Activation of gene expression in human neutrophils by high mobility group box 1 protein

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Activation of gene expression in human neutrophils by high mobility group box 1 protein. Am J Physiol Cell Physiol 284: C870–C879, 2003; 10.1152/ajpcell.00322.2002.—High mobility group box 1 (HMGB1) protein, a DNA binding protein that stabilizes nucleosomes and facilitates transcription, was recently identified as a late mediator of endotoxin lethality. High serum HMGB1 levels in patients with sepsis are associated with increased mortality, and administration of HMGB1 produces acute inflammation in animal models of lung injury and endotoxia. Neutrophils occupy a critical role in mediating the development of endotoxemia-associated acute lung injury, but previously it was not known whether HMGB1 could influence neutrophil activation. In the present experiments, we demonstrate that HMGB1 increases the nuclear translocation of NF-κB and enhances the expression of proinflammatory cytokines in human neutrophils. These proinflammatory effects of HMGB1 in neutrophils appear to involve the p38 MAPK, phosphatidylinositol 3-kinase/Akt, and ERK1/2 pathways. The mechanisms of HMGB1-induced neutrophil activation are distinct from endotoxin-induced signals, because HMGB1 leads to a different profile of gene expression, pattern of cytokine expression, and kinetics of p38 activation compared with LPS. These findings indicate that HMGB1 is an effective stimulus of neutrophil activation that can contribute to development of a proinflammatory phenotype in diseases characterized by excessively high levels of HMGB1.

p38 mitogen-activated protein kinase; phosphatidylinositol 3-kinase; Akt; extracellular signal regulated kinase 1/2; nuclear factor-κB; inflammation

ACTIVATED NEUTROPHILS produce cytokines, chemokines, and other proinflammatory mediators that participate in the development of acute inflammation. For instance, neutrophils are activated during endotoxin-induced shock, sepsis, and acute lung injury (2, 4, 27, 48). Bacterial products and cytokines can initiate acute inflammatory responses in neutrophils, and these pathways have been implicated in the development of tissue injury (2). Recent data implicated high mobility group box (HMGB) chromosomal proteins as cytokine-like mediators of delayed endotoxin lethality and acute lung injury (1, 3, 41).

There are three HMGB chromosomal proteins: HMGB1 (previously HMG1), HMGB2 (previously HMG2), and HMGB3 (previously HMG4 or HMG2). HMGBs are composed of three different domains, including homologous DNA binding boxes A and B and the COOH-terminal domain (6). HMGB1 appears to have two distinct functions in cellular systems. First, it has been shown to have an intracellular role as a regulator of transcription (7) and, second, an extracellular role in which it promotes tumor metastasis (37) and inflammation (41). Monocytes/macrophages stimulated by lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, or interleukin (IL)-1 secrete HMGB1 (41). Addition of HMGB1 to monocytes in culture induces the release of TNF-α, IL-1α, IL-1β, IL-1Ra, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, and MIP-1β, but not IL-10 or IL-12 (3). Intratracheal administration of HMGB1 produces acute lung injury, and antibodies against HMGB1 decrease LPS-induced lung edema and neutrophil accumulation (1). Anti-HMGB1 antibodies did not significantly reduce the levels of the proinflammatory cytokines TNF-α, IL-1β, or MIP-2 in LPS-induced acute lung injury, suggesting that HMGB1 occupies a more distal position in endotoxin-induced proinflammatory cascades (41). High serum HMGB1 levels accumulate in patients with sepsis, and significantly higher levels were found in lethal cases compared with survivors (41).

Although HMGB1 has been shown to activate macrophages and other cell populations, the signaling pathways involved have not been previously investigated. Here we establish that HMGB1 is an effective stimulus to neutrophil activation, primarily through the p38 MAPK pathway, leading to nuclear translocation of NF-κB and enhanced expression of proinflammatory cytokines.

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MATERIALS AND METHODS

Reagents. RPMI 1640 was obtained from Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gemini Bioproducts (Calabasas, CA). LPS (from Escherichia coli O111:B4) was obtained from Sigma Chemical (St. Louis, MO). Poly(di-dc)\cdot poly(dl-dc) and Percoll were purchased from Amer sham-Pharmacia (Piscataway, NJ). The Coomassie-Plus protein assay reagent and BCA protein assay reagent were purchased from Pierce (Rockford, IL). SB-202190, U0126, and LY-294002 were purchased from Calbiochem (La Jolla, CA). Antibodies for phospho (Ser276)-Akt, total Akt, phospho (Thr202/Tyr204)-p44/42, and total p44/42 MAPK were purchased from Cell Signaling Technology (Beverly, MA). Recombinant HMGB1 (rHMGB1) was prepared as described previously (3, 41).

