Activation of Extracellular Signal-Regulated Kinases, NF-κB, and Cyclic Adenosine 5′-Monophosphate Response Element-Binding Protein in Lung Neutrophils Occurs by Differing Mechanisms After Hemorrhage or Endotoxemia

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Activation of Extracellular Signal-Regulated Kinases, NF-κB, and Cyclic Adenosine 5′-Monophosphate Response Element-Binding Protein in Lung Neutrophils Occurs by Differing Mechanisms After Hemorrhage or Endotoxemia

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Acute lung injury is frequently associated with sepsis or blood loss and is characterized by a proinflammatory response and infiltration of activated neutrophils into the lungs. Hemorrhage or endotoxemia result in activation of cAMP response element-binding protein (CREB) and NF-κB in lung neutrophils as well as increased expression of proinflammatory cytokines, such as TNF-α and macrophage-inflammatory peptide-2, by these cells. Activation of the extracellular regulated kinase (ERK) pathway occurs in stress responses and is involved in CREB activation. In the present experiments, hemorrhage or endotoxemia produced increased activation of mitogen-activated protein kinase kinase (MEK)1/2 and ERK2 (p42), but not of ERK1 (p44), in lung neutrophils. ERK1, ERK2, and MEK1/2 were not activated in peripheral blood neutrophils after hemorrhage or endotoxemia. Inhibition of xanthine oxidase led to further increase in the activation of MEK1/2 and ERK2 in lung neutrophils after hemorrhage, but not after endotoxemia. α-Adrenergic blockade before hemorrhage resulted in increased activation in lung neutrophils of MEK1/2, ERK1, ERK2, and CREB, but decreased activation of NF-κB. In contrast, α-adrenergic blockade before endotoxemia was associated with decreased activation of MEK1/2, ERK2, and CREB, but increased activation of NF-κB. β-Adrenergic blockade before hemorrhage did not alter MEK1/2 or ERK1 activation in lung neutrophils, but decreased activation of ERK2 and CREB, while increasing activation of NF-κB. β-Adrenergic inhibition before endotoxemia did not affect activation of MEK1/2, ERK1, ERK2, CREB, or NF-κB. These data indicate that the pathways leading to lung neutrophil activation after hemorrhage are different from those induced by endotoxemia. The Journal of Immunology, 2001, 166: 522–530.

Acute lung injury (ALI) occurs frequently after sepsis, trauma, or blood loss (1). Neutrophil accumulation in the lungs, increased generation of reactive oxygen intermediates (ROI), and the release of proinflammatory cytokines contribute to the pulmonary inflammatory process that characterizes ALI (2–5). IL-1β, TNF-α, and IL-8 are elevated in the lungs of patients with ALI (6, 7). The promoter regions of the genes for each of these cytokines have binding sites for the transcriptional regulatory factor NF-κB, and activation of NF-κB appears to be important in modulating their expression (8, 9). Increased activation of NF-κB is present in the lung cell populations in experimental models of ALI and in patients with the acute respiratory distress syndrome (ARDS), a more severe form of ALI (10–12).

In murine models of acute lung injury associated with hemorrhage or endotoxemia, neutrophils that infiltrate into the lungs are important sources of IL-1β, TNF-α, and MIP-2 (13, 14). Data from our laboratory suggest that both xanthine oxidase derived ROI and catecholamines are involved in driving this proinflammatory cytokine response (14–16). Inhibition of xanthine oxidase prevents hemorrhage associated increases in lung neutrophil expression of IL-1β, TNF-α, and MIP-2 (14). In the setting of endotoxemia, exogenous administration of α1-adrenergic agonists inhibits proinflammatory cytokine expression in lung neutrophils (16).

NF-κB as well as another transcriptional factor, the cAMP response element-binding protein (CREB), are activated in lung, but not peripheral blood neutrophils, after hemorrhage or endotoxemia (14). If xanthine oxidase is inhibited, hemorrhage-induced CREB activation in lung neutrophils is further increased, but there is no change in NF-κB activation (14). Because NF-κB and CREB compete for the same KIX binding site on the coactivator molecule CREB-binding protein (CBP) and are transcriptionally active only if bound to CBP, increased CREB activation may result in diminished NF-κB-dependent transcription (17). Such competition between CREB and NF-κB for CBP binding provides a potential explanation for our previous results (14) that demonstrated inhibitory effects of xanthine oxidase blockade on hemorrhage-induced increases in the expression of proinflammatory cytokines, such as MIP-2 and TNF-α, the transcription of which is dependent on NF-κB.

