 7.6; 0.9% NaCl; 0.05% Tween 20; and 5% dried milk). Blocked membranes were then incubated overnight at 4°C with a rat monoclonal NF-kB p65 antibody or p50 antibody (1:100 dilution; Santa Cruz, Biotechnology, Santa Cruz, CA) in TBS-T with 5% milk. After incubation, membranes were washed for 15 min at room temperature in TBS-T, followed by five 5-min washes in TBS-T. After being washed, membranes were incubated for 1 h with a polyclonal goat anti-rat-IgG-horseradish peroxidase antibody (Bio-Rad) diluted to 1:2,500 in TBS-T. Membranes were then washed as described above and exposed to a mixture of luminol plus hydrogen peroxide under alkaline conditions (SuperSignal West Pico, Pierce-Endogen, Rockford, IL) for 5 min. The resulting chemiluminescent reaction was detected by Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). Experiments were repeated independently three times with groups as indicated in RESULTS. Equal loading of the samples was confirmed by the Bradford Assay and by Ponceau S staining of the PVDF membranes (Sigma) (7). Quantification of single band density was determined by using Quantity One Software (version 4.4.0, build 36; Bio-Rad) after conversion of radiographic film to TIFF files (8-bit grayscale) using a ScanJet 7400C (Hewlett-Packard, Palo Alto, CA) and reported in arbitrary units per square millimeter.

Isolation of the Cytosolic Fraction and Immunoblot Analysis for Cytochrome c

All steps were performed on ice. Isolated hearts were homogenized using a hand dounce at 4°C in an isolation buffer containing (in mmol/l) 10 N-(2-hydroxyethyl)piperazine-N-(4-butanesulfonic acid), 80 potassium chloride, 1 sodium ethylenediaminetetraacetic acid, 1 sodium ethyleneglycoltetraacetic acid, 4 DTT, and 250 sucrose and 50 µg/ml saponin and protease inhibitors (Complete Mini EDTA-free, Roche). The homogenate was centrifuged at 100,000 g for 1 h at 4°C, and the final supernatant was used as the cytosolic fraction. Protein concentrations were determined by using the Bio-Rad protein assay.

Protein (25 µg) from the cytosolic fraction was resolved on a 12% SDS-polyacrylamide gel. Proteins were electroblotted onto PVDF membrane (Bio-Rad) and were blocked for 1 h at room temperature in TBS-T with 5% nonfat dry milk. After the blocking stage, membranes were incubated with a rabbit polyclonal cytochrome c antibody (1:500) (Santa Cruz Biotechnology) for 12 h at 4°C. Membranes were then washed once in TBS-T for 15 min, followed by five 5-min washes. The membranes were then incubated for 1 h at room temperature with a polyclonal anti-rabbit IgG horseradish peroxidase-linked antibody (1:8,000) (Amersham, Piscataway, NJ). Membranes were then washed as described in Western Blot Analysis of p50 and p65 and exposed to a mixture of Luminol plus hydrogen peroxide under alkaline conditions (SuperSignal West Pico, Pierce-Endogen) for 5 min. The resulting chemiluminescent reaction was detected by Kodak X-OMAT AR film (Eastman Kodak). Experiments were repeated independently three times with groups as indicated in RESULTS.

Equal loading of the samples was confirmed by the Bradford Assay and by Ponceau S staining of the PVDF membranes (Sigma) (7). Loading of the control β-actin antibody (Novus Biologicals, Littleton, CO) was also used as an equal loading control. Quantification of single band density was determined by using Quantity One Software (version 4.4.0, build 36; Bio-Rad) after conversion of radiographic film to TIFF files (8-bit grayscale) using a ScanJet 7400C (Hewlett-Packard) and reported in arbitrary units per square millimeter.

Caspase-3 and -8 Activity Assay

Caspase-3 activity was evaluated by measuring relative DEVDase, or caspase, cleavage activity using the Clontech ApoAlert Caspase-3 Colorimetric Assay Kit (Clontech, Palo Alto, CA). Caspase-8 activity was evaluated using the Clontech ApoAlert Caspase-8 Colorimetric Assay Kit (Clontech). The caspase fluorescent assay kits detect...
caspase activation by assaying for the cleavage of a fluorescent substrate.

