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Pentoxifylline Attenuates Lung Injury and Modulates Transcription Factor Activity in Hemorrhagic Shock

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Background. **Evidence exists that resuscitation with Ringer's lactate (RL) contributes to postshock inflammation and lung injury. We hypothesized that the anti-inflammatory agent pentoxifylline (PTX) attenuates postresuscitative lung injury through modulation of transcription factors after hemorrhagic shock.**

Methods. **Male Sprague Dawley rats underwent a 1 h period of hypotension and resuscitation with RL (32 mL/kg) or RL + PTX (25 mg/kg). Lung sections were graded for histological injury and myeloperoxidase content. Cytokine-induced neutrophil chemoattractant concentration was determined by enzyme immunoassay. Matrix metalloproteinase-2 and -9 (MMP) activity was evaluated by zymography. Heme oxygenase-1, nuclear factor kappa B (NF-κB) p65 nuclear translocation, and cytoplasmic I-κB phosphorylation were assessed by Western blot. NF-κB and cAMP response element binding protein (CREB) DNA binding were determined by light shift chemiluminescent electrophoretic mobility shift assay.**

Results. **RL resuscitation led to statistically significant increases in all parameters of lung injury when compared with the negative control. The addition of PTX significantly decreased histology lung injury, myeloperoxidase content, cytokine-induced neutrophil chemoattractant by 48% ($P < 0.05$), heme oxygenase-1 expression by 50% ($P < 0.05$), MMP-2 activity by 70% ($P < 0.05$), MMP-9 activity by 44% ($P < 0.05$), cytoplasmic I-κB phosphorylation by 66% ($P < 0.01$), nuclear NF-κB p65 phosphorylation by 51% ($P < 0.05$), and NF-κB DNA binding by 42% ($P < 0.05$). In contrast, PTX increased CREB DNA binding by 69% when compared with RL alone ($P < 0.04$).**

Conclusions. **The addition of PTX to conventional RL infusion after shock significantly reduced histological lung injury and pulmonary neutrophil activity when compared to treatment with RL alone. The administration of PTX was also associated with diminished NF-κB and enhanced CREB activation. Therefore, the administration of PTX may serve as a novel therapeutic adjunct after hemorrhagic shock.**

Key Words: **pentoxifylline; neutrophil; NF-κB; CREB; hemorrhagic shock; Ringer's lactate; ischemia-reperfusion; acute lung injury.**

INTRODUCTION

Hemorrhagic shock is a major cause of death during the initial phases of trauma. For those who survive, the duration and depth of ischemia and subsequent reperfusion have been linked to the development of acute lung injury (ALI) and multisystem organ failure [1]. ALI and its most severe form, acute respiratory distress syndrome (ARDS), are characterized by disruption of the pulmonary endothelial barrier, which leads to interstitial edema, reduced lung compliance, and persistent hypoxia [2]. There is much evidence to suggest that the host's own neutrophil population plays a central role in the development of ALI and ARDS. More specifically, migration in response to chemokines such as interleukin-8 (IL-8), degranulation of proteolytic enzymes, and generation of the respiratory burst have all been recognized as critical neutrophil functions leading to resuscitation-induced lung injury [3, 4].

The rapid production of local chemokines and cytokines, including IL-8, after ischemia and reperfusion is

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preceded by and dependent on an increase in the corresponding mRNA transcripts and is a direct consequence of the initiation of proinflammatory gene transcription [5, 6]. The 5′ region of these particular genes have been shown to contain promoter regions that can bind transcriptional factors such as nuclear factor-kappa B (NF-κB) and cyclic-3′,5′-adenosine monophosphate (cAMP) response element binding protein (CREB), which are capable of modulating proinflammatory gene transcription in a positive or negative manner [7]. Therefore, strategies aimed at the modulation of neutrophil or transcriptional factor activation may reduce the incidence and severity of lung injury after shock.

The present standard of care for the treatment of hemorrhagic shock involves the intravenous infusion of large amounts crystalloids. However, there is recent evidence suggesting that the choice of postshock resuscitation strategy can also modulate the inflammatory response. Therefore, in terms of inflammation, the type of fluid used to restore perfusion may be just as important as restoring perfusion itself. Racemic Ringer's lactate (RL), the current clinical resuscitative fluid, has been shown to contribute to lung injury by augmenting neutrophil oxidative burst, adhesion molecule expression, endothelial dysfunction, and cellular apoptosis in animal models of ischemia-reperfusion [8–11].

Pentoxifylline (1-[5-oxohexyl]-3,7-dimethylxanthine; PTX), a methylxanthine derivative and nonspecific phosphodiesterase inhibitor, has been clinically used in the treatment of intermittent claudication in patients with peripheral and cerebrovascular atherosclerotic disease [12]. Through its hemorheologic properties, PTX enhances the deformability of red blood cells and as a result improves microvascular blood flow. Recently, studies examining the effects of PTX have been focused on its role as an immune modulator. In prospective, randomized, double-blind, placebo-controlled trials, the administration of PTX reduced mortality in neonatal sepsis and acute alcoholic hepatitis [13, 14]. PTX has been also shown to attenuate a variety proinflammatory neutrophil functions which are important in the development and propagation of host organ injury such as degranulation, adherence, and generation of the respiratory burst in animal models of ischemia-reperfusion and endotoxemia [15–19]. Since PTX alone is not capable of volume expansion, its role as an adjunct to standard RL resuscitation is an attractive resuscitation alternative. In this series, we hypothesized that the combination of RL and PTX attenuates posthemorrhagic shock resuscitation-induced lung injury through the modulation of neutrophil function and pulmonary transcription factor activation when compared to classic RL resuscitation in vivo.

MATERIALS AND METHODS

The experiment was approved by the University of California Animal Subjects Committee and was in accordance with guidelines established by the National Institutes of Health.

Experimental Hemorrhage Model

Male Sprague Dawley rats (300 to 400 g) were purchased from Harlan Sprague Dawley (Livermore, CA). A 12 h light and dark cycle was instituted, and food and water were provided ad libitum. Animals were anesthetized with nembutal (50 mg/kg) by intraperitoneal injection. A right inguinal incision was performed, and the femoral artery and vein were cannulated with polyethylene catheters (PE50). A venous catheter was used for injection of resuscitative fluids, and the arterial catheter was used to withdraw blood and monitor the mean arterial pressure (MAP). Blood was withdrawn over a period of 10 min until a MAP of 35 mmHg was obtained. Controlled hypotension was then maintained at 35 ± 5 mmHg for 1 h by withdrawal or reinfusion of blood as necessary.

The animals were randomly divided into three groups according to the treatment received. Sham animals ($n = 5$) underwent cannulation without shock or resuscitation and served as negative controls. RL resuscitated animals ($n = 7$) received 32 mL/kg of racemic RL. RL + PTX treated animals ($n = 7$) received 32 mL/kg racemic RL + 25 mg/kg of PTX (Sigma, St. Louis, MO). PTX was dissolved in the RL without the infusion of any additional fluid, and its dose was chosen based on studies from our laboratory demonstrating its safety and lack of hypotension at this concentration [20]. There was no significant difference between preshock, shock, and resuscitation MAP or the shed blood volume between the groups (12.1 mL and 11.8 mL for the RL and RL + PTX groups, respectively). After resuscitation with the respective fluid, all shed blood was reinfused. The body temperature of the animals was maintained at 37°C throughout the experiment with a heating blanket.