Although ROI and catecholamines appear to be involved in modulating proinflammatory cytokine expression and transcriptional factor activation in lung neutrophils after hemorrhage or endotoxemia, the mechanisms by which they do so have not been

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3 Abbreviations used in this paper: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; MIP, macrophage-inflammatory peptide; CREB, cAMP response element-binding protein; CRE, cAMP-responsive element; CBP, CREB-binding protein; ROI, reactive oxygen intermediates; ERK, extracellular signal-regulated kinases; MEK, mitogen-activated protein kinase kinase; BCA, bicinchoninic acid; PKA, protein kinase A; ELK-1, ETS domain protein.
determined. At least one possibility is that ROI and catecholamines affect intracellular mitogen-activated protein kinases involved in CREB and NF-κB activation. These serine/threonine kinases are present in all cell types and play a critical role in regulation of a wide variety of biological response mechanisms (18).

The extracellular signal-regulated kinases (ERK), particularly ERK1/ERK2 (also called p44/42 mitogen-activated protein kinases), play an important role in signal transduction pathways activated by diverse extracellular stimuli, including mitogens, growth factors, oxidative stress, and cytokines (19–24). ERK1/ERK2 are implicated in a variety of neutrophil functions, including cellular responses to stress (25, 26). Of particular importance, the ERK pathway has been shown to be involved in modulating the activation of transcriptional factors including CREB, NF-IL-6, and AP-1 (27–29). GTPases and kinases upstream to ERK1/ERK2 inactivation of transcriptional factors including CREB, NF-IL-6, and AP-1 are involved in modulating the growth factors, oxidative stress, and cytokines (19–24). ERK1/ERK2 are activated by diverse extracellular stimuli, including mitogens, growth factors, oxidative stress, and cytokines (19–24). GTPases and kinases upstream to ERK1/ERK2 inactivation of transcriptional factors including CREB, NF-IL-6, and AP-1 are involved in modulating the growth factors, oxidative stress, and cytokines (19–24). GTPases and kinases upstream to ERK1/ERK2 inactivation of transcriptional factors including CREB, NF-IL-6, and AP-1 are involved in modulating the growth factors, oxidative stress, and cytokines (19–24). GTPases and kinases upstream to ERK1/ERK2 inactivation of transcriptional factors including CREB, NF-IL-6, and AP-1 are involved in modulating the growth factors, oxidative stress, and cytokines (19–24). GTPases and kinases upstream to ERK1/ERK2 inactivation of transcriptional factors including CREB, NF-IL-6, and AP-1 are involved in modulating the growth factors, oxidative stress, and cytokines (19–24). GTPases and kinases upstream to ERK1/ERK2 inactivation of transcriptional factors including CREB, NF-IL-6, and AP-1 are involved in modulating the growth factors, oxidative stress, and cytokines (19–24).

**Materials and Methods**

**Mice**

Male BALB/c mice, 8–12 wk of age, were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were kept on a 12-h light/dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board-approved protocols.

**Materials**

Methoxyflurane was obtained from Schering-Plough Animal Health (Union, NJ). Escherichia coli 0111:B4 endotoxin, collagenase, DNase, propranolol, and phenolamine were obtained from Sigma (St. Louis, MO). The allopurinol-supplemented diet and tungsten-enriched molybdenum-deficient diet were purchased from ICN Biochemicals (Costa Mesa, CA). RPMI 1640, 25 mM HEPES, 1-glutamine was obtained from BioWhittaker Products (Walkersville, MD), and PBS and penicillin/streptomycin were purchased from Gemini Bioproducts (Calabasas, CA). Percoll was purchased from Amersham-Pharmacia (Piscataway, NJ). Anti-B220 and anti-Thy-1.2 magnetic beads were obtained from Dyan (Lake Success, NY). The Coomasie-Plus protein assay reagent and biocinchoninic acid (BCA) protein assay reagent were purchased from Pierce (Rockford, IL). Sequenase DNA polymerase was obtained from US Biochemicals (Cleveland, OH). Anti-phosphorylated CREB antiserum was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phosphorylated ERK1/ERK2, anti-phosphorylated-MEK1/2, anti-total ERK1/ERK2, anti-total MEK1/2, and anti-phosphorylated ELK-1 were purchased from New England Biolabs (Beverly, MA).