The caspase-3 and -8 fluorescent assays detect the shift in fluorescence emission of 7-amino-4-trifluoromethyl coumarin (AFC). After the substrate is cleaved by the protease, AFC emits a yellow-green fluorescence at 505 nm if excited at 400 nm. Caspase-3 and -8 assays were done according to the manufacturer’s protocol for 250 μg of heart lysate protein.

Preparation of Primary Cardiomyocyte Cultures

Rats received an intraperitoneal injection of heparin (2,000 U) 20–30 min before euthanasia. The rats were decapitated, and the hearts were harvested and placed in a petri dish containing ice-cold (4°C) heart medium containing 113 mM NaCl, 4.7 mM KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4, 12 mM NaHCO3, 10 mM KHCO3, 20 mM d-glucose, 0.5× MEM amino acids (50×, Gibco-BRL 11310–051), 10 mM HEPES, 30 mM taurine, 2.0 mM creatine, and 2.0 mM creatine. Hearts were cannulated via the aorta and perfused with room-temperature heart medium at a rate of 12 ml/min for a total of 5 min in a nonrecirculating mode. Enzymatic digestion was initiated by perfusing the heart with digestion solution, which contained 24.5 ml of heart medium described above plus 50 mg collagenase II (Worthington 4771, lot no. MOB3771), 50 mg BSA, fraction V (Gibco-BRL 11018–025), 0.5 ml trypsin (2.5%, 10× Gibco-BRL 15090–046), and 15 μM CaCl2. Enzymatic digestion was accomplished by recirculating this solution through the heart at a flow rate of 12 ml/min for 20 min. All solutions perfusing the heart were maintained at a constant temperature of 37°C. At the end of the enzymatic digestion, the ventricles were removed and mechanically disassociated in 6 ml enzymatic digestion solution containing a 6 ml aliquot of 2× BSA solution (2 mg BSA, fraction V to 100 ml of heart media). After mechanical disassociation with fine forceps, the tissue homogenate was filtered through a mesh filter into a conical tube. The cells adhering to the filter were collected by washing with an additional 10 ml aliquot of 1× BSA solution (100 ml of heart medium described above and 2 gm of BSA, fraction V). Cells were then allowed to pellet in the conical tube for 10 min. The supernatant was removed, and the pellet was resuspended in 10 ml of BSA. The cells were washed and pelleted further in BSA buffer with increasing increments of calcium (100 μM, 200 μM, and 500 μM to a final concentration of 1,000 μM). After the final pelleting step, the supernatant was removed and the pellet was resuspended in MEM [prepared by adding 10.8 g 1×MEM, Sigma M-1018, 11.9 mM NaHCO3, 10 mM HEPES, and 10 ml penicillin-streptomycin (100×, Gibco-BRL 1540–122) with 950 ml MilliQ water]; total volume was adjusted to 1 liter. At the time of MEM preparation, the medium was bubbled with 95% O2-5% CO2 for 15 min, and the pH was adjusted to 7.1 with 1 M NaOH. The solution was then filter sterilized and stored at 4°C until use. At the final concentration of calcium, the cell viability was measured, and cell suspensions with >85% viability were used for subsequent studies. Myocytes with a rod-like shape, clear-defined edges, and sharp striations were prepared with a final cell count of 5 × 10⁴ cells·ml⁻¹·well⁻¹ (18, 24).

Cytokine Secretion by Cardiomyocytes

Myocytes were pipetted into microtiter plates at 5 × 10⁴ cells·ml⁻¹·well⁻¹ (12 well cell culture cluster, Corning, Corning, NY) for 18 h (10% CO2 incubator at 37°C). Supernatants were collected to measure myocyte-secreted TNF-α, IL-1β, IL-6, and IL-10 (rat ELISA, Endogen, Woburn, MA). We previously examined the contribution of contaminating cells (nonmyocytes) in our cardiomyocyte preparations using flow cytometry, cell staining (hematoxylin and eosin), and light microscopy. We confirmed that <2% of the total cell number in a myocyte preparation was noncardiomyocytes. Because our preparations are 98% cardiomyocytes, we concluded that a majority of the inflammatory cytokines measured in the cardiomyocyte supernatant was indeed cardiomyocyte derived (18).