At the end of volume resuscitation, the catheters were removed, the incision was closed, and 1 mL of subcutaneous 0.125% bupivacaine was administered. Animals were euthanized at 4 or 24 h after the completion of shock and resuscitation via an intraperitoneal injection of nembutal (120 mg/kg). Immediately after sacrifice, animals underwent cardiac puncture for blood collection, and the injection of 10 mL of phosphate buffered saline (PBS; Irvine Scientific, Santa Ana, CA) into the right ventricle to clear the pulmonary circulation of blood cells. The left lung was stored in 10% PBS buffered formalin for histopathological examination, and the right lung was harvested, snap frozen in liquid nitrogen, and stored at −70°C for protein extraction.

Histopathological Examination

To assess the degree of lung damage, lung tissue from animals sacrificed 24 h after resuscitation was embedded in paraffin using an automated processing unit (Autotechnicon, Tarrytown, NY). Sections 5-μm thick were transferred onto glass slides and stained with hematoxylin and eosin ($n \geq 5$ per group; Richard Allen Scientific, Kalamazoo, MI). A pathologist blinded to the treatment groups subjected the lungs to microscopic examination and graded each slide on a scale of 0 to 3 (0 = normal, 3 = most severe). Separate ratings were estimated for pulmonary edema, intra-alveolar hemorrhage, congestion, and neutrophil infiltration.

Lung Myeloperoxidase Immunohistochemistry

Neutrophil accumulation in the lung at 24 h was assessed by staining tissue sections for myeloperoxidase (MPO). After deparaffinization, lung slides were incubated in Target retrieval solution (DAKO, Carpinteria, CA) for 20 min at 95°C and cooled to room

temperature ($n \geq 5$ per group). All subsequent steps were conducted at room temperature in a humid chamber.

Endogenous peroxide activity was quenched with 1.5% H_2O_2 for 5 min. Sections were blocked for 20 min in 1.5% goat serum in PBS and incubated for 2 h with rabbit polyclonal MPO antibody (Lab Vision Corp., Fremont, CA) diluted 1:100 in blocking solution. Sections were washed with PBS and incubated with biotinylated rabbit secondary antibody diluted 1:400 for 30 min. Specific labeling was detected with an Elite ABC peroxidase kit and DAB substrate (Vector Laboratories, Burlingame, CA). To ensure specific staining of MPO, a positive control slide obtained form Lab Vision Corporation and a negative control slide consisting of sections incubated with PBS instead of primary antibody were run in parallel with each set of experiments (data not shown). Twenty random high power fields were examined per animal to determine the number of cells staining positive for MPO.

Protein Extraction

Lung tissue was homogenized in 1 mL of ice-cold T-PER tissue protein extraction reagent containing 1% protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL). Homogenates were centrifuged at 10,000 $\times$ *g* for 5 min, and the supernatant fraction was aliquoted and stored at −70°C for analysis of cytokine-induced neutrophil chemoattractant (CINC), Heme oxygenase-1 (HO-1), and matrix metalloproteinase (MMP)-2 and -9 activity. To determine cytoplasmic I-κBα phosphorylation, NF-κB p65 subunit phosphorylation, nuclear NK-κB DNA binding, and nuclear CREB DNA binding, the extraction of lung cytoplasmic and nuclear proteins was performed using NE-PER nuclear and cytoplasmic extraction reagents containing 1% protease inhibitor cocktail per the manufacture's instructions (Pierce). The protein concentration of all extracts was determined using the bicinchoninic acid protein assay (Pierce). Absorbance was measured on a standard curve for albumin with a microplate reader (Molecular Devices, Sunnyvale, CA).

Determination of Lung CINC Content

CINC is a neutrophil chemoattractant present in rats whose functions are similar to that of human IL-8. Quantification of CINC was conducted through enzyme linked immunosorbent assay (ELISA). The wells of a 96-well immunoplate (NUNC Brand, Rochester, NY) were coated with capture antibody (goat anti-CINC; Peptide Institute, Osaka, Japan) diluted 1:396 in coating buffer, and incubated overnight at 4°C. Nonspecific binding sites were blocked with a buffer comprised of 5% nonfat dry milk (Sigma) in sterile PBS. The wells of the plate were washed with 0.05% Tween 20 (Fischer Scientific, Pittsburgh, PA) in PBS. Lung tissue lysates ($n \geq 5$ per group) were incubated on the plate at room temperature for 2 h. After washing, the secondary antibody (rabbit anti-CINC) was diluted 1:2000 in blocking buffer and dispensed onto the plate for incubation at room temperature. The conjugate, horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling, Beverly, MA) was added and incubated at room temperature for an additional 30 min. Immunopure TMB substrate kit (Pierce) was used for detection, and absorbance was read at 450 nm. Data are presented as ng/mL.

Detection of Lung Matrix Metalloproteinase Activity

MMP-2 and MMP-9 activity were measured in lung lysate samples by zymography under nonreducing conditions. MMP identity was verified by running human standards (Chemicon International, Temecula, CA) diluted 1:1000 in 2X Tris-Glycine Sample Buffer (Invitrogen, Carlsbad, CA). Two μg of each sample were diluted in sample buffer and loaded onto a Novex 10% Zymogram Gel copolymerized with gelatin (Invitrogen). Gels were run in Tris-Glycine SDS Running Buffer at 130 V for 90 min. After electrophoresis, gels were incubated in renaturing buffer (Invitrogen) for 30 min at room temperature. Gels were then incubated with developing buffer (Invitrogen) for 30 min at room temperature, followed by an overnight incubation with fresh developing buffer at 37°C. Gels were stained with Coomassie brilliant blue R-250 and rinsed frequently with destaining solution (50% methanol, 40% water, and 10% acetic acid). Band quantification was performed with UN-SCAN-IT gel digitizing software (Silk Scientific, Orem, UT). Data are expressed at the mean band pixel total ($n \geq 4$ per group).

Western Blot Analysis (HO-1, I-κBα, p65 NF-κB)

In separate experiments, lung tissue lysates (HO-1), cytoplasmic extracts (I-κBα), or nuclear extracts (p65 NF-κB) containing 10 μg of protein per sample were suspended in sodium dodecyl sulfate (SDS) sample buffer (Invitrogen) and collected by boiling the samples at 100°C for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis using 8% to 16% tris-glycine polyacrylamide gradient gels and subsequently transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked with 5% milk in tris-buffered saline/Tween 20 for 1 h. Primary antibodies specific for HO-1 (1:2000 Cell Signaling), phosphorylated I-κBα (1:250 Cell Signaling) or phosphorylated NF-κB p65 (1:500 Cell Signaling) were incubated with the membranes overnight at 4°C in BS/Tween supplemented with 3% bovine serum albumin along with the appropriate loading controls. The membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase-linked anti-rabbit IgG (1:2000) prepared in blocking solution. After thorough washing, the Pierce Supersignal West Pico Chemiluminescent Kit was applied for antibody detection with X-ray film. Data are expressed as the mean band pixel total after adjustment for the density of its respective control band ($n \geq 4$ per group).