**Models of hemorrhage and endotoxemia**

The murine hemorrhage model used in these experiments was reported previously (10, 13, 15). With this model, 30% of the calculated blood volume (∼0.55 ml for a 20-g mouse) is withdrawn during a 60-s period by cardiac puncture from a methoxyflurane-anesthetized mouse. The period of methoxyflurane anesthesia was <2 min in all of the cases. The mortality rate with this hemorrhage protocol is ∼12%.

The model of endotoxemia was used as reported previously (13, 16). Mice received an i.p. injection of LPS at a dose of 1 mg/kg in 0.2 ml PBS. This dose has previously been demonstrated to produce acute neutrophilic alveolitis, histologically consistent with acute lung injury in mice (32, 33).

**Allopurinol supplementation**

To assess the effects of xanthine oxidase on ERK1/ERK2 and MEK1/2 activation, mice were pair-fed an allopurinol-supplemented diet (2.5 g/kg chow) or a normal control diet for 1 wk before hemorrhage or endotoxemia (34).

**Tungsten feeding**

To assess further the effects of xanthine oxidase on ERK1/ERK2 and MEK1/2 activation, mice were pair-fed a tungsten-enriched, molybdenum-deficient diet (0.7 g sodium tungstate per kg chow) or a normal control diet for 3 wk before hemorrhage (35). Tungsten feeding inactivates xanthine oxidase by removing molybdenum, a crucial cofactor for xanthine oxidase activity (35).

**Interventions**

In designated experiments, mice were treated i.p. with 0.2 ml PBS (control), the α-adrenergic antagonist phenolamine (10 mg/kg), or the β-adrenergic antagonist propranolol (3 mg/kg) 30 min before either hemorrhage or LPS administration. These doses of phenolamine and propranolol have been used previously by our laboratory and result in complete α- and β-adrenergic blockade (16). Each drug was administered in a volume of 0.2 ml of PBS.

**Isolation of neutrophils**

Neutrophils from intraparenchymal pulmonary cell suspensions were isolated by a modification of the technique of Sugarawa and coworkers as previously used in our laboratory (13, 16, 36). In brief, the chest of the mouse was opened and the lung vascular bed was flushed with 2–3 ml chilled (4°C) PBS injected into the right ventricle. Lungs were then exsanguinated, avoiding the paratracheal lymph nodes and thymus, and washed twice in RPMI 1640 medium, 25 mM HEPES, 1-glutamine with penicillin/streptomycin. Intraparenchymal pulmonary cell suspensions were isolated by collagenase digestion, using techniques previously described by our laboratory (10, 13, 15). Essentially, the excised lungs were minced finely, and the tissue pieces were placed in RPMI 1640 containing 5% FBS, 20 U/ml collagenase, and 1 μg/ml DNase. After incubation for 60 min at 37°C, any remaining intact tissue was disrupted by passage through a 21-gauge needle. Tissue fragments and the majority of dead cells were removed by rapid filtration through a glass wool column, and cells were collected by centrifugation.

Peripheral blood neutrophils were isolated by techniques previously described by our laboratory (13, 16). In brief, mice were anesthetized with methoxyflurane and then exsanguinated. Blood was withdrawn by cardiac puncture and collected into a 1-ml syringe containing 5 U heparin. The blood was centrifuged, and the plasma was removed. The cell pellet was treated with Gey’s solution to lyse RBC. The remaining cells were washed in RPMI and collected by centrifugation.

To isolate lung or peripheral blood neutrophils, the pellets from either the intraparenchymal pulmonary cell or a peripheral blood cell suspension were resuspended in 2 ml PBS. If cells were to be used in either the EMSA or Western blotting, pellets from lung suspensions from three mice or blood suspensions from two mice were pooled in PBS. The lung cell suspension was layered onto 5 ml Percoll (density, 1.077) previously layered on 5 ml Percoll (density, 1.085). The peripheral blood cell suspension was layered onto 5 ml Percoll (density, 1.085), which previously had been layered on 5 ml Percoll (density, 1.088). The peripheral blood cell suspension was layered onto 5 ml Percoll (density, 1.085), which previously had been layered on 5 ml Percoll (density, 1.088). After centrifugation at 600 × g for 25 min at 18°C, the neutrophil-rich fraction was collected from the interface between the two Percoll layers and washed with RPMI. For EMSA or Western blots, lung or blood neutrophils were pooled from four Percoll gradients. Neutrophils were further purified by the removal of T and B cells with anti-B220 and anti-Thy-1.2 magnetic beads using the manufacturer’s protocol. Viability, as determined by trypan blue exclusion, was consistently >98%. Neutrophil purity, as determined by Wright’s staining cytospin preparations, was >95%.