Intracellular Calcium and Sodium Concentration Measurements

Separate aliquots of cells were loaded with either fura-2 AM (Molecular Probes, Carlsbad, CA) for 45 min or sodium-binding benzofuran isophthalate (SBFI) (Molecular Probes) for 1 h at room temperature in the dark. Myocytes were then suspended in 1.0 mM calcium containing MEM, washed to remove extracellular dye, and placed on a glass slide on the stage of a Nikon inverted microscope. The microscope was interfaced with Groomey optics. This InCyt Im2 Fluorescence Imaging System (Intracellular Imaging, Cincinnati, OH) allowed alternation between the 340- and 380-nm excitation wavelengths. Images were captured by monochrome charge-coupled device camera equipped with a TV relay lens. InCyt Im2 Image software allowed measurement of intracellular calcium and sodium concentrations from the ratio of the two fluorescent signals generated from the two excitation wavelengths (340 nm/380 nm). The calibration procedure included measuring fluorescence ratio with buffers containing different concentrations of either calcium or sodium. At each wavelength, the fluorescence emissions were collected for 1-min intervals, and the time between data collection was 1 to 2 min. Because quiescent or noncontracting myocytes were used in these studies, the calcium levels measured reflect diastolic levels.

Isolated Coronary Perfused Hearts

Twenty-four hours after intratracheal challenge, rats from each experimental group (n = 8 to 9 rats/group) were heparinized, and a blood sample was collected to measure circulating cytokines and to determine the presence or absence of circulating bacteria. One lung was harvested and placed in buffered formalin for examination by a veterinarian pathologist who was unaware of the origin of tissue with regard to group assignment. The remaining lung was lavaged with 25 ml of ice-cold phosphate buffer solution, and bronchoalveolar lavage samples were used to determine presence or absence of bacteria. Hearts were removed and placed on ice in ice-cold (4°C) Krebs-Henseleit bicarbonate-buffered solution containing (in mM) 118 NaCl, 4.7 KCl, 21 NaHCO3, 1.25 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 11 glucose (95% O2-5% CO2; pH 7.4; P02, 550 mmHg; and PC02, 38 mmHg). The ascending aorta was cannulated, and the coronary circulation was perfused (Krebs-Henseleit bicarbonate, flow rate of 6 ml/min, ISMATEC model 7335–30, Cole Palmer, Chicago, IL). A pressure transducer connected to the pressure tubing between the heart and the heating coil was used to measure coronary perfusion pressure; effluent was collected and measured to confirm coronary flow rate. In vitro contractile function was monitored by placing a latex balloon attached to a polyethylene tube into the left ventricular chamber through an apical stab wound. Left ventricular pressure (LVP) was measured with a Statham P23 ID pressure transducer attached to the balloon cannula. Left ventricular maximum rate of LVP rise and fall (±dP/dt(max) values were obtained using an electronic differentiator (model 7P20C, Grass Instruments). All variables were recorded on an ink-writing Grass polygraph (model 7DWL8P); a Grass tachycardiograph (model 7P4F) was used to monitor heart rate, and a Grass Poly VIEW Data Acquisition System was used to convert acquired data into digital form.

Statistical Analysis

All values are expressed as means ± SE. ANOVA was used to assess an overall difference among the groups for each of the variables. Levene’s test for equality of variance was employed to suggest the multiple comparison procedure to be used. If equality of variance among the four groups was suggested, multiple comparison procedures were performed (Bonferroni); if inequality of variance was suggested by Levene’s test, Tamhane multiple comparisons, which do
Hemodynamic and metabolic responses to sepsis

<table>
<thead>
<tr>
<th>Group 1, Sham</th>
<th>Group 2, Sham + Antioxidants</th>
<th>Group 3, Sepsis</th>
<th>Group 4, Sepsis + Antioxidants</th>
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<tr>
<td>MAP, mmHg</td>
<td>150 ± 4</td>
<td>149 ± 5</td>
<td>128 ± 2*</td>
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<td>HR, beats/min</td>
<td>523 ± 12</td>
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<td>Base excess, mM</td>
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<td>TNF-α, pg/ml</td>
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Values are means ± SE. MAP, mean arterial pressure; HR, heart rate; PaCO2, arterial PaCO2; Ca2++, serum-ionized calcium. *P < 0.05, difference from respective sham-operated groups. †P < 0.05, antioxidant vitamin-related difference (group 4 vs. group 3).