NF-κB and CREB Electrophoretic Mobility Shift Assay

The nonradioactive light shift chemiluminescent electrophoretic mobility shift assay (EMSA) Kit was used to detect DNA-transcription factor interactions. The 3′ biotin end-labeled oligonucleotide used as a probe for the NF-κB EMSA was a 42-bp double-stranded construct (5′-TTGTTACAAG*GGGACTTTCC*GCTG*GGGACTTTCC*-AGGGAGGC-3′) containing two randomly repeated NF-κB binding sites (underlined). The 3′ biotin end-labeled oligonucleotide used in the CREB EMSA was a 23-bp double stranded construct (5′-TTTTCG-AGCTC*TGACGTCA*GAGC-5”). Specificity for each experiment was determined by competition assay with the addition of 200 M excess of unlabeled double-stranded NF-κB or CRE oligonucleotide, respectively.

Nuclear extracts (10 μg) were incubated with 5 nM NF-κB or CRE probe (1× binding buffer, 150 mmol/L KCl, 0.1 mmol/L EDTA, 2.5 mmol/L DTT, 0.05% NP40, 10% glycerol, and 50 ng/mL poly[dI-dC]) and subjected to 6% DNA retardation gel at 100 V for 90 min. To determine binding specificity, supershift analysis was performed with the addition of 2 μg of phosphorylated p65 or phosphorylated CREB antibody (Cell Signaling) to each sample. Gels were electrophoretically transferred at 380 mA for 1 h on ice to a positively charged nylon membrane (Roche Applied Science, Indianapolis, IN) and immediately UV cross-linked for 15 min with a UV transilluminator equipped with a 312-nm bulb. Streptavidin-horseradish peroxidase conjugate and the light shift chemiluminescent substrate were used to detect the biotin end-labeled DNA. The nylon membranes were then exposed to X-ray film for 1 to 3 min for detection. Data are expressed at the mean band pixel total ($n \geq 4$ per group).

Statistical Analysis

All values are expressed as the mean + the SEM of *n* observations, where *n* represents the number of animals in each group. Each assay was performed in duplicate or triplicate where appropriate. Statistical significance of differences among groups was determined by analysis of variance with Bonferroni correction. A *P*-value <0.05 was considered statistically significant.

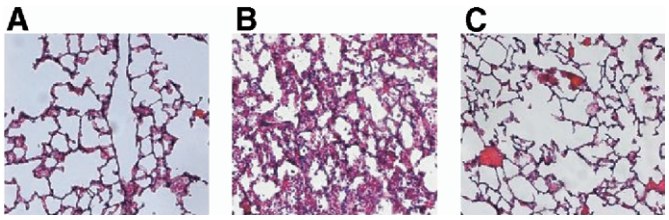

FIG. 1. Resuscitation-induced lung injury. Lungs were harvested 24 h after shock and resuscitation and evaluated by a blinded pathologist who was unaware of the treatment each animal received ($n \geq 5$ per group). (A) Demonstrates the normal appearance of lung specimens from sham animals. (B) Shows the appearance of lungs from RL-resuscitated animals. Note the marked inflammatory infiltrate, alveolar-capillary membrane thickening, hyaline membrane formation, and areas of alveolar edema. (C) Shows the appearance of lungs from the RL + PTX treated animals. Note the similarity in lung morphology to sham animals (100× magnification). (Color version of figure is available online.)

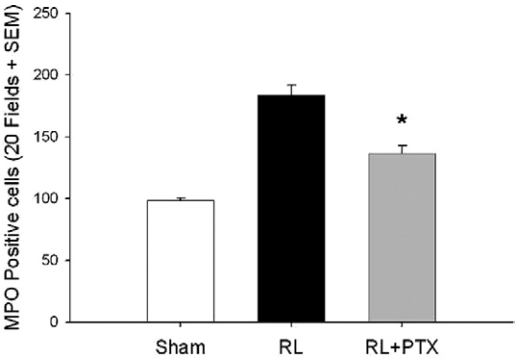

FIG. 2. Lung myeloperoxidase immune staining. MPO immune staining 24 h after shock and resuscitation was used as a marker of pulmonary neutrophil infiltration. Positive cells were counted as described in Methods ($n \geq 5$ per group). RL-treated animals displayed a significantly greater number of MPO cells when compared with sham or RT + PTX animals. *, $P < 0.05$ *versus* RL.

RESULTS

Histological Lung Injury

Lung specimens from those animals treated with standard RL resuscitation developed significant histological changes including cellular infiltration, edema, and alveolar-capillary membrane thickening (Fig. 1). In contrast, the addition of PTX to RL infusion after shock attenuated the severity of this injury to a level observed in the sham animals. The severity scores for interstitial inflammation, neutrophil infiltration, and congestion were all markedly higher in the RL group (11.4 ± 2) than either the sham or RL + PTX groups (6.4 ± 1.2 and 7.7 ± 0.9, respectively; $P < 0.05$).

Myeloperoxidase Content

Neutrophil infiltration into the lung tissue was increased in RL resuscitated animals at 24 h (183 ± 8 positive cells/20 fields) when compared to sham (98 ± 1.8 positive cells/20 fields; $P < 0.05$). The concomitant administration of PTX with RL resulted in a 25% decrease in MPO stained cells (136 ± 6 positive cells/20 fields; $P < 0.05$) when compared with RL infusion alone (Fig. 2).

Cytokine-Induced Neutrophil Chemoattractant Levels

In humans, IL-8 regulates chemotaxis and aids in neutrophil recruitment and activation in the lung during ischemia-reperfusion [21]. Since IL-8 does not exist in rodent species, other chemokines such as CINC, which fulfill the same biological functions, must instead be measured. Administration of RL resulted in a marked increase pulmonary CINC (67.7 ± 7.1 ng/mL; $P < 0.05$) at 4 h and 24 h (31.9 ± 1.6; $P < 0.01$) over both the sham and RL + PTX counterparts (Fig. 3). The concentration of CINC did not differ between the sham group and those treated with RL + PTX at 4 h (26.1 ± 4.1 *versus* 25.5 ± 3.3 ng/mL) and 24 h (9 ± 0.7 *versus* 13.1 ± 3.7 ng/mL), suggesting that the beneficial effects of PTX administration persist over time.

Matrix Metalloproteinase Activity

The matrix metalloproteinases are released upon neutrophil degranulation and aid in neutrophil sequestration in the lung tissue. Pulmonary MMP-2 activity observed after RL resuscitation was significantly elevated when compared with the sham group at both 4 h (27343 ± 2612 *versus* 36485 ± 2612; $P < 0.05$) and 24 h (36883 ± 1242 *versus* 402501 ± 1586; $P < 0.01$). In contrast, RL + PTX resuscitation resulted in comparable levels of MMP-2 activity to that of the control group at both time points (Fig. 4).

