Alveolar macrophage activation after trauma-hemorrhage and sepsis is dependent on NF-κB and MAPK/ERK mechanisms

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Jarrar, Doraid, Joachim F. Kuebler, Loring W. Rue, III, Sadis Matalon, Ping Wang, Kirby I. Bland, and Irshad H. Chaudry. Alveolar macrophage activation after trauma-hemorrhage and sepsis is dependent on NF-κB and MAPK/ERK mechanisms. Am J Physiol Lung Cell Mol Physiol 283: L799–L805, 2002; 10.1152/ajplung.00465.2001.—The acute respiratory distress syndrome (ARDS) is a major cause of morbidity after injury. We hypothesized that alveolar macrophage (AMΦ) chemokine and cytokine release after hemorrhage and sepsis is regulated by NF-κB and MAPK. Adult male rats underwent soft tissue trauma and hemorrhagic shock (~90 min) followed by crystalloid resuscitation. Sepsis was induced by cecal ligation and puncture (CLP) 20 h after resuscitation. AMΦ were harvested, and TNF-α, IL-6, and macrophage inflammatory protein (MIP)-2 release and serum IL-6 and TNF-α levels were measured at 5 h after HCLP. Lung tissues were analyzed for activation of NF-κB, myeloperoxidase activity, and wet/dry weight ratio. In control animals, AMΦ were stimulated with LPS with or without inhibitors of NF-κB and MAPK. Serum TNF-α and IL-6 levels and spontaneous AMΦ TNF-α and MIP-2 release were elevated (P < 0.05) after HCLP, concomitantly with the development of lung edema and leukocyte activation. Activation of NF-κB increased in lungs from the hemorrhage and CLP group compared with shams. Inhibition of NF-κB or the upstream MAPK significantly decreased LPS-stimulated AMΦ activation. Because enhanced release of inflammatory mediators by AMΦ may contribute to ARDS after severe trauma, inhibition of intracellular signaling pathways represents a target to attenuate organ injury under those conditions.

mitogen-activated protein kinase; leukocytes; macrophage inflammatory protein; acute respiratory distress syndrome; extracellular signal-regulated kinase; nuclear factor-κB

TRAUMATIC INJURIES and the ensuing sepsis and septic shock are the leading causes of death in the ages 1–44 years in the United States (3, 18). Recent studies by Heckbert et al. (15) have shown that 39% of the trauma patients with a documented episode of hypotension during or shortly after the incident develop infectious complications. Under those conditions, the respiratory system is the most frequently affected organ system, and lung dysfunction is the first step in the development of multiple organ failure (4). Thus the acute respiratory distress syndrome (ARDS) is a major cause of death in surgical intensive care units with an incidence of ~10–14 cases per 100,000 people and an associated mortality rate of 36–52% (35). Although the exact sequence of events leading to the clinical picture of ARDS remains unknown, experimental and clinical studies have suggested that the migration of polymorphonuclear granulocytes (PMN) into the lung tissue plays a key role in the cascade of events leading to ARDS (21, 28, 37).

Studies have suggested that macrophage-derived chemokines, such as the macrophage inflammatory protein-2 (MIP-2), play an important role in mediating PMN influx into the lung interstitium (20, 32, 36). In addition to the extensive influx of monocytes (17), activation of nuclear transcriptional regulatory factors such as nuclear factor-κB (NF-κB) in alveolar macrophages (AMΦ), which has been reported in patients suffering from ARDS (22, 24, 33), is believed to be involved in the activation of the local immune reaction (1, 42). Studies in different experimental animal models have shown that systemic stressors, such as endotoxemia, hemorrhagic shock, or sepsis per se, can modulate the in vivo activity of AMΦ and their reactivity to a subsequent in vitro stimulation (9, 12, 39). However, in the usual clinical situation, the patients who are most susceptible to multiple organ failure are the ones who encounter several sequential insults after the initial injury (4, 37).

In light of these findings, the aim of our study was to examine the inflammatory response of the lung parenchyma as well as macrophage activity in a “two-hit” rat model of sequential injuries. We hypothesized that after soft tissue trauma, severe hemorrhagic shock, and subsequent induction of polymicrobial sepsis, characteristic signs of tissue damage and inflammation, such as edema formation, neutrophil accumulation, and activation of NF-κB in lung tissues are detectable, and, if so, we wondered whether there is any correlation in the activation of AMΦ as characterized...
by their increased chemokine and cytokine release. In a second set of experiments, we investigated whether the activation of AM, mimicked by in vitro LPS stimulation, could be abolished by inhibitors of NF-kB and MAPKs.