RESULTS

There was no evidence of infection (negative blood and bronchoalveolar cultures) in sham-operated rats given vehicle (group 1) and sham-operated rats given antioxidant vitamins (group 2), and there were no deaths in these experimental groups. Development of sepsis 24 h after intratracheal administration of S. pneumoniae was confirmed by positive blood cultures as well as histological evidence of pulmonary infection and inflammation. A pathological examination of pulmonary tissue confirmed mild to moderate pulmonary infiltrates, neutrophil emigration, and moderate edema in all rats given S. pneumoniae, regardless of antioxidant vitamin therapy. There were two deaths out of 18 animals (11%) 24 h after bacterial challenge alone (group 3). There were no deaths in the septic group given antioxidant vitamins (group 4). S. pneumoniae CFUs in blood cultures from group 3 (1 × 10^7 CFU/ml) were higher than values measured in group 4 (2.8 × 10^4 CFU/ml blood). Bronchoalveolar lavage cultures were also higher in group 3 (6.4 × 10^3 CFU/ml) compared with those in group 4 (1.3 × 10^3 CFU/ml).

Twenty-four hours after intratracheal administration of S. pneumoniae in the absence of vitamin therapy (group 3), mean arterial blood pressure (MAP) was significantly lower than values measured in time-matched, sham-operated rats (P < 0.05), despite fluid resuscitation to maintain circulating volume and preload (Table 1). Sepsis in group 3 was associated with metabolic acidosis as indicated by a rise in blood lactate levels and changes in base excess. Free fraction of serum-ionized calcium levels also fell in vehicle-treated sepsis (Table 1).

Antioxidant vitamin therapy in sepsis (group 4) improved MAP values over values measured in sepsis in the absence of vitamin therapy (group 3, P < 0.05), despite identical volume of fluid resuscitation in all septic rats. In addition, blood lactate levels were lower in antioxidant vitamin-treated sepsis compared with values measured in vehicle-treated sepsis, but changes in base excess persisted in antioxidant vitamin-treated sepsis. Sepsis-related fall in serum-ionized calcium levels was evident 24 h after septic challenge despite vitamin treatment.

Fig. 1. Vehicle-treated, pneumonia-related sepsis promoted NF-κB activation as indicated by representative gel shift assay, and this effect was attenuated by antioxidant vitamin therapy. Densitometric analysis of multiple gel shifts was performed, and data are shown as means ± SE (bottom). All cardiac tissue was collected at 24-h postseptic insult. Groups, as labeled, include (as described in MATERIALS AND METHODS) sham-operated animals; sham-operated animals treated with antioxidant vitamins; sepsis, animals treated with Streptococcus pneumoniae; and sepsis + vitamins, animals treated with S. pneumoniae + antioxidant vitamins. *P < 0.05, significant difference from sham-operated rats.
Plasma cytokines (TNF-α, IL-1β, and IL-6), measured to provide an index of inflammatory status, were elevated in vehicle-treated sepsis (group 3, Table 1). Systemic inflammation was significantly attenuated by antioxidant vitamin treatment of sepsis (group 4, Table 1).

Myocardial NF-κB Activation in Sepsis

NF-κB nuclear translocation occurred 24 h after *S. pneumoniae* challenge as indicated by the representative gel shift assay shown in Fig. 1. Densitometric analysis of six to seven gel shift assays from five to six rats per experimental group is shown in the lower portion of Fig. 1. These data are consistent with our previous report that LPS challenge in adult mice promoted myocardial NF-κB activation (15). Antioxidant vitamin therapy initiated before *S. pneumoniae* challenge significantly inhibited myocardial NF-κB nuclear translocation, reducing NF-κB nuclear translocation by as much as 38% from that observed after sepsis in the absence of antioxidant vitamin therapy. Moderate activation of myocardial NF-κB in untreated sham-operated animals (group 1) was attributed to the stress of anesthesia and handling.

To confirm the translocation of NF-κB to the nucleus by another approach, immunoblots were used to measure sepsis-related changes in nuclear cytoplasmic p50 and p65 distribution. Cytosolic cellular fractions were isolated from the hearts of either sham-operated animals, sham-operated animals treated with antioxidant vitamins, septic animals, or septic animals treated with antioxidant vitamins. As seen in Fig. 2, protein levels of both p50 and p65 increased significantly in both the cytoplasmic and nuclear fractions in untreated septic animals (group 3). No similar increase in protein concentration in either fraction was detected in either the sham-operated or the sham-operated animals treated with antioxidant vitamins. In addition, treatment with antioxidant vitamins ablated the sepsis-related increase in protein levels of p50 and p65 (group 4).