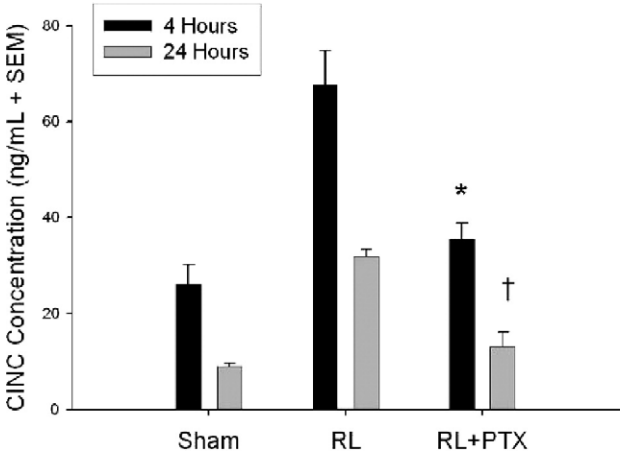

FIG. 3. Effects of resuscitation strategy on pulmonary CINC levels. The pulmonary concentration of CINC was determined by ELISA as described in Methods. Data are presented as the mean ± SEM in ng/mL ($n \geq 5$ per group). RL + PTX resuscitation after hemorrhagic shock (32 mL/kg RL + 25 mg/kg PTX) resulted in a 48% decrease at 4 h and a 40% decrease at 24 h when compared with RL (32 mL/kg). *, $P < 0.05$ *versus* RL and †, $P < 0.01$ *versus* RL.

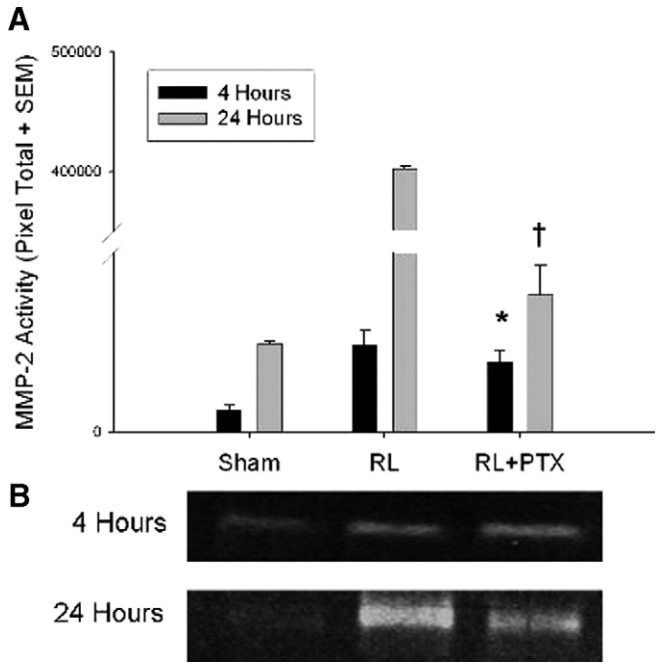

FIG. 4. Effect of resuscitation strategy on MMP-2 activity. MMP-2 activity was determined by zymography as described in Methods. Data are presented as the mean ± SEM ($n \geq 4$ per group). RL+PTX resuscitation after hemorrhagic shock (32 mL/kg RL + 25 mg/kg PTX) resulted in a 70% decrease at 4 h and a 84% decrease at 24 h in MMP-2 activity when compared with RL (32 mL/kg). *, $P < 0.05$ *versus* RL and †, $P < 0.01$ *versus* RL. B, Representative zymograms of MMP-2 activity at 4 and 24 h.

MMP-9 activity was also markedly elevated in the group receiving RL infusion when compared with either the sham or RL + PTX group at 4 or 24 h ($P < 0.05$). There was no significant difference between MMP-9 activity in the sham or RL + PTX treatment at 4 h (Fig. 5). At the 24 h time point, MMP-9 activity was greater in the RL + PTX treatment arm when compared with sham (350774 ± 22624 *versus* 157224 ± 28140; $P < 0.05$). Despite the increase, MMP-9 activity was still significantly lower than observed in the animals that received RL alone ($P < 0.05$).

Heme Oxygenase-1 Content

HO-1 serves as a marker of the relative degree of oxidative stress within tissues, and its expression is induced by the presence of reactive oxygen species generated during reperfusion [22, 23]. In animals resuscitated with RL, a marked increase in HO-1 expression was seen in lung extracts at 24 h (2.42 ± 0.12; $P < 0.05$; Fig. 6). In contrast, the concomitant administration of PTX with RL infusion did not result in the induction of HO-1 with levels paralleling that observed in the sham group (1.25 ± 0.13 *versus* 1.22 ± 0.27, respectively).

NF-κB Activation

In its inactive state, NF-κB remains quiescent in the cytoplasm associated with the inhibitory protein, I-κB. Reactive oxygen species generated through reperfusion is one stimulus capable of activating the cascade responsible for I-κBα phosphorylation which, in turn, allows for ubiquitination and proteolytic degradation of this inhibitory subunit and results in nuclear translocation of the p50-p65 NF-κB heterodimer [24–26]. Resuscitation with RL led to an 88% increase in pulmonary cytoplasmic I-κBα phosphorylation over the sham group at 4 h (6.36 ± 0.73 *versus* 1.74 ± 0.21) while the total amounts of I-κBα remained unchanged (Fig. 7). This marked increase in I-κBα phosphorylation was not observed with RL + PTX resuscitation (2.16 ± 0.36).

To assess whether the attenuation I-κBα phosphorylation observed with HSPTX after hemorrhagic shock affected nuclear translocation of NF-κB, we analyzed the relative amounts of NF-κB p65 subunit phosphorylation in lung nuclear extracts. As expected, RL-treated animals had a significantly higher degree of nuclear translocation over that of the sham group (50034 ± 5997 *versus* 11863 ± 4036; Fig. 8). There was a 41% reduction in p65 phosphorylation in the RL + PTX treated group (29898 ± 8060) when compared with their RL-treated counterparts ($P < 0.05$).

EMSA of nuclear extracts 4 h after shock and resuscitation was performed to determine if the modulation in NF-κB nuclear translocation reflected its binding to

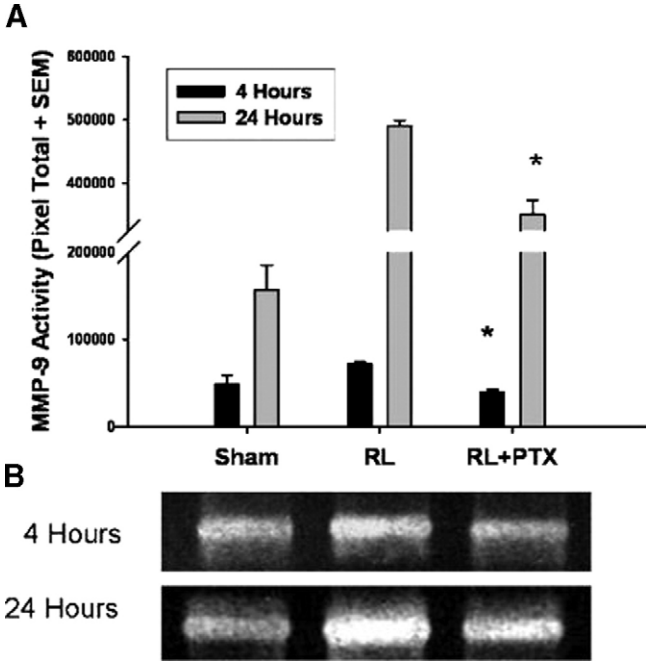

FIG. 5. (A) Effect of resuscitation strategy on MMP-9 activity. MMP-9 activity was determined by zymography as described in Methods. Data are presented as the mean ± SEM ($n \geq 4$ per group). RL + PTX resuscitation after hemorrhagic shock (32 mL/kg RL + 25 mg/kg PTX) resulted in a 44% decrease at 4 h and a 25% decrease at 24 h in MMP-9 activity when compared with RL (32 mL/kg). *, $P < 0.05$ *versus* RL. (B) Representative zymograms of MMP-9 activity at 4 and 24 h.

