IκB overexpression in cardiomyocytes prevents NF-κB translocation and provides cardioprotection in trauma

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ROBIN C. NGUYEN,2 BRETT GIROIR,2 AND JURETA W. HORTON. IκB overexpression in cardiomyocytes prevents NF-κB translocation and provides cardioprotection in trauma. Am J Physiol Heart Circ Physiol 284: H804–H814, 2003; 10.1152/ajpheart.00394.2001.—This study examined the effects of either IκBα overexpression (transgenic mice) or N-acetyl-leucinyl-leucinyl-norleucinal (ALLN) administration (proteosome inhibitor in wild-type mice) on cardiomyocyte secretion of tumor necrosis factor-α (TNF-α) and on cardiac performance after burn trauma. Transgenic mice were divided into four experimental groups. IκBα overexpressing mice were given a third-degree scald burn over 40% of the total body surface area or wild-type littermates were given either a scald or sham burn to provide appropriate controls. Pharmacological studies included ALLN (20 mg/kg) administration in either burned wild-type mice or wild-type shams. Burn trauma in wild-type mice promoted nuclear factor-κB (NF-κB) nuclear translocation, cardiomyocyte secretion of TNF-α, and impaired cardiac performance. IκBα overexpression or ALLN treatment of burn trauma prevented NF-κB activation in cardiac tissue, prevented cardiomyocyte secretion of TNF-α, and ablated burn-mediated cardiac contractile dysfunction. These data suggest that NF-κB activation and inflammatory cytokine secretion play a significant role in postburn myocardial abnormalities.

N-acetyl-leucinyl-leucinyl-norleucinal; collagenase digestion; cardiac contractile function; fura 2-AM

MAJOR BURN TRAUMA is characterized by a rapid and continual loss of isotonic fluid into the burn wound, producing a significant decrease in circulating volume. The systemic derangements that occur secondary to fluid shifts into the burn wound include low cardiac output, decreased peripheral perfusion, impaired renal and pulmonary function, and hematological abnormalities (7, 8). Initially, the cardiac dysfunction that was shown to occur after burn trauma was attributed to this decrease in plasma volume and a Frank-Starling mechanism. However, in 1957, Dobson and Warner (14) discovered that after burn trauma, cardiac output fell significantly before changes in plasma volume. Cardiac contractile dysfunction after burn trauma was confirmed subsequently using isolated ventricular muscle preparations (17). Later studies by Baxter and colleagues (6) described a myocardial depressant factor in the serum of human burn subjects. More recently, Ferrara and colleagues (16) described the presence of a myocardial depressant factor in the lymph after experimental burn. Both experimental and clinical studies of burn trauma have correlated the extent of cardiac injury (indicated by a rise in cardiac troponin I levels in the serum) with the percentage of the total body surface area (TBSA) burn (1, 34). Cardiac troponin I is a highly specific and sensitive indicator of myocardial ischemia and injury, but is undetectable in the serum of normovolemic, unburned subjects (2).

While there is considerable evidence that thermal injury impairs cardiac systolic and diastolic function, recent attention has focused on the hypothesis that burn trauma initiates a systemic inflammatory response that contributes to myocardial injury and dysfunction. We and others (27, 30, 41) have documented that cardiac myocytes themselves secrete inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 in response to a variety of insults, including burn trauma, ischemia-reperfusion, and sepsis. Local myocardial inflammatory cytokine levels may be an important component of postburn cardiac contractile dysfunction because TNF-α directly depresses cardiac contraction and relaxation and induces cardiac myocyte apoptosis (26, 30, 32). Transgenic mice overexpressing TNF-α exclusively in the myocardium have myocardial TNF-α levels sufficient to cause dilated cardiomyopathy and severe congestive heart failure (13). Anti-TNF-α strategies that specifically interrupt or inhibit TNF-α synthesis have(600,746),(994,842)