Because it has been demonstrated that NF-κB activity can be correlated with cell damage and apoptosis, we chose to examine another variable of cellular integrity and a marker of the endogenous pathway of apoptosis—translocation of cytochrome c from the mitochondrial intermembrane space to the cytosol. When compared with sham-operated animals, animals with untreated sepsis had a significant increase in cytosolic cytochrome c. Antioxidant vitamin treatment before sepsis ablated the increase in cytosolic cytochrome c levels, suggesting that treatment with antioxidant vitamins contributed to the maintenance of cellular membrane integrity (Fig. 3).

To determine whether this cell membrane insult in the septic group correlated with programmed cellular death (apoptosis),
Cardiomyocyte Secretion of Inflammatory Cytokines

As shown in Fig. 5, cardiomyocytes prepared from rats 24 h after untreated S. pneumoniae challenge (group 3, sepsis alone) secreted significantly more pro- and anti-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-10 compared with cytokine levels secreted by myocytes prepared from respective sham-operated groups (P < 0.05). Antioxidant vitamin therapy significantly attenuated the sepsis-related cardiomyocyte inflammatory cytokine responses, producing cardiomyocyte cytokine levels that were comparable with those measured in sham-operated rats and significantly less than cytokine levels secreted by myocytes prepared from rats given S. pneumoniae challenge in the absence of antioxidant vitamins (P < 0.05).

Cardiomyocyte Calcium and Sodium Levels

Several studies have suggested that cardiomyocyte accumulation of calcium and sodium promotes NF-κB nuclear translocation and programmed cell death. In addition, others have suggested that myocyte calcium/sodium status provides an additional measure of sepsis-related myocyte dysfunction and injury. Thus additional aliquots of cardiomyocytes from each of the four experimental groups were prepared 24 h after S.

Left Ventricular Performance

Myocardial contraction and relaxation defects were evident 24 h after untreated S. pneumoniae challenge; LVP and ±dP/dtmax values measured during stabilization of the hearts (perfused at a constant left ventricular end-diastolic volume, constant coronary flow rate, and constant heart rate) were significantly lower in the sepsis group (group 3) compared with values measured in sham-operated animals (groups 1 and 2, Table 2). Contractile abnormalities in the sepsis group were further indicated by the downward shift of left ventricular function curves as preload was incrementally increased. In vehicle-treated sepsis, LVP and ±dP/dt responses to either incremental increases in LV volume (Fig. 6A) or to increases in...
perfusate calcium (Fig. 6B) were significantly lower compared with values measured in sham-operated groups. Antioxidant vitamin therapy attenuated the sepsis-related myocardial contraction and relaxation defects but failed to return LVP and \( \frac{dP}{dt_{\text{max}}} \) responses to values measured in sham-operated rats. LVP and \( \frac{dP}{dt_{\text{max}}} \) responses to increases in either left ventricular volume (Fig. 6A) or incremental increases in perfusate calcium levels (Fig. 6B) were nearly identical in sham-operated animals given antioxidant vitamins compared with values measured in sham-operated animals given vehicle alone.

**DISCUSSION**

Our rodent model of *S. pneumoniae* challenge to produce sepsis confirmed that myocardial oxidative stress occurs during sepsis and that it plays a role in the development of cardiac dysfunction. Our model produced sepsis as confirmed by the presence of bacteria in the systemic circulation 24 h after intratracheal administration of *S. pneumoniae*. A systemic inflammatory response was evident from both the rise in circulating cytokines, as well as increased cardiomyocyte se-
creatinulation of pro- and anti-inflammatory cytokines. We further showed that treatment with antioxidant vitamins alleviated both the systemic and myocardial inflammatory cytokine response and that it inhibited myocardial NF-κB nuclear translocation, prevented cardiomyocyte calcium and sodium accumulation, and decreased both caspase-3 and -8 myocardial activity. All of these changes after antioxidant treatment before the onset of sepsis led to a significant improvement in cardiac function compared with vehicle-treated septic animals. Although the clinical symptoms of sepsis are well understood, the specific delineation of pathways, such as the oxidative stress mechanisms, is essential to the development of therapeutic interventions that can reduce risk of adverse outcome and improve survival.