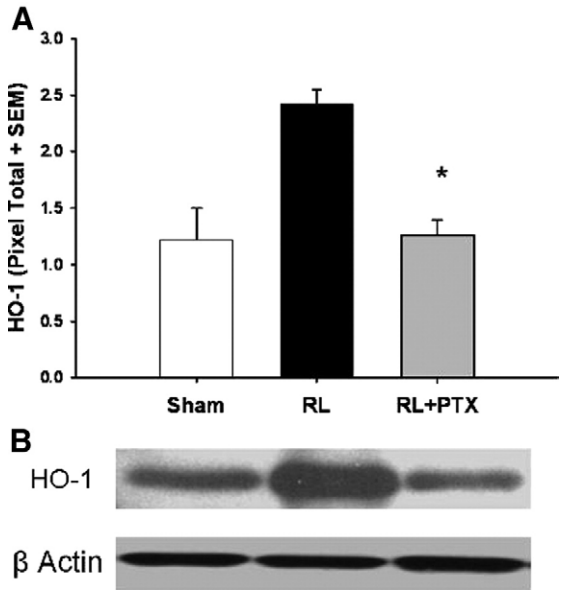

FIG. 6. (A) Pulmonary Heme oxygenase-1 content 24 h after hemorrhagic shock and resuscitation with RL (32 mL/kg) or RL + PTX (32 mL/kg RL + 25 mg/kg PTX). Results are expressed as the mean ± SEM ($n \geq 4$ per group). The RL + PTX-treated animals had significantly decreased HO-1 levels when compared to those animals who received RL alone. *, $P < 0.05$ *versus* RL. (B) Representative Western blots of HO-1 content and β Actin control.

DNA. RL administration resulted in a marked increase in NF-κB DNA binding, while the addition of PTX to RL attenuated this effect to a level comparable to the sham group (Fig. 9). Additionally, supershift with phosphorylated p65 antibody verified binding of the transcriptionally active subunit of NF-κB.

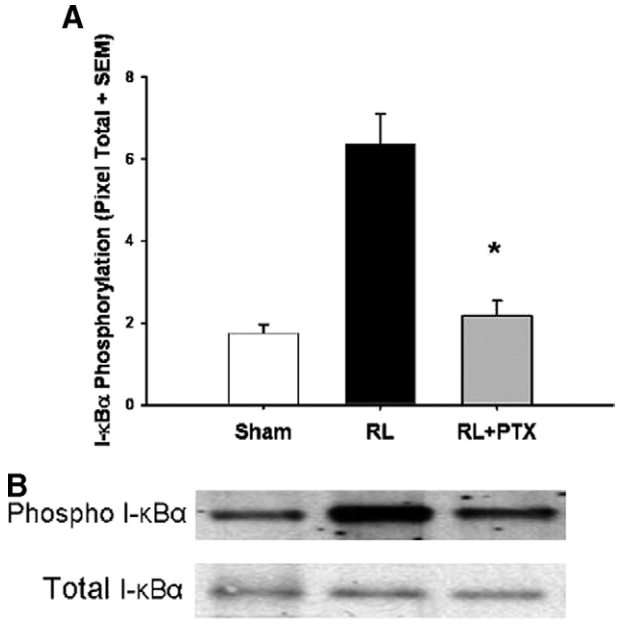

FIG. 7. (A) Cytoplasmic I-κBα phosphorylation was determined 4 h after shock and resuscitation with RL (32 mL/kg) or RL + PTX (32 mL/kg RL + 25 mg/kg PTX). Results are expressed as the mean ± SEM ($n \geq 4$ per group). The RL + PTX-treated animals had 66% less I-κBα phosphorylation than their RL-treated counterparts. *, $P < 0.01$ *versus* RL. (B) Representative Western blots of phosphorylated and total I-κBα phosphorylation.

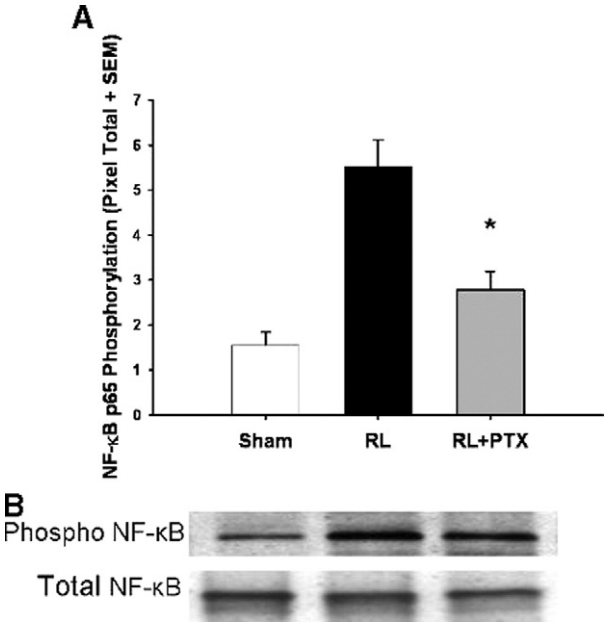

FIG. 8. (A) Nuclear NF-κB p65 subunit phosphorylation was determined after 4 h after shock and resuscitation with either RL (32 mL/kg) or RL + PTX (32 mL/kg RL + 25 mg/kg PTX). Results are expressed as the mean ± SEM ($n \geq 4$ per group). The RL+PTX-treated animals had 51% less p65 phosphorylation than their RL-treated counterparts. *, $P < 0.05$ *versus* RL. (B) Representative Western blots of total and NF-κB p65 subunit phosphorylation.

CREB Electrophoretic Mobility Shift Assay

EMSA of nuclear extracts of lung tissue from both the sham and RL treated groups demonstrated comparable CRE DNA binding (183 ± 8.2 *versus* 148 ± 32.3, respectively). The addition of PTX to standard RL resuscitation markedly elevated CRE DNA binding by 69% ($P < 0.04$). Supershift analysis confirmed DNA binding of CREB phosphorylated at site serine 133 (Fig. 10).