**Preparation of nuclear extracts**

Nuclear extracts were prepared as previously described (11, 15). In brief, 2–9 × 106 intraparenchymal pulmonary neutrophils, pooled from 8 or 12 mice, were incubated on ice for 15 min in buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, pH 7.9). After cytoplasm was removed from the nuclei by 15 passages through a 25-gauge needle, the nuclei were centrifuged at 4°C for 6 min at 600 × g. The nuclear pellet was then incubated on ice for 15 min in buffer C (20 mM HEPES (pH 7.9), 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol) (37), after which the extract was centrifuged at 4°C for 10 min at 12,000 × g. The supernatant was collected, divided into aliquots, and stored at −86°C. Protein concentration was determined by using the Coomasie-Plus Protein Assay Reagent standardized to BSA, according to the manufacturer’s protocol.

**EMSA analysis**

Activation of the transcriptional factors, NF-κB and CREB, was determined by EMSA analysis, as described previously by our laboratory (11, 15). The Journal of Immunology 523
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15). The cDNA sequence of the Ig gene (38) and CAMP-responsive element (CRE) conserved element (39) were used. Synthetic double-stranded sequences (with enhancer motifs underlined) were fill in labeled with [γ-32P]dATP using Sequenase DNA polymerase: 5'-TTTTT GAGCTGCGACATCCCAGC-3', 5'-GCTGCGACCCTCTTGAAGGGCTG TTTTT-3'; CRE: 5'-TTTTCGACGCTTCAGCTGACAGGC-3', 3'-GCT CGAAGCTGACGTTCGTTTT-5'.

DNA-binding reaction mixtures of 20 μl contained 1 μg nuclear extract, 10 nM Tris-Cl (pH 7.5), 50 mM EDTA, 0.5 mM DTT, 1 mM MgCl2, 4% glycerol, 0.08 μl poly(dC)poly(dC), and 0.7 mmol 32P-labeled double-stranded oligonucleotide. For supershift experiments, 1 μl of anti-CRE antibody was added to the binding reaction mixture just before the 20-min incubation. After the samples were incubated at room temperature for 20 min, they were loaded onto a 4% polyacrylamide gel. Protein was electrotransferred to a chemiluminescent membrane and then blocked with 5% nonfat dry milk, Tris-buffered saline, with 0.1% Tween. After blocking, the membrane was incubated overnight at 4°C with a rabbit polyclonal specific Ab to phosphorylated ERK1/ERK2 or phosphorylated MK1/2 using a dilution of 1:1000 followed by peroxidase-conjugated anti-rabbit Ig. The membranes were washed three times with Tris-buffered saline, then stripped using Immuno Pure IgG Elution Buffer (Pierce, Rockford, IL) and reprobed with anti-total ERK1/ERK2 or MEK1/2 Abs. Denitrometry was performed using a chemiluminescence system and software (Bio-Rad, Hercules, CA) to determine the ratio between phosphorylated and total kinase.

Immunoprecipitation

For immunoprecipitation of phospho-ERK1/ERK2, lung neutrophils were resuspended in ice cold lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 300 μM p-nitrophenyl phosphate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.3) for 15 min. The protein concentration of each sample was assayed using the BCA protein assay kit standardized to BSA, according to manufacturer's protocol. Briefly, 20 to 30 μg of protein were loaded on a 10% Tris-HCl-SDS-polyacrylamide gel. Protein was electrotransferred to a chemiluminescent membrane and then blocked with 5% nonfat dry milk, Tris-buffered saline, with 0.1% Tween. After blocking, the membrane was incubated overnight at 4°C with a rabbit polyclonal specific Ab to phosphorylated ERK1/ERK2 or phosphorylated MK1/2 using a dilution of 1:1000 followed by peroxidase-conjugated anti-rabbit Ig. The membranes were washed three times with Tris-buffered saline, then stripped using Immuno Pure IgG Elution Buffer (Pierce, Rockford, IL) and reprobed with anti-total ERK1/ERK2 or MEK1/2 Abs. Denitrometry was performed using a chemiluminescence system and software (Bio-Rad, Hercules, CA) to determine the ratio between phosphorylated and total kinase.