MATERIALS AND METHODS

**Experimental procedures.** The previously described nonheparinized model of trauma and hemorrhage in the rat (19, 40) was used with minor modifications. Briefly, male Sprague-Dawley rats (250–300 g; Charles River Labs, Wilmington, MA) fasted overnight before the experiments but were allowed water ad libitum. The animals were anesthetized by methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL) inhalation, and a 5-cm midline incision was performed to induce soft tissue trauma. After this, the abdomen was closed in layers, and the wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) throughout the surgical procedure to reduce postoperative pain. The catheters were then placed in both femoral arteries and the right femoral vein (polyethylene (PE-50) tubing; Becton Dickinson, Sparks, MD). Upon awakening, the rats were bled to and maintained at a mean arterial pressure (MAP) of 40 mmHg until the animals could not maintain a MAP of 40 mmHg unless extra fluid in the form of Ringer lactate was administered. This time was termed as maximum bleed-out, and the amount of withdrawn blood was noted. The animals were then maintained at MAP of 40 mmHg until 40% of the maximum bleed-out volume was returned in the form of Ringer lactate. The rats were then resuscitated with four times the volume of the withdrawn blood in the form of Ringer lactate over 60 min, and shed blood was not used for resuscitation. The catheters were then removed, the vessels were ligated, and the skin incisions were closed with sutures. Sham-operated animals underwent the same groin dissection, which included the ligation of both femoral arteries and the right vein; however, neither hemorrhage nor resuscitation was carried out.

After returning to the cages, the rats were allowed food and water ad libitum. At 20 h after the completion of fluid resuscitation or sham operation, the animals were again anesthetized with methoxyflurane, and polymicrobial sepsis was induced by cecal ligation and puncture (CLP) as described by Chaudry et al. (8). Briefly, after a 2-cm midline incision was made, the cecum was exposed, ligated proximal to the ileocecal valve, punctured twice with an 18-gauge needle, and returned to the abdominal cavity. The abdominal cavity was then closed in layers, and the rats were resuscitated with saline solution subcutaneously (3 ml/100 g body wt). Our previous studies have shown that blood cultures are positive for Escherichia coli, Streptococcus bovis, Proteus mirabilis, Enterococcus faecalis, and Bacteroides fragilis within 1 h after CLP (41). Sham-operated animals underwent the same surgical procedure except that the cecum was neither ligated nor punctured.

**Isolation of AM.** At 5 h after CLP, AM were collected from the bronchopulmonary lavage fluid as described by Leeper-Woodford et al. (23). The animals were anesthetized with pentobarbital intraperitoneally and exsanguinated. The lungs were lavaged with a total of 50 ml of phosphate-buffered saline (Organon). The cell fractions were then washed, counted, and suspended (10^6 cells/ml) in Dulbecco’s modified Eagle’s medium (DMEM) in 24-well plates. After 2 h of incubation (37°C at 5% CO2), nonadherent cells were removed, and 1 ml of fresh DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin was added to the adhered AM. After an incubation period of 4 h, the supernatants were harvested and frozen at −70°C until assayed. In additional groups of control animals, isolated AM were stimulated with LPS (10 μg/ml; Sigma, St. Louis, MO) in the presence of either the MAPK inhibitors SB-203580 and PD-98059 (10 μmol/l) or 200 μmol/l of pyrrolidinedithiocarbamate (PDTC, an inhibitor of NF-κB translocation; all purchased from Sigma).

**Measurement of proinflammatory cytokines and chemokines.** The levels of TNF-α, IL-6, and MIP-2 in the supernatants, TNF-α, and IL-6 and in the serum were determined by an ELISA (PharMingen, Biosource) according to the manufacturer’s instructions.

**Myeloperoxidase activity.** Myeloperoxidase (MPO) activity in the lungs was determined as described by Lukaszewicz et al. (28). All reagents were purchased from Sigma. Briefly, lungs were excised, rinsed with saline, and frozen in liquid nitrogen at −70°C until assayed. Frozen lung samples were thawed and homogenized in 10 volumes of 20 mmol/l potassium phosphate, pH 7.4, for 30 s. The samples were then centrifuged at 14,000 rpm for 30 min at 4°C. The pellets were resuspended in 10 volumes of 50 mmol/l potassium phosphate, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide. Samples were kept on ice and sonicated with a probe sonicator at two-thirds the maximum setting for ~40 s and centrifuged at 14,000 rpm at 4°C for 10 min. Supernatants were then added to a 96-well plate at 5 μl/well and 196 μl of reaction buffer containing 530 mmol/l o-dianisidine and 150 mmol/l H2O2 in (added immediately before use) 50 mmol/l potassium phosphate, pH 6.0. Light absorbances at 490 and 620 nm (reference wavelength) were read and compared with those obtained in wells containing a known activity of MPO standard purified from human leukocytes (Sigma, activity as declared on batch) (28). Protein content in the samples was determined by the Bradford assay (Bio-Rad).