DISCUSSION

Despite marked improvements in hospital care over the past four decades, the mortality rate for ALI and ARDS in trauma patients remains near 40% [27]. To date, no pharmacologic therapy has demonstrated a survival benefit in multicenter trials. Recently, conventional fluid resuscitation with RL has been shown to contribute to neutrophil activation and end organ injury [8–11]. Consequently, there has been increased interest in alternative strategies or pharmacologic adjuncts that can be given at the start of resuscitation, which have the capacity to attenuate more then one component of inflammatory response. In this series,

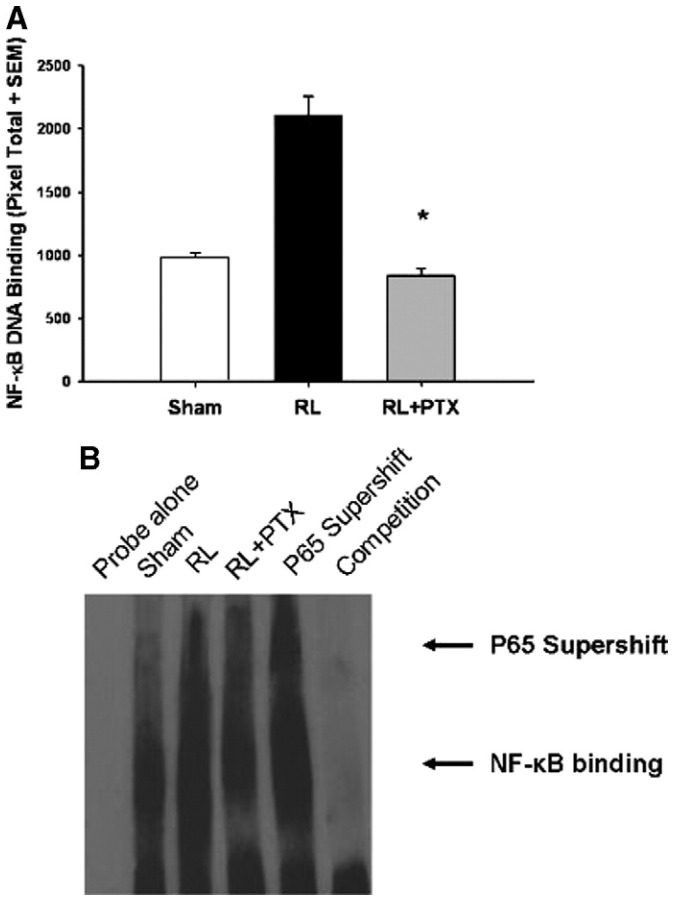

FIG. 9. (A) Effect of resuscitation strategy on NF-κB binding to DNA. The binding of NF-κB to DNA was determined by EMSA as described in Methods 4 h after resuscitation with either RL (32 mL/kg) or RL + PTX (32 mL/kg RL + 25 mg/kg PTX). Results are expressed as the mean ± SEM ($n \geq 4$ per group). RL + PTX-treated animals had 42% less NF-κB binding than their RL-treated counterparts. *, $P < 0.05$ *versus* RL. (B) Representative EMSA of NF-κB binding to DNA.

the reduction in resuscitation-induced pulmonary inflammation observed with the concomitant administration of PTX to standard RL resuscitation was associated with the down-regulation of multiple aspects of neutrophil function.

One component of neutrophil activation that is critical to the pathogenesis of inflammation is neutrophil degranulation. Under normal circumstances, degranulation allows for the controlled and targeted release of enzymes such as MMP-2 and MMP-9, which help facilitate neutrophil sequestration into the lung through their proteolytic actions on the endothelium and pulmonary basement membrane. In the case of ALI, the uncontrolled release of MMPs results in tissue damage, and their relative levels of expression have been shown to correlate with the severity of ARDS in humans [28, 29]. We observed a marked reduction in both MMP-2 and MMP-9 activity in those animals resuscitated with PTX + RL when compared with standard RL infusion. This finding is supported by previous work from our laboratory demonstrating the beneficial effects of PTX on neutrophil degranulation in vitro and MMP activity in an animal model of sepsis [20, 30]. In addition to the release of MMPs, neutrophils are capable of releasing large quantities of reactive oxygen species such as superoxide anions and hydrogen peroxide in a process known as the respiratory burst [31]. The overproduction of these toxic species results in considerable degree of oxidative stress and tissue damage, and inhibition of their production has been shown to alleviate organ injury after hemorrhagic shock [32]. Although we did not measure oxidative burst directly, we demonstrated a significant decrease in pulmonary HO-1, a relative marker of oxidative stress, when PTX was added to RL resuscitation.

Another factor that may account for the attenuation in pulmonary inflammation seen in animals that received PTX as part of their resuscitation regimen is the substantial reduction in the number of neutrophils present in the lungs when compared with those that

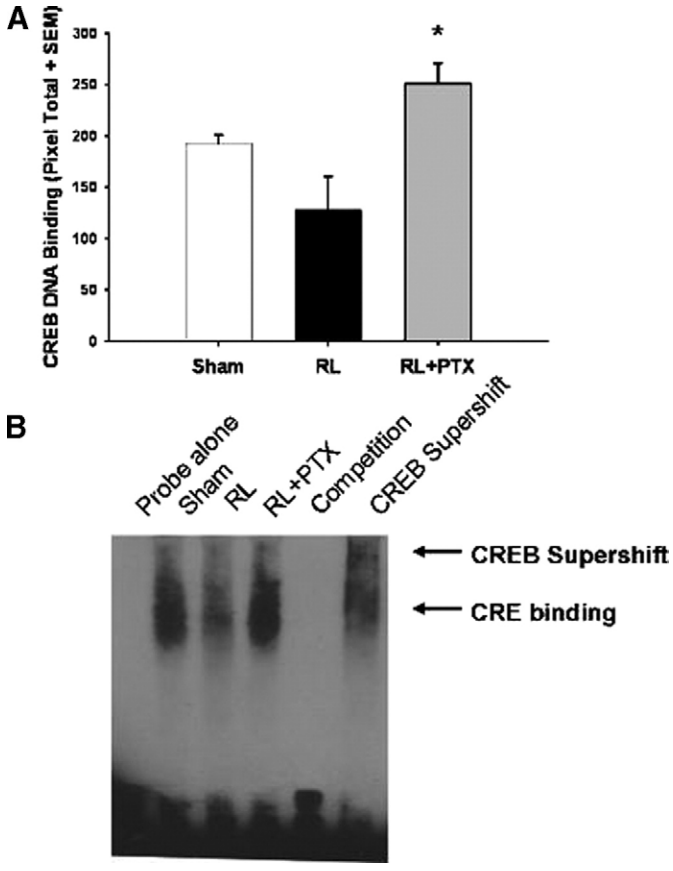

FIG. 10. (A) Effect of resuscitation strategy on CREB binding to DNA. The binding of CREB to DNA was determined by EMSA as described in Methods 4 h after resuscitation with either RL (32 mL/kg) or RL + PTX (32 mL/kg RL + 25 mg/kg PTX). Results are expressed as the mean ± SEM ($n \geq 4$ per group). RL + PTX-treated animals had 69% greater CREB binding than their RL-treated counterparts. *, $P < 0.04$ *versus* RL. (B) Representative EMSA of CREB binding to DNA.

received RL alone. Previous studies examining the effects of sepsis and hemorrhage have found inhibition of intercellular adhesion molecule-1 expression with the administration of PTX, making the attenuation of neutrophil adhesion to the endothelium of the lung a plausible mechanism for the decrease in neutrophil number [19, 20, 33, 34]. Another possible explanation for the reduction in cell number may be due to the attenuation in CINC, seen in this study with PTX. IL-8, the homologue to CINC in humans, which functions as a neutrophil-specific pulmonary chemotactic factor and has been shown to be up-regulated in trauma patients who go on to develop ARDS [35]. Moreover, Mukaida *et al.* demonstrated that inhibition of IL-8 through specific antibodies resulted in reduction of ARDS in a rabbit endotoxemia model [36].