Western blot analysis

Whole cell extracts from lung neutrophils were denatured in ice cold lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 300 μM p-nitrophenyl phosphate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.3) for 15 min. The protein concentration of each sample was assayed using the BCA protein assay kit standardized to BSA, according to manufacturer's protocol. Briefly, 20 to 30 μg of protein were loaded on a 10% Tris-HCl-SDS-polyacrylamide gel. Protein was electrotransferred to a chemiluminescent membrane and then blocked with 5% nonfat dry milk, Tris-buffered saline, with 0.1% Tween. After blocking, the membrane was incubated overnight at 4°C with a rabbit polyclonal specific Ab to phosphorylated ERK1/ERK2 or phosphorylated MK1/2 using a dilution of 1:1000 followed by peroxidase-conjugated anti-rabbit Ig. The membranes were washed three times with Tris-buffered saline, then stripped using Immuno Pure IgG Elution Buffer (Pierce, Rockford, IL) and reprobed with anti-total ERK1/ERK2 or MEK1/2 Abs. Denitrometry was performed using a chemiluminescence system and software (Bio-Rad, Hercules, CA) to determine the ratio between phosphorylated and total kinase.

Results

Activation of MEK1/2 and ERK1/ERK2 in lung neutrophils after hemorrhage or endotoxemia

Hemorrhage or endotoxemia leads to increased numbers of neutrophils in the perivascular and interstitial spaces of the lungs (13). To determine whether the ERK1/ERK2 pathway was activated in lung or peripheral blood neutrophils after hemorrhage or endotoxemia, we used Western blotting to examine levels of total and phosphorylated forms of MEK1/2 and ERK1/ERK2 in neutrophils isolated 1 h after hemorrhage or endotoxemia. As shown in Fig. 1A, neither hemorrhage nor endotoxemia affected activation of MEK1/2, ERK1, or ERK2 in peripheral blood neutrophils. In contrast, MEK1/2 and ERK2 (p42), but not ERK1 (p44), were activated after hemorrhage or endotoxemia in lung neutrophils (Fig. 1B).

The effects of xanthine oxidase inhibition on the activation of MEK1/2 and ERK1/ERK2 in lung neutrophils after hemorrhage or endotoxemia

We previously found that inhibition of xanthine oxidase prevented hemorrhage, but not endotoxin-induced increases in TNF-α, MIP-2, and IL-1β mRNA levels among lung neutrophils (14). To assess the effects of inhibition of xanthine oxidase on MEK1/2 and ERK1/ERK2 activation after hemorrhage or endotoxemia, mice were fed an allopurinol-enriched diet, and then hemorrhaged or given endotoxin. As shown in Fig. 2A, hemorrhage-induced activation of MEK1/2 and ERK2 was further increased in lung neutrophils from mice fed an allopurinol-enriched diet compared with those given control diets.

To confirm the enhancing effects of xanthine oxidase blockade on hemorrhage-induced ERK activation, cell extracts from lung neutrophils were immunoprecipitated and their ability to phosphorylate Elk-1, the immediate downstream ERK1/2 substrate, was directly determined. These experiments also demonstrated that ERK1/2 activity was further increased after hemorrhage in lung neutrophils from mice fed an allopurinol-containing diet when compared with mice fed a control diet (Fig. 2B).

To verify that xanthine oxidase blockade, rather than nonspecific effects of allopurinol feeding, was responsible for the increase in MEK1/2 and ERK2 activation found in lung neutrophils after hemorrhage, tungsten feeding (11, 34, 35) was used as an alternate method to inhibit xanthine oxidase. As was the case with allopurinol feeding, increased phosphorylated MEK1/2 and ERK2, but not phosphorylated ERK1, was present after hemorrhage in lung neutrophils from tungsten-fed mice, as compared with animals given a control diet (Fig. 2C).

In contrast to the increased activation of MEK1/2 and ERK2 associated with xanthine oxidase blockade in lung neutrophils from hemorrhaged animals, no changes in the levels of phosphorylated MEK1/2 or ERK2 were found in allopurinol-fed mice after endotoxin administration (Fig. 3). Allopurinol feeding had no effects on ERK1 activation after either hemorrhage or endotoxemia.