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(25, 29) preliminary studies suggest that burn trauma promotes nuclear translocation of the transcription factor nuclear factor (NF)-κB in the myocardium. In unstimulated cells, NF-κB exists as a latent cytoplasmic complex bound to an inhibitory protein, IκBα (5). In models of sepsis, studies have shown that lipopolysaccharide (LPS) binding to LPS-binding protein and subsequently to cell surface CD14/TLR receptors initiates a signaling pattern mediated through the Ras/Raf-1 MAPK pathway, leading to phosphorylation and degradation of IκBα and subsequent release of NF-κB (18). NF-κB then translocates to the nucleus, where it binds to specific NF-κB recognition sequences found in the 5′ flanking regions of diverse genes involved in inflammatory responses such as TNF-α, inducible nitric oxide synthase and adhesion molecules, thus regulating their synthesis (4, 5). Recent studies have shown that measures of increased NF-κB binding activity in peripheral blood monocytes predicted fatal outcome in septic patients. These investigators further showed that inhibition of NF-κB, by overexpression of its inhibitor IκBα in an endotoxemic mouse model, increased survival (10). Similarly, Shames and colleagues (38) showed that high levels of IκBα diminished cardiac NF-κB activation and attenuated myocardial TNF-α production after LPS challenge. Therefore, NF-κB may play a pivotal role in regulating myocardial synthesis and secretion of inflammatory proteins and may provide a new target for therapeutic intervention.

The purpose of this present study was to examine the cardiac effects of two strategies which have been shown to inhibit NF-κB. If this transcription factor plays a pivotal role in cardiac myocyte secretion of inflammatory cytokines and burn-related cardiac dysfunction, then strategies that inhibit this transcription factor would be expected to improve postburn cardiac performance. We used both a pharmacological and a transgenic approach to address this issue. Specifically, the ability of the proteosome inhibitor N-acetyl-leucinyl-leucinyl-norleucinal (ALLN) to inhibit cardiomyocyte secretion of TNF-α and to ablate postburn cardiac dysfunction was examined in C57BL/6J mice. In addition, transgenic mice that overexpress a nondegradable (nonphosphorylatable) form of IκBα (IκBαΔN) specifically in the heart were given burn injury, and the effects of IκBα overexpression on myocyte TNF-α secretion and ventricular performance were examined. The data suggest that therapeutic strategies that specifically target activation of the nuclear transcription factor NF-κB limit burn-mediated cardiomyocyte secretion of TNF-α and improve cardiac contractile performance.

**MATERIALS AND METHODS**

**Experimental Animals**

All animals were used in accordance with the guidelines established by the University of Texas Southwestern Medical Center Institutional Animal Care and Research Advisory committee and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animals were housed in a temperature-controlled environment with 12:12-h light-dark cycles, and food and water were available at will. Mice were acclimated for 7–10 days before inclusion in the studies described herein.

To determine the cardiac effects of pharmacological inhibition of NF-κB, C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Adult male and female mice weighing 25–30 g were used for the studies described. To pursue a molecular approach for inhibiting cardiac NF-κB, IκBαDN mice were developed at University of Texas Southwestern Medical Center at Dallas. Transgenic mice overexpressed IκBαDN under the control of the cardiomyocyte-specific α-myosin heavy chain promoter exclusively in the heart but not in other tissues. Overexpression of IκBαDN has been shown previously to inhibit cardiac NF-κB activation after LPS exposure (23).

**Burn Trauma Protocol**

All mice were weighed immediately before anesthesia. After adequate anesthesia (methoxyflurane) was achieved, the side and back of each mouse were closely clipped and carefully shaved from the base of the tail to the base of the neck. The animals were then randomly assigned to sham burn or burn groups. In those animals designated for burn trauma, a cutaneous burn injury was produced >40% of the TBSA by applying brass probes (1 × 2 cm with a 3 mm thickness) heated to 100°C in boiling water to the side and back of the animals for 5 s. The TBSA was calculated using murine-specific data (22), and this calculation was verified by removing the animal pelt and measuring the actual burned area. The percentage of burned area was then calculated based on the animal’s measured TBSA. The animals designated for sham burn group received identical regimens of anesthesia and handling, but no burn injury was given. After the burn trauma was completed, the mice were given lactated Ringer solution (4 ml·kg⁻¹·percent burn⁻¹ ip). One-half of the calculated volume of lactated Ringer was given immediately postburn, and the remaining fluid was given intraperitoneally 8 h postburn. All animals received analgesic (0.05 mg/kg im buprenorphine) every 8 h after burn trauma. The animals were monitored closely for the first 8 h after burn trauma to determine adequate recovery from the anesthesia, animal responsiveness to external stimuli, the absence of pain, and the ability to consume food and water.