**EMSA.** Active NF-κB isoforms in nuclear extracts of whole lungs were detected by EMSA. The NF-κB oligonucleotide containing the consensus sequence gactgaggggacctctcctc (Stratagene, La Jolla, CA) was end labeled by incubation of the oligonucleotide with [γ-32P]ATP (≥6,000 Ci/mmole; New England Nuclear) and T4 polynucleotide kinase according to the manufacturer’s instructions (Stratagene). Purification of the labeled oligonucleotide was carried out with a NucTrap probe purification columns (Stratagene). Each 25-μl assay contained 20 ng of nuclear extracts, ~20,000 counts/min (cpm) of radiolabeled double-stranded target oligonucleotide, distilled H2O, and incubation buffer (Stratagene). After a 30-min incubation period on ice, 2 μl of 0.1% (w/vol in water) bromphenol blue dye were added to the reaction, and each of the samples was loaded onto a 6% DNA retardation gel (Novex, Carlsbad, CA). The gels were run at 20–25 mA for ~40 min at 4°C in a Tris borate-EDTA buffer. After electrophoresis, the band intensities were quantified using a phosphorimager (Packard Instruments, Meriden, CT) to analyze each lane. After this, gels were dried and exposed for autoradiography.

For supershift assays, antibodies to the NF-κB isoforms p50 and p65 were added to the binding reaction mixture and incubated at room temperature for 30 min subsequent to the incubation of the labeled oligonucleotide probe with the nuclear extracts. The antibodies used were specific to the iso-
form indicated and cross-reactive with rat protein. After incubation, the reaction mixture was electrophoresed as described above.

**Determination of lung water content.** In a separate cohort, animals were exsanguinated at the end of the experiment; the lungs were excised, weighed, and then dried for 24 h at 95°C. Lung water content (%) was calculated as (wet wt – dry wt)/(wet wt) × 100.

**Histology of lung tissues.** The alterations in lung morphology were examined in a separate group of sham-operated animals and in animals after trauma-hemorrhage and induction of subsequent sepsis (HCLP). Lung tissues were harvested and fixed in 10% neutral buffered formalin (Sigma) and later embedded in paraffin. The tissues were then sectioned at a thickness of 5 μm and stained with hematoxylin and eosin, and slides were evaluated by light microscopy and documented by photographs. Several sections from each lung from various lobes were examined, and all were consistent with the presence of significant lung injury.

**Statistical analysis.** The results are presented as means ± SE. One-way ANOVA and Student-Newman-Keuls test for multiple comparisons were used, and the differences were considered significant at a P value ≤0.05.

**RESULTS**

**Serum levels of proinflammatory cytokine.** The results in Fig. 1A indicate that after HCLP, serum levels of IL-6 were found to be 1,228 ± 317 pg/ml. Serum levels of IL-6 were undetectable in sham-operated animals, whereas serum levels of TNF-α were 15 ± 6 pg/ml, and they increased by 700% (P < 0.05) after HCLP (Fig. 1B).

**AMΦ cytokine and MIP-2 release.** As shown in Fig. 2A, spontaneous TNF-α secretion in cultured AMΦ from sham-operated animals was 369 ± 120 pg/ml, and the secretion was significantly higher in AMΦ from HCLP animals (by 186%; P < 0.05). Unstimulated MIP-2 secretion in AMΦ from sham-operated animals was found to be 1,113 ± 246 pg/ml, and the secretion increased by 190% after HCLP (Fig. 2B; P < 0.05). Spontaneous, unstimulated IL-6 release was undetectable in both groups.

**Lung MPO activity and lung water content.** MPO activity in lungs harvested from sham-operated animals was found to be 0.17 ± 0.06 U/mg protein, and this increased by 348% (P < 0.05) after HCLP (Fig. 3A). Water content in control animals was 77.3 ± 1.1%, and it increased to 79.7 ± 0.2% after HCLP (P < 0.05; Fig. 3B).