In addition to the decline in neutrophil number and function seen with PTX-supplemented resuscitation, we also observed alterations in the activation of the transcription factors NF-κB and CREB when compared with RL. The transcriptional factor NF-κB consists of homo- or heterodimers of members of the Rel family proteins, p50, p52, p65 (RelA), cRel, and RelB. Its activity has been shown to correlate with severity of illness in systemic inflammatory response syndrome patients and was significantly increased in nonsurvivors when compared with survivors of septic shock [37, 38]. In conditions such as ischemia-reperfusion, active NF-κB can bind to the promotor regions of proinflammatory genes. Molecular studies examining IL-8 gene activation have shown that the majority of IL-8 gene transcription is mediated by the activation of NF-κB, and that inhibition of NF-κB using antisense oligonucleotides inhibits IL-8 production in vitro [39, 40]. Therefore, the attenuation in IL-8 observed in this study may be associated with the down-regulation in NF-κB activity seen with the administration of PTX.

CREB, a member of the basic domain-leucine zipper transcription factor class, is involved in cell proliferation, differentiation, and adaptive responses. Stimulus-induced activation of CREB can occur secondary to a variety of cellular stressors, including ischemia-reperfusion, appears to function in growth factor and stress-inducible gene expression [41]. In contrast to NF-κB, CREB-DNA binding was significantly up-regulated with the addition of PTX to RL resuscitation, thereby possibly shifting transcription in lung from a pro- to an anti-inflammatory state.

From what is known about the actions of PTX, it may be capable of transcription factor modulation through numerous mechanisms. While the generation of reactive oxygen species has been shown to activate NF-κB, [42, 43], it is possible that PTX may attenuate NF-κB activation through its role as an inhibitor of the respiratory burst and oxygen radical production [44, 45]. A second potential mechanism may be due to the intracellular effects of PTX. PTX administration results in the rapid accumulation of cAMP and activation of protein kinase A (PKA) [15, 46]. Phosphorylation of CREB at serine 133 is primarily the result of a PKA activation and is required for CREB activation [47]. In contrast, numerous studies have demonstrated that agents that increase cAMP, such as PTX, dibutyryl cAMP, and forskolin lead to inhibition of NF-κB-dependent proinflammatory gene expression through a PKA-dependent mechanism [48–51]. A third possibility involves the competition between transcription factors for coactivation. The phosphorylated forms of both CREB and NF-κB require coactivation with CBP for the initiation of optimal transcriptional activity [52, 53]. The coactivator CBP and its paralogue p300 function as a physical bridge between the activator and the basal transcription machinery, facilitating the initiation of transcription [47]. The phosphorylated forms of NF-κB and CREB have been shown to interact with the same site in the N-terminus of CBP (also called the KIX region) [54, 55]. Since CBP is present in the nucleus of cells in finite amounts, PTX may modulate the competition of transcription factors for CBP toward CREB and subsequently reduce NF-κB-dependent transcription.

The effects of PTX on other additional transcription factors, such as activating protein-1 (AP-1), cannot be ruled out by our current experiments. Since activation of AP-1 and its components have been implicated in inflammation, and based on the fact that AP-1 can also bind CBP and recognize similar sites in the promotor regions of proinflammatory genes such as tumor necrosis factor-alpha, it is possible that PTX may affect AP-1 activity in addition to CREB and NF-κB [56, 57]. Further studies are needed to clarify these issues.

While the effects demonstrated with the adjunctive use of PTX with resuscitation after shock are promising, further extended studies evaluating the impact of PTX on mortality after trauma must be completed prior to human use. In summary, the attenuation in pulmonary inflammation observed with addition of PTX to RL is associated with attenuation of neutrophil activity and modulation of local transcription factor activation. This novel resuscitation strategy may have future therapeutic potential in the treatment of resuscitation-induced lung injury after hemorrhagic shock.

REFERENCES

1. Zallen G, Moore EE, Johnson JL, et al. Posthemorrhagic shock mesenteric lymph primes circulating neutrophils and provokes lung injury. J Surg Res 1999;83:83.
2. Aldridge AJ. Role of the neutrophil in septic shock and the adult respiratory distress syndrome. Eur J Surg 2002;168:204.
3. Lee WL, Downey GP. Neutrophil activation and acute lung injury. Curr Opin Crit Care 2001;7:1.