**Experimental Groups**

Molecular approach to inhibiting NF-κB. Genetically engineered mice overexpressing IκBα in the heart were randomly divided into sham and burn groups. Appropriate wild-type groups were included, resulting in the following four experimental groups: 1) wild-type shams, 2) wild-type mice given burn injury over 40% TBSA and fluid resuscitation, 3) IκBα overexpressing sham-burned mice, and 4) IκBα overexpressing mice given burn and fluid resuscitation, as described for group 2. All mice were euthanized 24 h postburn, and the hearts were harvested to examine cardiac mechanical function (Langendorff perfusion, N = 7–8 mice/group), to examine cardiac myocyte secretion of TNF-α (cardiomyocytes isolated from 4–5 mice per group), or to examine NF-κB activation (4 mice per group per time period).

Pharmacological inhibition of NF-κB. C57BL/6J mice were divided into four experimental groups. Group 1 included sham-burned mice receiving the proteosome inhibitor ALLN (20 mg/kg, Sigma; St. Louis, MO) suspended in 0.3 ml of saline and given intraperitoneally. Group 2 included sham-
burned mice receiving vehicle only (0.3 ml saline ip). Group 3 included mice given burn injury over 40% TBSA, fluid resuscitation (lactated Ringer, 4 ml · kg⁻¹ · percent burn⁻¹ ip), and ALLN (20 mg/kg ip as described for group 1). Group 4 included mice receiving burn injury and fluid resuscitation as described for group 3, but treated with vehicle only (0.3 ml ip saline).

**Measures of Cardiac Contraction and Relaxation (Langendorff Model)**

To examine cardiac contraction and relaxation, mice from each experimental group were anticoagulated with 200 units of heparin sodium (Elkins-Sinn; Cherry Hill, NJ) and cervicalized. The heart was rapidly removed and placed in a petri dish containing ice-cold (4°C) Krebs-Henseleit bicarbonate buffered solution composed of (in mM) 118 NaCl, 4.7 KCl, 21 NaHCO₃, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 11 glucose. All solutions were prepared before each day with demineralized deionized water and bubbled with 95% O₂-5% CO₂ (pH 7.4; P O₂, 500 mmHg). A cannula placed in the ascending aorta was connected via glass tubing to a buffer-filled reservoir for perfusion of the coronary circulation at a constant flow rate. Hearts were suspended in a temperature-controlled chamber (38°C), and a constant flow pump (Holter model 911, Critikon; Tampa, FL) was used to maintain perfusion of the coronary arteries by retrograde perfusion of the aortic stump cannula as previously described (29). Krebs buffer was passed through a bubble trap and a heating coil before delivery to the aorta. A pressure transducer connected to the pressure tubing between the heart and the heating coil was used to measure coronary perfusion pressure. Coronary effluent was collected in a graduated cylinder and measured to confirm coronary flow rate. Contractile function was assessed by measuring left ventricular (LV) pressure (LVP) and the positive and negative first derivative of pressure over time (±dp/dt) responses to increases in either perfusate calcium or coronary flow. LVP was measured with a Statham pressure transducer (model P23 ID, Gould Instruments; Oxnard, CA) attached to an intraventricular cannula, and ±dp/dt was obtained using an electronic differentiator (model 7P20C; Grass Instruments; Quincy, MA) and recorded (model 7DWL8P; Grass Recording Instruments). Data from the Grass recorder was input to a Dell Pentium computer, and a Grass Poly VIEW Data Acquisition System was used to convert acquired data into digital form.

At the time of death, blood was collected from each mouse (0.5–0.6 ml) and placed on ice until centrifugation; plasma samples were then snap-frozen and stored at −80°C for measurement of circulating TNF-α levels with the use of ELISA kits (Endogen; Woburn, MA) and using the protocol provided by the manufacturer.