Effects of α- and β-adrenergic blockade on the activation of MEK1/2 and ERK1/ERK2 in lung neutrophils after hemorrhage or endotoxemia

Catecholamine concentrations in the pulmonary and systemic circulation are increased after either hemorrhage or endotoxemia (15, 16, 40). In previous studies, we demonstrated that modulation of adrenergic stimuli could affect hemorrhage or endotoxin associated increases in cytokine expression among lung cell populations (15, 16). To assess the effects of modulation of adrenergic stimuli on MEK1/2 and ERK1/ERK2 activation in lung neutrophils after...
hemorrhage or endotoxemia, mice were given either the β-adrenergic antagonist propranolol, the α-adrenergic antagonist phentolamine, or PBS, as a control, before endotoxin administration or hemorrhage.

As shown in Fig. 4A, β-blockade before hemorrhage produced a decrease in ERK2 activation in lung neutrophils compared with PBS-treated, hemorrhaged controls. No alterations in the activation of ERK1 or MEK1/2 were found when β-adrenergic blockade was instituted before hemorrhage. In contrast, lung neutrophils from mice given the α-adrenergic antagonist phentolamine before hemorrhage showed an increase in MEK1/2 and ERK2 activation compared with that found in PBS treated hemorrhaged controls (Fig. 4A). ERK1 activation was also increased when α-adrenergic blockade was instituted before hemorrhage.

To confirm the effects of adrenergic modulation on ERK activation after hemorrhage, we directly determined the ability of ERK1/ERK2 to phosphorylate ELK-1, using immunoprecipitated cell extracts from lung neutrophils of mice given propranolol or phentolamine before hemorrhage. Hemorrhage-induced phosphorylation of ELK-1 by ERK1/ERK2 in lung neutrophils was further increased by α-adrenergic blockade, but decreased by β-adrenergic inhibition (Fig. 4B).

The effects of α- or β-adrenergic blockade on endotoxemia-associated activation of MEK1/2, ERK1, and ERK2 in lung neutrophils were different from those found after hemorrhage. In particular, treatment with propranolol before endotoxin administration did not affect levels of phosphorylated MEK1/2, ERK1, or ERK2 in lung neutrophils (Fig. 4C). In contrast, administration of phentolamine before endotoxemia resulted in a decrease in the activation of MEK1/2 and ERK2 (Fig. 4C). There were no effects of α- or β-adrenergic blockade on ERK1 activation in lung neutrophils after endotoxemia. The inhibitory effects of α-adrenergic blockade on ERK activation were confirmed directly by determining the ability of ERK1/ERK2 to phosphorylate ELK-1, using immunoprecipitated cell extracts from lung neutrophils of mice given phentolamine before endotoxemia (Fig. 4D).

Effects of α- and β-adrenergic blockade on CREB and NF-κB activation in lung neutrophils after hemorrhage or endotoxemia

ERK1/ERK2 has been demonstrated in vitro to be involved in intracellular signaling cascades leading to increases in levels of the transcriptionally active, serine 133-phosphorylated form of CREB (26–28). In contrast, ERK1/ERK2 does not appear to have an important direct role in affecting NF-κB activation. However, the in vivo roles of ERK1/ERK2 in modulating NF-κB or CREB activation have not been fully elucidated.

The above results showed that α- or β-adrenergic blockade can modulate hemorrhage- or endotoxemia-induced increases in ERK2 activity in lung neutrophils. If ERK2 is involved in CREB phosphorylation or NF-κB activation in vivo, then interventions that...
increase or decrease ERK2 activation would also be expected to have parallel effects on nuclear levels of NF-κB or of phosphorylated CREB. To examine this issue, mice were treated with either the β-adrenergic antagonist propranolol, the α-adrenergic antagonist phentolamine, or PBS before hemorrhage or endotoxemia, and then the amounts of NF-κB, transcriptionally inactive unphosphorylated CREB, or of the transcriptionally active serine 133-phosphorylated form of CREB in nuclear extracts were determined.

Propranolol treatment before hemorrhage resulted in decreased levels of serine 133-phosphorylated CREB but increased amounts of NF-κB in nuclear extracts from lung neutrophils compared with PBS-treated, hemorrhaged controls (Fig. 5). In contrast, the amounts of unphosphorylated and serine 133-phosphorylated CREB were increased, whereas those of NF-κB were decreased, in nuclear extracts from lung neutrophils of mice treated with phentolamine before hemorrhage.