4. Windsor AC, Mullen PG, Fowler AA, et al. Role of the neutrophil in adult respiratory distress syndrome. Br J Surg 1993;80:10.
5. Bazzoni F, Cassatella MA, Laudanna C, et al. Phagocytosis of opsonized yeast induces tumor necrosis factor-α mRNA accumulation and protein release by human polymorphonuclear leukocytes. J Leukoc Biol 1991;50:223.
6. Strieter RM, Kasahara K, Allen RM, et al. Cytokine-induced neutrophil-derived interleukin-8. Am J Path 1992;141:397.
7. Sha WC. Regulation of immune responses by NF-κB/Rel transcription factors. J Exp Med 1998;187:143.
8. Alam HB, Stanton K, Koustova E, et al. Effect of different resuscitation strategies on neutrophil activation in a swine model of hemorrhagic shock. Resuscitation 2004;60:91.
9. Alam HB, Sun L, Ruff P, et al. E- and P-selectin expression depends on the resuscitation fluid used in hemorrhaged rats. J Surg Res 2002;94:145.
10. Savage SA, Fitzpatrick CM, Kashyap V, et al. Endothelial dysfunction after lactated Ringer's solution resuscitation for hemorrhagic shock. J Trauma 2005;59:284.
11. Ayuste EC, Chen H, Koustova E, et al. Hepatic and pulmonary apoptosis after hemorrhagic shock in swine can be reduced through modifications in conventional ringer's solution. J Trauma 2006;60:52.
12. Porter JM, Cutler BS, Le BY, et al. Pentoxifylline efficacy in the treatment of intermittent claudication: Multicenter controlled double-blind trial with objective assessment of chronic occlusive arterial disease patients. Am J Heart 1982;104:66.
13. Lauterbach R, Pawlik D, Kowalczyk D, et al. Effect of the immunomodulating agent, pentoxifylline, in the treatment of sepsis in prematurely delivered infants: A placebo-controlled, double-blind trial. Crit Care Med 1999;27:807.
14. Akriviadis E, Botla R, Briggs W, et al. Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: A double-blind, placebo-controlled trial. Gastroenterology 2000; 119:1637.
15. Coimbra R, Melbostad H, Hoyt DB. Effects of phosphodiesterase inhibition on the inflammatory response after shock: Role of pentoxifylline. J Trauma 2004;56:442.
16. Michetti C, Coimbra R, Hoyt DB, et al. Pentoxifylline reduces acute lung injury in chronic endotoxemia. J Surg Res 2003;115:92.
17. van Leenen D, van der Poll T, Levi M, et al. Pentoxifylline attenuates neutrophil activation in experimental endotoxemia in chimpanzees. J Immunol 1993;151:2318.
18. Coe DA, Freischlag JA, Johnson D, et al. Pentoxifylline prevents endothelial damage due to ischemia and reperfusion injury. J Surg Res 1997;67:21.
19. Barroso-Aranda J, Schmid-Schonbein GW. Pentoxifylline pretreatment decreases the pool of circulating activated neutrophils, in-vivo adhesion to endothelium, and improves survival from hemorrhagic shock. Biorheology 1990;27:401.
20. Coimbra R, Melbostad H, Loomis WH, et al. LPS-induced acute lung injury is attenuated by phosphodiesterase inhibition: Effects on proinflammatory mediators, metalloproteinases, NF-κB, and ICAM-1 expression. J Trauma 2006;60:115.
21. Remick DG. Interleukin-8. Crit Care Med 2005;33:S466.
22. Bauer M, Bauer I. Heme oxygenase-1: Redox regulation and role in the hepatic response to oxidative stress. Antioxid Redox Signal 2002;4:749.
23. Ryter SW, Otterbein LE, Morse D, et al. Heme oxygenase/carbon monoxide signaling pathways: Regulation and functional significance. Mol Cell Biochem 2002;249:234.
24. McDonald PP, Bald A, Cassatella MA. Activation of the NF-κB pathway by inflammatory stimuli in human neutrophils. Blood 1997;89:3421.
25. Renard P, Percherancier Y, Kroll M, et al. Inducible NF-κB activation is permitted by simultaneous degradation of nuclear IκBα. J Biol Chem 2002;275:15193.
26. Schmitz ML, Baeuerle PA. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J 1991;10:3805.
27. Levitt JE, Matthay MA. Treatment of acute lung injury: Historical perspective and potential future therapies. Semin Respir Crit Care Med 2006;27:426.
28. Corbel M, Biochot E, Lagente V. Role of gelatinases MMP-2 and MMP-9 in tissue remodeling following acute lung injury. Braz J Med Biol Res 2000;155:43.
29. Torii K, Iida K, Miyazaki Y, et al. Higher concentrations of matrix metalloproteinases in bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 1997;155:43.
30. Deree J, Lall R, Melbostad H, et al. Neutrophil degranulation and the effects of phosphodiesterase inhibition. J Surg Res 2006;133:22.
31. Redl H, Gasser H, Schlag, et al. Involvement of oxygen radicals in shock related cell injury. Br Med Bull 1993;49:556.
32. Abdelrahman M, Mazzon E, Bauer M, et al. Inhibitors of the NADPH oxidase reduce the organ injury in hemorrhagic shock. Shock 2005;23:107.
33. Krakauer T. Pentoxifylline inhibits ICAM-1 expression and chemokines production induced by proinflammatory cytokines in human pulmonary epithelial cells. Immunopharmacology 2000;46:253.
34. Yada-Langui MM, Anjos-Valotta EA, Sannomiya P, et al. Resuscitation affects microcirculatory polymorphonuclear leukocyte behavior after hemorrhagic shock: Role if hypertonic saline and pentoxifylline. Exp Biol Med 2004;229:684.
35. Donnelly SC, Strieter RM, Kunkel SL, et al. Interleukin-8 and the development of adult respiratory distress syndrome in at-risk patient groups. Lancet 1993;341:643.
36. Mukaida N, Matsumoto T, Yokoi K, et al. Inhibition of neutrophil mediated acute inflammatory injury by an antibody against interleukin-8. Inflamm Res 1998;3:S151.
37. Nakamori Y, Koh T, Ogura H, et al. Enhanced expression of intranuclear NF-κB in primed polymorphonuclear leukocytes in systemic inflammatory response syndrome patients. J Trauma 2003;54:253.
38. Arnalich F, Garcia-Palomero E, Lopez J, et al. Predictive value of nuclear factor κB activity and plasma cytokine levels in patients with sepsis. Infect Immun 2000;68:1942.
39. Mukaida N, Okamoto S, Ishikawa Y, et al. Molecular mechanism of interleukin-8 gene expression. J Leuk Biol 1994;56:554.
40. Yoshida S, Ono M, Shono T, et al. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol 1997;17:4015.
41. Shaywitz AJ, Greenberg ME. CREB: A stimulus induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 1999;68:821.
42. Ndengele MM, Muscoli C, Wang ZQ, et al. Superoxide potentiates NF-κB activation and modulates endotoxin-induced cytokine production in alveolar macrophages. Shock 2005;23:186.
43. Christman JW, Lancaster LH, Blackwell TS. Nuclear factor κB: A pivotal role in the systemic inflammatory response syndrome and new target for therapy. Intens Care Med 1998;24:1131.
44. Coimbra R, Loomis WH, Melbostad. LPS-stimulated PMN activation and proinflammatory mediator synthesis is downregulated by phosphodiesterase inhibition: Role of pentoxifylline. J Trauma 2004;57:1157.

45. Josaki K, Contrino J, Kristie J, et al. Pentoxifylline-induced modulation of human leukocyte function in vitro. Am J Path 1990;136:623.
46. Nielson CP, Crowley JJ, Morgan ME, et al. Polymorphonuclear leukocyte inhibition by therapeutic concentrations of theophylline is mediated by cyclic-3′, 5′-adenosine monophosphate. Am J Respir Dis 1988;137:25.
47. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation dependent factor CREB. Nat Rev Mol Cell Biol 2001;8:599.
48. Coimbra R, Melbostad H, Loomis W, et al. Phosphodiesterase inhibition decreases nuclear factor-κB activation and shifts the cytokine response toward anti-inflammatory activity in acute endotoxemia. J Trauma 2005;59:575.
49. Ollivier V, Parry GC, Cobb RR, et al. Elevated cyclic AMP inhibits NK-κB-mediated transcription in human monocytic cells and endothelial cells. J Biol Chem 1996;271:20828.
50. Takahashi N, Tetsuka T, Uranishi H, et al. Inhibition of the NF-κB transcriptional activity by protein kinase A. Eur J Biochem 2002;269:4559.
51. Minguet S, Huber M, Rosenkranz L, et al. Adenosine and cAMP are potent inhibitors of the NF-κB pathway downstream on immunoreceptors. Eur J Immunol 2005;35:31.
52. Perkins ND, Felzien LK, Betts JC, et al. Regulation of NF-κB by cyclin-dependent kinases associated with the p300 coactivator. Science 1997;275:523.
53. Shenkar R, Abraham E. Mechanisms of lung neutrophil activation after hemorrhage or endotoxemia: Roles of reactive oxygen intermediates, NF-κB, and cyclic AMP response element binding protein. J Immunol 1999;163:954.
54. Sheppard KA, Rose DW, Haque ZK, et al. Transcriptional activation of NF-κB requires multiple coactivators. Mol Cell Biol 1999;19:6367.
55. Shaywitz AJ, Dove SL, Kornhauser JM, et al. Magnitude of the CREB-dependent transcriptional response is determined by the strength of the interaction between the kinase-inducible domain of CREB and the KIX domain of CREB binding protein. Mol Cell Biol 2000;20:9409.
56. Newell CL, Deisseroth AB, Lopez-Berestein G. Interaction of nuclear proteins with an AP-1/CRE-like promoter sequence in the human TNF-α gene. J Leuk Biol 1994;56:27.
57. Janknecht R. The versatile functions of the transcriptional coactivators p300 and CBP and their roles in disease. Histol Histopathol 2002;17:657.