**Cardiac NF-κB Activation**

**Nuclear protein extraction.** A modified procedure based on the method of Grabellus et al. (20) was used. All steps were performed on ice. Hearts were thawed in the presence of buffer A (10 mM HEPES, pH 7.9; 10 mM KCl, 1.5 mM MgCl₂, 0.1% Nonidet P-40, and protease inhibitors plus 0.5 mM DTT); the proteinase included a protease inhibitor cocktail (Sigma) containing the following: 20 mM 4-(2-aminophenyl)benzenesulfonylfluoride, 14 μM trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane, 1,300 μM bostatin, 10 μM leupeptin, 3 μM aprotinin, and 10 mM sodium EDTA. The hearts were allowed to thaw on ice for 20 min and were then suspended in 500 μl of buffer A per heart. The tissue was then homogenized with the use of a dounce homogenizer by hand. The cells were then allowed to incubate for 20 min on ice and 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 then resuspended in 200 μl of ice-cold buffer C (20 mM HEPES, pH 7.9; 420 mM NaCl, 1.8 mM MgCl₂, 0.2 mM EDTA, pH 8; and 25% glycerol) and protease inhibitor as described above plus 0.5 mM DTT. The pellets were completely resuspended by being pipetted and were incubated for 20 min on ice with gentle resuspension of the pellets every 2 min. After incubation, the pellets were centrifuged at full speed for 5 min at 4°C. The supernatant was collected (nuclear fraction) and frozen at −80°C. Protein concentrations were determined with the use of a protein assay (Bio-Rad; Hercules, CA). Nuclear and cytosolic extracts were stored at −80°C.

**EMSA.** Double-stranded oligonucleotides corresponding to the consensus NF-κB binding site of the κ-light chain enhancer (5’AGITGAGGGGACTTTCCCAGGC-3’) were purchased from Promega Biotech (Madison, WI). In a total volume of 10 μl, 3 pmol of oligonucleotide, 10 units of T4 polynucleotide kinase in 1× forward buffer (GIBCO-BRL; 100 mM Tris · HCl, pH 8.0; 1 mM MgCl₂; and 1 mM CaCl₂) were treated with 1 Ci of [α-32P]ATP (DuPont/NECN, Boston, MA) were incubated at 37°C for 60 min. The reaction was stopped by the addition of 1 μl of 0.5 mM EDTA. The volume was brought up to 50 μl with the addition of a buffer composed of 10 mM Tris·HCl (pH 7.5) 10 mM NaCl, and 1 mM EDTA. The labeled probe was separated from unbound ATP with 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.

Twofour micrograms of nuclear proteins were incubated in 1× gel shift buffer (100 mM HEPES, pH 7.6; 250 mM KCl, 5 mM DTT, 5 mM EDTA, and 25% glycerol). In addition, these were incubated with polydeoxyinosinic-deoxycytidylic acid (dI-dC) and 2 μl of probe between 400,000 and 500,000 counts/min. The final reaction volume was combined with water to 20 μl. The reactions were incubated at room temperature for 20 min, and then 2 μl of loading buffer added. The 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 (0.5%) composed of (in mM) 25 Tris·HCl, 25 boric acid, and 0.5 EDTA. Finally, the gels were dried and exposed to X-ray film. Competition analyses were performed by the inclusion of a 50 M excess of unlabeled double-stranded DNA oligonucleotide in the binding reaction. Nonspecific competitor DNA contained an AP-1 binding element.

**Western Blot Analysis**

Protein extracts (20 μg), both 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% mercaptoethanol, and 10% bromophenol blue). The samples were boiled for 5 min and resolved on a 12.5% polyacrylamide gel. The gels were then transferred to polyvinylidene difluoride membranes (Perkin-Elmer Life Sciences; Boston, MA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline-Tween 20 (TBS-T; 120 mM Tris, pH 7.6, 0.9% NaCl, and 0.05% Tween 20) and 5% dried milk. Blocked membranes were then incubated overnight at 4°C with monoclonal NF-κB p65 antibody or p50 antibody (BD Transduction Laboratories; San Diego, CA) (1:100 dilution) in TBS-T and 5% milk. After being incubated, membranes were washed for 15 min at room temperature in TBS-T, followed by five 5-min washes in TBS-T. After the washes, the membranes were incubated for 1 h in secondary...
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 X-ray film. Experiments were repeated independently three times with groups as indicated in the results.