Numerous studies have confirmed the release of inflammatory cytokines during sepsis and their correlation with negative outcome. The complex regulation of proinflammatory cytokine synthesis and the synthesis of counterregulatory mediators including anti-inflammatory cytokines as well as receptor antagonists have prevented a clear definition of the contributing role of inflammatory cytokine responses in sepsis-related morbidity and mortality. Recent studies have suggested that programmed cell death (apoptosis) also contributes to a negative outcome in sepsis. Cell death has been shown to play a role in impaired cardiac function in models of heart failure, including myocarditis (21), ischemia/reperfusion injury (6), and congestive heart failure (31). Two main apoptotic pathways have been described: the first integrates different proapoptotic signals at a mitochondrial level, triggering the release of cytochrome c; and the second is independent of the mitochondria and involves the recruitment and activation of caspases. Mitochondria contribute directly to apoptotic signaling via generation of reactive oxygen species. In our study, administration of antioxidant vitamins before septic challenge ablated release of cytochrome c into the cytosol as measured by immunoblots, suggesting preservation of mitochondrial function and resolution of proapoptotic signaling at the mitochondrial level. Sepsis resulted in activation of both caspase-3 and -8 by 2-h postinfection, well before the onset of cardiac dysfunction, which we have previously demonstrated occurs by 16- to 24-h postinsult. We show that antioxidant vitamins significantly reduced sepsis-related rise in caspase-3 and -8 in the myocardium at all time points examined, confirming the resolution of sepsis-related caspase-initiated myocardial apoptosis.

In this present study, translocation of NF-κB was similarly ablated in the myocardium of septic animals given antioxidant vitamin therapy. A number of studies have examined regulation/activation of NF-κB as a pivotal modulator of immune-regulatory genes as well as organ dysfunction in sepsis. The role of NF-κB in cytokine transcription has been well defined; in unstimulated cells, NF-κB is retained in the cell cytoplasm due to NF-κB binding of inhibitory protein termed IκB. Many proinflammatory cytokines and stress-related mediators have NF-κB binding sites in their promoter region, and the pivotal role of NF-κB in inflammatory responses has been confirmed in a variety of animal models and in numerous injury states including endotoxemia, trauma, hemorrhage, and burn injury (2, 33, 38). Parsey and colleagues (29) described a significant correlation between increased NF-κB activation in peripheral blood mononuclear cells and poor outcome in sepsis. Similarly, Bohrer and colleagues (4) described that an increase in NF-κB binding activity in peripheral blood mononuclear cells predicted fatal outcome in patients with sepsis. We have described previously that NF-κB nuclear translocation plays a regulatory role in the myocardial synthesis of inflammatory cytokines (7, 15, 24, 39) and have shown further that limiting NF-κB activation prevented the inflammatory cytokine secretion associated with injury (7, 15). In this present study, antioxidant vitamin therapy abrogated sepsis-related systemic as well as myocardial inflammatory cytokine responses. Sun and Andersson (38) reviewed the role of NF-κB in both animal and clinical sepsis, suggesting that therapeutic strategies that modulate NF-κB rather than inhibit the function of this transcriptional regulatory protein may be of great clinical consequence. One concern in this regard is that NF-κB inhibition may accelerate apoptosis of several cell types, further disrupting recovery responses to injury. Although antioxidant vitamin therapy inhibited myocardial NF-κB translocation in our study, there was no NF-κB inhibition-related acceleration of apoptosis as indicated by both cytochrome c release and caspase-3 and -8 data.

In our study, antioxidant vitamin therapy prevented the sepsis-related cardiomyocyte accumulation of calcium and sodium. Our interest in the effects of antioxidant therapy on cardiomyocyte calcium handling was related to a recent report (34) that an increase in intracellular calcium promotes phosphorylation and degradation of IκBα and -β, resulting in NF-κB activation. Because we have shown previously that a rise in intracellular calcium/sodium is an early event in sepsis.