Mice given propranolol before endotoxin administration showed neither increase nor decrease in activation of CREB or NF-κB when compared with PBS-treated, endotoxemic controls (Fig. 5). However, compared with control endotoxemic mice, α-adrenergic blockade with phentolamine before endotoxemia decreased levels of both unphosphorylated and serine 133-phosphorylated CREB, whereas those of NF-κB were increased in nuclear extracts from lung neutrophils (Fig. 5). In contrast, even though levels of unphosphorylated CREB were decreased in mice given propranolol before endotoxemia, the amounts of transcriptionally active serine 133-phosphorylated CREB were not different from those present in control mice, treated with endotoxin only.

Discussion
In the present experiments, activation of MEK1/2 and ERK2, but not ERK1, was increased in lung neutrophils after hemorrhage and
endotoxemia. However, these effects of hemorrhage or endotoxemia on MEK1/2 and ERK2 activation appeared to occur through distinct mechanisms. Xanthine oxidase-derived ROI were inhibitory of MEK1/2 and ERK2 activity after blood loss but did not modulate activation of these kinases after endotoxemia. Similarly, endogenous α-adrenergic stimuli inhibited MEK1/2 and ERK2 activation after hemorrhage but increased such activation after endotoxemia. Endogenous β-adrenergic stimulation up-regulated activation of ERK2 after hemorrhage but had no effect on ERK2 activity after endotoxemia.

Although MEK1/2 activation generally paralleled that of ERK2 in these experiments, this was not always the case. Inhibition of β-adrenergic stimulation decreased hemorrhage-induced activation of ERK2 but did not affect that of MEK1/2. Because MEK1/2 is upstream of ERK2, alterations, or the lack thereof, in MEK1/2 activity would be expected to be reflected in similar changes in ERK2 activity. However, kinases other than MEK1/2 can modify ERK2 activity (31, 41), and involvement of such pathways is a likely mechanism for the disparity in MEK 1/2 and ERK2 activation in mice treated with the β-adrenergic inhibitor propranolol before hemorrhage.

In vitro studies showed that ERK1/ERK2 can phosphorylate serine 133 of CREB, thereby producing the transcriptionally active form of CREB (26–28, 42). In the present experiments, we found that interventions that increased or decreased ERK2 activation after hemorrhage or endotoxemia, such as modulation of xanthine oxidase activity or the effects of endogenously released catecholamines, produced parallel changes in the levels of phosphorylated CREB in lung neutrophils. These results suggest that ERK2 has a regulatory role in activating CREB in vivo in lung neutrophils after hemorrhage or endotoxemia.
In the present experiments, xanthine oxidase blockade resulted in further increases in ERK2 activity after hemorrhage, while having no apparent effect after endotoxemia. These results are consistent with those of our previous experiments (14) in which inhibition of xanthine oxidase produced additional increases of CREB activation in lung neutrophils after hemorrhage, but not endotoxemia. Our studies therefore suggest that xanthine oxidase activation, presumably due to the ischemia/reperfusion injury that accompanies hemorrhage, is more important after blood loss than endotoxemia.

In vitro studies have previously demonstrated that xanthine oxidase-derived ROI can activate ERK1/ERK2 (43). Yet, the present experiments, showing that xanthine oxidase inhibition resulted in increased ERK2 activity after hemorrhage, appear to contradict those in vitro findings. In addition to the obvious distinction between the present in vivo studies and the previously performed in vitro experiments, where supraphysiological levels of ROI are present, the apparently disparate results may also reflect the different cell types examined. The in vitro experiments showed that the activating effects of ROI on ERK1/ERK2 were cell type dependent, with ERK1/ERK2 being unaffected by ROI in some cell populations (44). The response of ERK1/ERK2 in neutrophils to ROI has not previously been examined. Additionally, transcriptional regulatory mechanisms may be affected by ROI in a dose-specific manner (45). For example, even though in vitro experiments showed that large amounts of ROI increased degradation of the NF-κB-regulatory protein IκB-α (46), we found that xanthine oxidase inhibited IκB-α expression in the lung in vivo, because IκB-α levels increased when xanthine oxidase was blocked (47). A similar in vivo inhibitory effect of xanthine oxidase on ERK2 activity in lung neutrophils is suggested by the present experiments.