Primary Cardiomyocyte Preparation

All pipettes, plates, test tubes, and other equipment used for preparation and culture of cardiomyocytes were sterile. The mice were heparinized and decerebrated, and the heart was removed through a medial sternotomy with the use of sterile techniques. The isolated heart was immediately placed in ice-cold calcium-free Tyrode solution containing (in mM) 136 NaCl, 5.36 KCl, 0.56 MgCl₂, 0.33 NaH₂PO₄, 10 HEPES, and 10 glucose. The aorta was cannulated within 60–80 s, and the excised heart was perfused with calcium-free Tyrode solution using a Langendorff perfusion apparatus. The Tyrode solution was equilibrated with 95% O₂-5% CO₂ during perfusion of the heart. Perfusion was maintained for 5 min and then continued for an additional 8 min with the use of a collagenase solution that contained 80 ml of calcium-free Tyrode, 20 mg collagenase A (0.05%; Boehringer-Mannheim; Indianapolis, IN), and 2 mg of protease (Polysaccharide XIV, Sigma) with continuous oxygenation. After this enzymatic digestion, the heart was removed from the cannula, and the ventricular tissue was separated from the base of the heart in a petri dish containing Tyrode solution with 100 mM calcium where gentle mincing increased cell dispersion over 5 min. The myocyte suspension was then filtered, and the cells were allowed to settle. The supernatant was removed, and the cells were resuspended in 50 ml Tyrode solution; the cells were allowed to settle. The supernatant was removed, 10-fold excess AP-1 oligonucleotide had no effect (data not shown). As shown in Fig. 1B, ALLN administration in burned animals inhibited burn-mediated NF-κB activation at all time periods studied. Similarly, IκB overexpression specifically in cardiomyocytes ablated burn-related NF-κB activation (Fig. 1C); there was no evidence of NF-κB nuclear translocation at any time postburn in the IκBα overexpressing mice. Because multiple independent gel shifts were performed on several animals (N = 4–5) from each experimental group at each time postburn, the gel shifts were examined by densitometric analysis and the data are summarized as means ± SE (shown below each group). Western blots were also performed to confirm NF-κB activation by translocation of p50 and p65 to the nucleus. As shown in Fig. 2, A and B, top, representative Western blots are shown, whereas Fig. 2, A and B, bottom, summarize the data from multiple Western blot experiments. Translocation of p50 and p65 from the cytosol to the nucleus was confirmed 2, 4, and 24 h after burn injury in wild-type mice; this translocation of NF-κB dimer components did not occur after burn trauma in ALLN-treated or IκB-overexpressor mice (Fig. 2).

Cardiomyocyte Secretion of TNF-α in IκBα Transgenic Mice

To determine whether IκBα overexpression in cardiomyocytes altered myocyte secretion of TNF-α, hearts harvested from each of the four experimental groups were perfused with collagenase containing buffer to prepare cardiomyocytes. As shown in Fig. 3, myocytes prepared from wild-type burns secreted significantly more TNF-α (177 ± 16 pg per 5 × 10⁴ myocytes) compared with cytokines secreted by cardiomyocytes prepared from wild-type sham (70 ± 4 pg per 5 × 10⁴ myocytes, P < 0.05). These data confirm previous reports (19, 27, 29, 41) showing, in several species, that burn trauma promotes TNF-α secretion by cardiomyocytes per se. In contrast, cardiomyocytes prepared from IκBα overexpressing mice given burn injury secreted significantly less TNF-α (15 ± 1 pg per 5 × 10⁴ myocytes) compared with that secreted by wild-type burns (177 ± 16 pg per 5 × 10⁴ myocytes) (Fig. 3A).

In vitro studies examining cardiomyocyte responsiveness to LPS challenge (either 0, 10, 25, or 50 μg/ml) are shown in Fig. 3B. Myocytes from wild-type shams and burns responded to LPS challenge with a dose-related increase in TNF-α secretion (P < 0.05). How-
ever, myocytes prepared from wild-type burned mice secreted significantly more TNF-α at each LPS dose compared with responses measured in myocytes from wild-type sham-burned mice \((P < 0.05)\). In contrast, cardiomyocytes prepared from IkBα overexpressing mice given either sham burn or burn injury secreted minimal TNF-α at all LPS doses.

Cardiomyocyte Secretion of Inflammatory Cytokines in ALLN-Treated Animals

Figure 4 summarizes TNF-α levels measured in the supernatants of myocytes prepared from 1) vehicle-treated shams, 2) vehicle-treated burns, 3) ALLN-treated shams, and 4) ALLN-treated burns. Cardiomyocyte secretion of TNF-α was significantly higher in vehicle-treated burns \((P < 0.05)\) compared with that measured in vehicle-treated shams. ALLN treatment in burn trauma significantly reduced burn-mediated cardiomyocyte secretion of the inflammatory cytokine (Fig. 4A). In addition, cardiomyocytes from all four experimental groups were challenged in vitro with 0, 10, or 25 μg of LPS per \(5 \times 10^4\) myocytes for 18 h. As shown in Fig. 4B, myocytes from both vehicle-treated shams and vehicle-treated burns responded to LPS with a dose-related increase in TNF-α. However, myocytes prepared from vehicle-treated burns secreted significantly more TNF-α at all LPS doses compared with the responses measured in myocytes prepared from vehicle-treated shams \((P < 0.05)\). ALLN treatment of burn trauma significantly attenuated LPS-induced secretion of TNF-α by cardiomyocytes prepared from both burn and sham animals; myocytes from ALLN-treated animals (both sham and burn) secreted minimal TNF-α at all LPS doses.