### Table 2. Anti-oxidant vitamin treatment in sepsis challenged rats

<table>
<thead>
<tr>
<th></th>
<th>Group 1, Sham</th>
<th>Group 2, Sham + Antioxidants</th>
<th>Group 3, Sepsis</th>
<th>Group 4, Sepsis + Antioxidants</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVP, mmHg</td>
<td>88.6±2.9</td>
<td>87.9±2.1</td>
<td>65.8±2.6*</td>
<td>74.3±2.2†</td>
</tr>
<tr>
<td>+dP/dt(max), mmHg/s</td>
<td>2.190±30</td>
<td>2.050±47</td>
<td>1.356±53*</td>
<td>1.772±26†</td>
</tr>
<tr>
<td>-dP/dt(max), mmHg/s</td>
<td>1.775±71</td>
<td>1.716±51</td>
<td>1.166±59*</td>
<td>1.511±36†</td>
</tr>
<tr>
<td>dP40, mmHg/s</td>
<td>1.980±25</td>
<td>1.855±42</td>
<td>1.184±53*</td>
<td>1.394±54†</td>
</tr>
<tr>
<td>TPP, ms</td>
<td>91.4±4.6</td>
<td>99.4±2.7</td>
<td>90.5±1.7</td>
<td>97.1±3.6</td>
</tr>
<tr>
<td>Time to 90% relax, ms</td>
<td>85.0±6.3</td>
<td>97.9±3.9</td>
<td>84.7±1.6</td>
<td>93.1±4.7</td>
</tr>
<tr>
<td>Time to +dP/dt(max), ms</td>
<td>57.0±4.4</td>
<td>62.3±2.3</td>
<td>53.5±1.0</td>
<td>61.4±2.9</td>
</tr>
<tr>
<td>Time to -dP/dt(max), ms</td>
<td>51.6±2.1</td>
<td>54.3±1.3</td>
<td>51.1±0.8</td>
<td>56.4±1.1</td>
</tr>
<tr>
<td>CPP, mmHg</td>
<td>49.3±3.6</td>
<td>51.0±4.3</td>
<td>47.4±2.6</td>
<td>59.9±8.2</td>
</tr>
<tr>
<td>CVR, mmHg/ml</td>
<td>9.87±0.7</td>
<td>9.5±1.0</td>
<td>9.6±0.5</td>
<td>12.0±1.6</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>256±19</td>
<td>294±6</td>
<td>261±7</td>
<td>256±8</td>
</tr>
</tbody>
</table>

Values are means ± SE. LVP, left ventricular pressure; ±dP/dt(max), maximum rate of LVP rise and fall, respectively; dP40, developed pressure at 40 mmHg; TPP, time to peak pressure; CPP, coronary perfusion pressure; CVR, coronary vascular resistance. *P < 0.05, difference from respective sham-operated groups. †P < 0.05, antioxidant vitamin-related difference (group 4 vs. group 3).
or injury, preceding left ventricular contractile dysfunction, it is possible that cardiomyocyte accumulation of calcium contributes to NF-κB activation, thus triggering downstream events such as cardiomyocyte cytokine secretion, cytokine-mediated injury to cardiomyocytes, cellular apoptosis, and progressive myocardial contractile abnormalities. Antioxidant therapy attenuated myocardial contractile dysfunction that occurred after sepsis. Despite resolution of inflammatory cytokine responses suggested that other inflammatory mediators, including prostaglandins and perhaps late mediators of organ dysfunction such as migration inhibitory factor (MIF) (10, 12) and high-mobility group box-1 (HGMB1) (27), contribute to the myocardial performance defects that are seen in severe sepsis.

In summary, recent attention has focused on numerous therapeutic strategies that target inflammatory signaling in a variety of injury and sepsis models; however, our choice of
antioxidant vitamins was based on the fact that this therapeutic approach is readily available for clinical use, and antioxidant vitamins have been shown to provide significant organ protection in a variety of injury and disease states. This present study confirms that antioxidant vitamin therapy, given before a major insult, decreases the proinflammatory cytokine cascade and attenuates the myocyte apoptosis that are evoked by pneumonia-related sepsis. This therapeutic approach attenuated myocardial contraction and relaxation defects; the persistence of sepsis-related myocardial depression after antioxidant vitamin therapy in group 4 may be related to release of late mediators of septic shock, including MIF and HGMB1. These data also suggest that further studies are warranted.

GRANTS

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