**Histological alterations after HCLP.** Representative histological lung biopsy findings in control and experimental animals are shown in Fig. 4. Figure 4A represents the normal lung architecture observed in sham controls. Figure 4B represents the histological findings in the lungs of rats subjected to trauma-hemorrhage and CLP. Diffuse alveolitis is observed with intense neutrophil accumulation, infiltrates, and widening of the alveolar septa by the inflammatory process.

**Activation of NF-κB in lung tissue.** As shown in Fig. 5, the activity of NF-κB in nuclear extracts of whole lungs was significantly increased in HCLP animals.
compared with sham controls (P < 0.05). Supershift assays demonstrated that NF-κB complexes contain p65 and p50 subunits. Figure 5A shows representative blots of EMSA, whereas Fig. 5B shows cpm from three animals in each group. The subsequent supershift analysis is shown in Fig. 5C.

Effects of NF-κB and MAPK inhibition on TNF-α release by AMΦ. In additional experiments, AMΦ were isolated from sham-operated animals and stimulated in vitro with LPS (10 μg/ml). Such AMΦ (10⁶ cells/ml) released 2,120 ± 120 pg/ml of TNF-α (Fig. 6). The addition of the specific MAPK inhibitor SB-203580/PD-98059 and PDTC decreased TNF-α production by 73.3 and 71.9%, respectively (P < 0.05).

DISCUSSION

Previous studies from our laboratory have shown that although rodents in the early stage of sepsis without undergoing prior trauma-hemorrhage show the characteristic signs of a systemic inflammatory response, activation of the AMΦ population is not detectable in the early stage of sepsis (2, 10). Our present results indicate that serum levels of the proinflammatory cytokines IL-6 and TNF-α were significantly elevated at 5 h after trauma-hemorrhage and CLP. Moreover, neutrophil activation as assessed by MPO activity in whole lung tissue, as well as lung water content, was significantly increased in this two-hit injury model compared with sham-operated animals. The accumulation of neutrophils was associated with the activation of NF-κB transcriptional activity in lung tissues as assessed by its binding to the consensus sequence. The activated NF-κB complex was supershifted with p65 antibodies, indicating that the complex was composed of both p50 and p65. These findings are in keeping with the fact that the p50/p65 heterodimer is responsible for the transcriptional activity (38), i.e., the inflammatory response, whereas p50/p50 homodimers have been associated with inhibition of stress gene transcription (6). These findings, indicating a local inflammatory response in the lungs, were associated with the histological alterations in trauma-hemorrhage and septic animals, which included edema formation, intra-alveolar hemorrhage, and PMN accu-
mulation. Moreover, the release of TNF-α and MIP-2 in cultured AMΦ in the absence of any stimuli (e.g., LPS) was markedly increased 5 h after the induction of sepsis. Together, these results demonstrate that after a combination of trauma, hemorrhagic shock, and a subsequent sepsis, the local inflammatory response of the lung tissue is associated with an increased release of cytokines in the AMΦ.

Nwariaku et al. (34) reported a depression of the AMΦ activity for 5 days after hemorrhagic shock with a reduced release of TNF-α after the in vitro stimulation with LPS. In our two-hit model, the amount of spontaneous TNF-α release by isolated macrophages as well as in the bronchoalveolar lavage (BAL) fluid was significantly elevated after HCLP, indicating a different response of the AMΦ to in vitro stimulation with LPS compared with the in vivo situation of sepsis. The increase in MIP-2 levels in the BAL fluid and in the supernatant of cultured, isolated AMΦ associated with the increased PMN sequestration into the lung tissue suggests that activation of the local monocyte population contributes to the sequestration of PMNs and the sustained inflammatory reaction within the lung tissue under those conditions. However, in the present study, measurement of arterial blood gases was not performed, and thus it remains unknown whether pulmonary functional impairment occurred in our double-hit model of trauma-hemorrhage and subsequent sepsis.

To further examine the cellular mechanisms involved in the inflammatory activation of AMΦ, we assessed the effects of inhibitors of NF-κB activation (PDTC) and MAPK (SB-203580 and PD-98059) on the release of TNF-α and MIP-2 in isolated AMΦ stimulated with LPS. The combined inhibition of P38 and P42/44 was used, since our preliminary studies indicated that maximal suppression of cytokine release was obtained by the combination of both antagonists.