Cardiac Contractile Performance in IkBα Transgenics and Wild-Type Littermates

Our first concern was that IkBα overexpression in the heart could alter some aspect of ventricular performance per se. To address this issue, hearts were isolated from IkBα transgenic mice and wild-type littermates in the absence of burn trauma; hearts were
perfused in vitro via Langendorff apparatus. Ventricular performances in these two groups were compared as either the coronary flow rate or the perfusate calcium was incrementally increased. LV function measured in IκBα transgenic mice and wild-type mice was nearly identical as coronary flow rate was incrementally increased from 1 to 4 ml/min (Fig. 5). Similarly, there were no significant differences in ventricular responsiveness to increases in perfusate calcium (from 1 to 8 mM) in IκBα overexpressing mice compared with wild-type littermates (Fig. 6).

As shown in Fig. 5, burn trauma in wild-type mice produced significant cardiac contractile dysfunction as indicated by the reduced LVP and ±dP/dt max responses to incremental increases in coronary flow rate and compared with responses measured in wild-type shams at each level of coronary flow. In contrast, burn trauma in the IκBα overexpressing mice produced significantly less cardiac contractile dysfunction at all levels of coronary flow rate compared with that shown in wild-type burned littermates (Fig. 5). Whereas burn trauma in wild-type littermates significantly impaired LVP and ±dP/dt responses to increases in perfusate calcium compared with that measured in wild-type shams (Fig. 6), cardiac IκBα overexpression provided significant cardioprotection as indicated by the significantly higher LVP and ±dP/dt at each concentration of perfusate calcium compared with responses measured in wild-type burns.

Cardiac Contractile Performance in ALLN-Treated Burns

To further explore the role of NF-κB activation in postburn cardiac contractile dysfunction and myocyte cytokine secretion, the proteosome inhibitor ALLN was administered immediately after burn trauma in wild-type mice (both sham and burn). Wild-type shams as well as wild-type burns given fluid resuscitation plus vehicle (no ALLN therapy) were included to provide appropriate time-matched controls. As shown in Fig. 7, cardiac contraction and relaxation deficits were evident in hearts harvested from mice given burn injury plus vehicle and compared with function calculated for sham-burned mice. LVP and ±dP/dt max increased significantly in all mice as either coronary flow rate (Fig. 7) or perfusate calcium (Fig. 8) was increased. However, all measures of cardiac performance were significantly lower in vehicle-treated burns at each level of coronary flow rate or at each level of perfusate calcium compared with values measured in the time matched shams. In contrast, ALLN given immediately after
burn trauma significantly improved cardiac contractile responsiveness to increases in either coronary flow rate (Fig. 7) or perfusate calcium (Fig. 8) compared with those values measured in vehicle-treated burns. Measures of contractile performance (LVP and ±dP/dt max) were always higher in ALLN-treated burned mice compared with values measured in burns given standard fluid resuscitation and vehicle.

Fig. 3. A: tumor necrosis factor-α (TNF-α) was measured in cardiomyocyte supernatants prepared from 1) sham-burned WT, 2) sham-burned IκBα overexpressing mice, 3) burned WT mice, and 4) burned IκB overexpressing mice. TNF-α secretion by cardiomyocytes was measured after incubation of myocytes (CO2 incubator for 18 h) in the absence of any in vitro challenge. *P < 0.05, significant difference among groups (ANOVA, Newman Keuls); †P < 0.05, significant difference in sham WT versus IκB sham or burn WT vs. IκB burn. B: cardiomyocytes (5 × 10⁴ per microliter well) from the four experimental groups were challenged in vitro with either 0, 10, 25, or 50 μg of lipopolysaccharide (LPS) for 18 h. Secreted TNF-α protein was measured by ELISA. *P < 0.05, significant difference at each LPS condition compared with baseline (zero LPS) within an experimental group; †P < 0.05, significant effect of IκB overexpression in either sham or burns compared with the respective WT. All values are means ± SE.