In the present work, β-adrenergic blockade decreased ERK2 and CREB activation after hemorrhage, but not endotoxemia. In previous experiments (15), as well as in these studies, we found that β-adrenergic inhibition increased NF-κB activation in lung cells after hemorrhage. β-Adrenergic stimulation increases intracellular levels of cyclic AMP that subsequently can activate protein kinase A (PKA) (48). Activation of CREB and inhibition of nuclear translocation of NF-κB can occur through PKA-dependent pathways (49, 50). However, although the effects of β-adrenergic stimuli on CREB and NF-κB may involve cAMP-regulated changes in PKA activation, it seems unlikely that PKA is involved in ERK activation.

Crespo et al. (51) found that β-adrenergic stimulation led to increased ERK1/ERK2 activity via a Ras-dependent pathway and that PKA-stimulating agents did not lead to ERK1/ERK2 activation.

We previously demonstrated that α-adrenergic blockade decreased NF-κB activation and proinflammatory cytokines in the lung after hemorrhage but had opposite effects after endotoxemia, where such therapy increased IL-1β, MIP-2, and TNF-α expression in lung neutrophils (15, 16). A similar pattern was seen in the present experiments, where α-adrenergic blockade decreased NF-κB activation in lung neutrophils after hemorrhage but resulted in increased NF-κB activation after endotoxemia. Additionally, we found that α-adrenergic blockade further increased MEK1/2 and ERK2 activity after hemorrhage but decreased such activation after endotoxemia. α-Adrenergic stimulation increases intracellular Ca2+, which has been demonstrated to activate ERK1/ERK2 (52), as well as NF-κB (53). However, the differing effects of α-adrenergic stimuli on MEK1/2, ERK2, CREB, and NF-κB after hemorrhage or endotoxemia imply that each of these pathophysiological insults initiates additional regulatory mechanisms that result in distinct patterns of activation for MEK1/2, ERK2, and transcriptional factors.

In the present experiments, interventions that resulted in increased or decreased amounts of serine 133-phosphorylated CREB
in lung neutrophils had opposite effects on nuclear levels of NF-kB. Although there are reports that ERK1/ERK2 can be indirectly involved in inducing nuclear translocation of NF-kB (54), there is no evidence to suggest that ERK1/ERK2 can inhibit NF-kB activation. A possible explanation for the inverse relationship between ERK2 activity and NF-kB activation involves enhanced turnover of NF-kB that is free in the cell and not bound to either CBP or IxB-like molecules. Because serine 133-phosphorylated CREB can displace NF-kB from the KIX-binding site of CBP (17), increases in phosphorylated CREB due to ERK2 activation may result in diminished nuclear levels of NF-kB as freed, unbound NF-kB is degraded (55). Under these conditions, activation of ERK2 does not need to have a direct effect on NF-kB but rather may affect alterations in NF-kB levels indirectly through increasing amounts of serine 133-phosphorylated CREB.

Our findings may have important implications for the care and treatment of patients with acute lung injury and ARDS. At present, acute lung injury and ARDS are considered to be a single entity, diagnosed by a constellation of clinical findings, including an abnormal chest radiograph, consistent with noncardiogenic pulmonary edema, and hypoxemia (56). Yet, the present experiments, as well as other recent data (14), demonstrate that hemorrhage or endotoxemia activate distinct intracellular signaling pathways in lung neutrophils that play a central role in the pathophysiologic process of acute lung injury. In particular, our studies show that ROI and catecholamines have different modulatory effects on transcriptional regulatory mechanisms involving MEK1/2, ERK2, NF-kB, CREB, and proinflammatory cytokine expression after hemorrhage or endotoxemia. The present results therefore suggest that not only the initial inciting events but also subsequent intracellular signaling leading to the development of acute lung injury are distinct after hemorrhage or endotoxemia. Similarily, these findings indicate that different therapies may be necessary to correct dysregulated proinflammatory responses leading to acute lung injury after hemorrhage or endotoxemia.

In the clinical setting, it is often difficult to distinguish a single etiology that leads to the development of acute lung injury. For example, patients who initially suffered blood loss may subsequently become infected, and the relative contributions of hemorrhage or endotoxemia to the pathogenesis of acute lung injury are unclear. The present results would suggest that patterns of MEK1/2, ERK2, CREB, and NF-kB activation could serve to distinguish between groups with distinct intracellular mechanisms producing acute lung injury. Although differences in transcriptional regulatory mechanisms were found at early time points in these experimental models of acute lung injury, additional work is necessary to establish their relevance in the clinical setting, where patients are usually seen at later stages in their clinical course.

References