**Fig. 5.** Effects of HCLP on the activation of whole lung tissue NF-κB DNA binding. As shown in A and B, after injury NF-κB activation was significantly increased compared with control animals. Moreover, supershift analysis using antibodies specific for the subunits p50 and p65 demonstrates that the NF-κB complex is predominantly composed of p65 (C). cpm, Counts/min; oligo, oligonucleotide. *P < 0.05 vs. sham.

**Fig. 6.** In additional experiments, AMΦ from control animals were harvested and stimulated with LPS. The addition of pyrrolidinedithiocarbamate (PDTC, blockade of NF-κB translocation) or the MAPK inhibitors SB-203580 (SB) and PD-98059 (PD) decreased TNF-α secretion significantly. *P < 0.05 vs. LPS.

**Fig. 7.** AMΦ from control animals were harvested and stimulated with LPS. PDTC or the MAPK inhibitors SB-203580 and PD-98059 markedly decreased LPS-stimulation MIP-2 secretion. *P < 0.05 vs. LPS.
These observations are supported by studies of Carter et al. (7), who have shown that the release of cytokines by AMϕ is regulated by both kinases. Our results indicate that inhibition of NF-κB translocation (PDTC) decreased TNF-α and MIP-2 release more than four-fold, although it has to be mentioned that PDTC, despite being repeatedly used as an NF-κB antagonist, has other effects that could contribute to the observed results, such as being a strong antioxidant (31). Similarly, inhibition of the upstream MAPK P38 and P42/44 resulted in a comparable decrease in cytokine and chemokine release. Thus these signal-transducing kinases appear to be upstream regulators of the proinflammatory cytokine release by AMϕ.

Perpetuation of the systemic inflammatory response syndrome is thought to be a major contributor to the ARDS (11, 12). Once ARDS is manifested, it appears that a vicious cycle of increased Fio2 demands and positive end-expiratory pressure to sustained acceptable peripheral oxygen delivery produces further lung damage (16). Understanding the inflammatory milieu might help to interrupt this cascade of events and improve outcome once a diagnosis of ARDS is made. Lentsch et al. (24) have recently shown that AMϕ play an essential role in recruiting neutrophils and perpetuating lung injury. Moreover, they confirmed that among other cytokines and chemokines, TNF-α plays a central role in recruiting PMN to the lung tissues. By depleting rat lungs of AMϕ, they were able to show that in whole lung tissue NF-κB was suppressed in a model of immunoglobulin G-induced lung injury. Furthermore, NF-κB activation was restored after instillation of TNF-α into the lungs (24). Studies by Lindsey et al. (25, 26) have shown that after pulmonary atelectasis in rats, cytokine production by isolated AMϕ was significantly increased. In contrast, polymicrobial sepsis per se in the absence of prior trauma-hemorrhage did not activate AMϕ to release inflammatory mediators (2). Moreover, Fan et al. (12) have demonstrated that hemorrhage per se does not activate AMϕ; however, this primes this cell population for an exaggerated response to a subsequent stimulus. In light of these findings, our data suggest that priming by antecedent shock might be essential for an increased release of inflammatory mediators after subsequent stimuli, such as sepsis. This suggestion appears important, since even minor direct lung injuries that are commonly observed in critically ill patients, such as 1-h pulmonary atelectasis, can trigger significant activation of the lung immune cells (25, 26). The release of proinflammatory cytokines by AMϕ appears to involve the activation of the signal-transducing MAPK system and to be transcriptionally regulated by the activation of NF-κB. Recent studies have indicated that inhibition of those factors can reduce the inflammatory response and reduce lung injury in rats after LPS infusion or intratracheal allergen administration (13, 14, 27, 29, 43). The second part of our studies using isolated AMϕ from control animals and inhibition of P38 and p44/42 as well as NF-κB, resulting in a depression of cytokine and chemokine release, suggests that both pathways are crucial for AMϕ activation. Indeed, several investigators have shown that MAPK act as upstream activators of NF-κB (5). However, Means et al. (30) have shown that distinct MAPK pathways are utilized in different macrophage populations. It therefore remains to be determined whether this mechanism of activation is specific for AMϕ without affecting other cell populations such as peritoneal macrophages.

In summary, our results support the hypothesis that, due to their release of MIP-2 and TNF-α, AMϕ play a key role in PMN recruitment in the lungs after multiple injuries, such as HCLP and subsequent sepsis. Because our data demonstrate that the production of MIP-2 and TNF-α is dependent on NF-κB and upstream kinases, inhibitions of these pathways by the administration of specific inhibitors could be a novel approach in the treatment or the prevention of ARDS in the critically ill patient.

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