Fig. 4. TNF-α was measured in cardiomyocytes prepared from all four experimental groups: 1) sham burns, 2) burns given standard fluid resuscitation, 3) ALLN-treated shams, and 4) ALLN-treated burns. A: cardiomyocyte secretion of TNF-α in the absence of in vitro LPS challenge. *P < 0.05, significant difference among groups at (ANOVA, Newman Keuls). B: cardiomyocytes from all four experimental groups were challenged with 0, 10, or 25 μg of LPS for 18 h. Secreted TNF-α protein was measured by ELISA. *P < 0.05, significant difference at each LPS condition compared with baseline (zero LPS) within an experimental group; †P < 0.05, significant difference in burned WT vs. IκB overexpressing burns. All values are means ± SE.
Circulating TNF-α Levels

TNF-α levels, measured 24 h postburn in wild-type mice, were elevated (32 ± 1 pg/ml) compared with values measured in wild-type control mice (15.9 ± 0.8 pg/ml, \( P < 0.05 \)). ALLN administration (20 ± 2 pg/ml) and IkB overexpression (15 ± 1 pg/ml) attenuated the burn-related rise in circulating TNF-α.

DISCUSSION

Both the pharmacological and the transgenic approach used in this present study inhibited NF-κB activation, confirming previous reports in this regard (23, 37). In addition, both IkB overexpression in cardiac myocytes and ALLN treatment of burned mice decreased TNF-α secretion by cardiac myocytes and
provided cardioprotection after burn trauma. Overexpression of the nondegradable form of IkBα was limited to the cardiac myocytes, and we have shown previously that this molecular approach prevented LPS-mediated activation of NF-κB in a murine model of sepsis (23). These data are consistent with previous reports (3, 11, 12) showing that overexpressing nondegradable IkBα inhibits NF-κB activation. In this regard, Börhrer and colleagues (10) showed that injection of an IkBα expression plasmid in mice reduced LPS-induced NF-κB activation in polymorphonuclear blood cells and improved survival of septic mice.

It was expected that ALLN administration would reduce the burn-related rise in circulating TNF-α levels, confirming that systemic administration of this NF-κB inhibitor interrupted TNF-α synthesis by several cell populations, including Kupffer’s cells, alveolar macrophages, and cardiomyocytes. Overexpression of IkB, specifically in cardiomyocytes, was expected to have little effect on TNF-α secretion by nonmyocyte

![Fig. 7. LV function curves were calculated for groups of sham-burned mice (Sham, group 1) and burned mice given standard fluid resuscitation and no pharmacological intervention (Burn, group 2). In addition, groups of mice given sham burns plus ALLN (group 3) and burns treated with ALLN (group 4) were included. LVP and dP/dtmax were plotted against incremental increases in coronary flow rate. All values are means ± SE. *P < 0.05, significant difference among groups (ANOVA, Newman Keuls).](http://ajpheart.physiology.org/)

![Fig. 8. LV function curves were calculated for groups of sham-burned mice (Sham, group 1) and burned mice given standard fluid resuscitation and no pharmacological intervention (Burn, group 2). In addition, groups of mice given sham burns plus ALLN (group 3) and burns treated with ALLN (group 4) were included. LVP and dP/dtmax were plotted against incremental increases in calcium. All values are means ± SE. *P < 0.05, significant difference among groups (ANOVA, Newman Keuls).](http://ajpheart.physiology.org/)
cell populations, and the mechanism underlying the significant fall in systemic TNF levels seen late in IκB overexpressor burned mice is unclear.

The transgenic mouse line used in our study was characterized by expression of a murine nonphosphorylatable nondegradable IκBα coding sequence (IκBαΔN) driven by the murine α-myosin heavy chain promoter. This promoter is steadily activated only in cardiac myocytes, and thus nondegradable IκBα is constitutively and exclusively expressed in the heart (21). Our finding of significant cardiac contractile dysfunction in hearts prepared 24 h after burn trauma in wild-type littermates is consistent with previous reports (15, 19, 28, 29, 35) of cardiac contraction and relaxation deficits after thermal trauma in rats, rabbits, guinea pigs, sheep, and humans. Overexpression of IκBα in cardiomyocytes significantly attenuated burn-induced cardiac contractile dysfunction despite an identical percent of TBSA burn and identical anesthetic regimens used for all burned animals. Significant differences in cardiac performance in the burned wild-type mice compared with the burned IκBα overexpressing mice could not be attributed to either differences in in vitro handling of the hearts or perfusion stabilization times; in addition, heart rates and coronary flow rates were nearly identical in all burned mice.

Our pharmacological approach to inhibiting burn-mediated cardiac NF-κB activation used a new class of proteasome inhibitor shown previously to inhibit NF-κB activation in several nonmyocyte cell types (33, 37). Our preliminary studies examined the effects of several doses of ALLN on cardiac function and cardiomyocyte viability (unpublished data). The ALLN dose selected by us was consistent with that reported in adult female Swiss-Webster mice and was shown by Schow and Jolly (37) to inhibit NF-κB activation by inhibiting IκBα degradation and inhibiting the proteolytic processing of p105. In our study, ALLN effectively inhibited NF-κB activation in cardiac tissue, prevented burn-related TNF-α secretion by cardiomyocytes, and improved postburn cardiac performance.

TNF-α synthesis and secretion by several cell types such as macrophages is dependent on NF-κB activation. We have shown previously that cardiomyocytes prepared from either adult rats or guinea pigs secrete significant TNF-α after burn injury (19, 29), and these previous studies have shown that in vitro LPS challenge in cardiac myocytes exacerbated burn-related secretion of inflammatory cytokines (29). Data from this present study show that in vivo administration of ALLN immediately after burn injury prevented both burn-mediated and LPS-mediated TNF-α secretion by cardiac myocytes, a result that was identical to the finding that IκBα overexpression inhibited cardiomyocyte secretion of TNF-α. It could be argued that in vivo administration of ALLN in burned mice altered several aspects of the postburn inflammatory cascade, down-regulating the overall inflammatory response and secondarily improving cardiac performance. However, our studies in mice with IκBα overexpression exclusively in the heart suggested that the doses of ALLN used in our study specifically targeted TNF-α secretion within the myocardium.

We attributed the improved cardiac performance after burn trauma in ALLN-treated animals and IκBα overexpressing mice to the decreased TNF-α secretion by cardiomyocytes. The negative inotropic effects of TNF-α are well recognized. Kumar and colleagues (31) and Yokoyama and colleagues (41) showed significant cardiodepression with TNF-α challenge in isolated ventricular muscle preps or Langendorff-perfused hearts. These data are consistent with our previous finding that TNF-α added to the perfusate (Langendorff model) of naive hearts produced significant cardiodepression (26). That TNF-α mediates, in part, the myocardial depression seen in burn trauma, sepsis, and ischemia reperfusion has been supported by the finding that anti-TNF-α strategies, which inhibit or neutralize TNF-α provide significant cardioprotection (9, 19, 24, 36, 39, 40). It is likely that secretion of the inflammatory mediator TNF-α by cardiac myocytes produces local or compartmentalized levels of TNF-α that far exceed those measured in the systemic circulation. In addition, local synthesis and accumulation of TNF-α in the myocardium may not be rapidly and effectively neutralized by soluble receptors or other endogenous scavenging mechanisms.

While we have shown previously that NF-κB activation is one signaling event involved in the inflammatory cascade that culminates in cardiomyocyte secretion of inflammatory cytokines (29), we clearly recognize that other transcription factors (i.e., AP-1) may also play a role. However, the data presented in this present study suggest that therapeutic strategies that specifically target some aspects of the signaling cascade that regulate myocardial secretion of TNF-α may be useful in the treatment of disease and injury that are characterized by profound cardiac contractile depression.

In summary, data from this present study show that IκBα overexpression specifically in cardiomyocytes prevented burn-mediated cardiac NF-κB activation, prevented cardiomyocyte secretion of TNF-α, and improved burn-mediated cardiac contractile performance. Parallel pharmacological studies confirmed that the proteasome inhibitor ALLN inhibited postburn NF-κB nuclear migration in the myocardium, prevented inflammatory cytokine secretion by cardiomyocytes, and significantly improved myocardial performance. Targeting specific aspects of the signal transduction mechanisms that regulate inflammatory cytokine synthesis by specific cell populations may provide novel and useful therapeutic modalities to treat patient populations with significant cardiac contraction and relaxation deficits.

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REFERENCES

INHIBITING NF-κB IN TRAUMA