Isolation and culture of human neutrophils. Peripheral blood was obtained from healthy volunteers under a protocol approved by the University of Colorado Health Sciences Center Institutional Review Board. Neutrophils (purity >99%) were isolated by plasma-Percoll gradients after dextran sedimentation of erythrocytes (14). Neutrophils were resuspended in RPMI 1640 at a final concentration of 5 x 10^6 cells/ml and stimulated with 0-1,000 ng/ml rHMGB1 or 100 ng/ml LPS. The p38 MAPK inhibitor SB-202190 (30 μM), the MEK1/2 inhibitor U0126 (10 μM), or the phosphatidylinositol 3-kinase (PI 3-K) inhibitor LY-294002 (100 μM) was added to the neutrophil cultures for 1 h before HMGB1 or LPS stimulation. Neutrophil viability as assessed by trypan blue staining was consistently >95% after 1 h in control or LPS-stimulated cultures as well as under conditions where LY-294002 or U0126 was added to the cells for 1 h. The concentrations of kinase inhibitors have previously been used by ourselves and others (8, 32, 33, 50, 51). Specificity of these concentrations of inhibitors was verified by demonstrating that LY-294002 did not affect LPS-induced activation of SAPK/JNK or MEK1/2 and that U0126 did not alter LPS-associated SAPK/JNK activation in neutrophils. Cultures were supplemented with polymyxin B (10 μg/ml) to block any effects of contaminating endotoxin.

Gene array analysis. RNA was isolated from neutrophils using an RNeasy kit according to the manufacturer’s protocol (Qiagen, Valencia, CA). Total RNA (20 μg) was converted to double-stranded cDNA (ds-cDNA) using the Superscript Choice System (Life Technologies). An oligo-dT primer containing a T7 RNA polymerase promoter (Genset, Kents Store, VA) was utilized. After second-strand synthesis, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol, and the ds-cDNA was recovered by ethanol precipitation. In vitro transcription was performed to generate biotin-labeled cRNA using a RNA transcript labeling kit (Enzo, Farmingdale, NY), and 3.3 μl of ds-cDNA template were transcribed in the presence of a mixture of biotin-labeled ribonucleotides. Biotin-labeled cRNA was purified using an RNeasy affinity column. To ensure optimal hybridization to the oligonucleotide array, we performed fragmentation of cRNA such that the fragments were between 35 and 200 bases in length by incubating the cRNA at 94°C for 35 min in fragmentation buffer. The sample was then added to a hybridization solution containing 100 mM MES, 1 M NaCl, and 20 mM EDTA in the presence of 0.01% Tween 20. The final concentration of the fragmented cRNA was 0.05 μg/μl. Hybridization was performed by incubating 200 μl of the sample. The sample was loaded on a test chip to determine the quality of mRNA by using known housekeeping genes. After passing the test chip, samples were loaded on Affymetrix GeneChip Human Genome U95Av2 (12,626 genes) chips (Affymetrix, Santa Clara, CA). Hybridization occurred at 45°C for 16 h using GeneChip Hybridization Oven 640 (Affymetrix). After hybridization, the hybridization solutions were removed, and the arrays were washed and stained with streptavidin-phycocerythrin using a GeneChipFluorides Staining Protocol 400 (Affymetrix). Arrays were read at a resolution of 6 μm using an HP Gene Array Scanner (Affymetrix). Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described previously (13, 25).

Real-time quantitative RT-PCR. Total cellular RNA was isolated from human neutrophils using Trizol reagent (Life Technologies), as recommended by the manufacturer. Real-time RT-PCR was performed with specific primers and probes corresponding to the proinflammatory cytokine genes IL-1β, IL-8, and TNF-α. For each mRNA detection, a fluorogenic probe and two primers for PCR (forward and reverse) were synthesized (Perkin-Elmer Life Sciences). An internal oligonucleotide probe was labeled with the fluorescent dyes carboxyfluorescein (FAM) at the 5′-end and N,N′N′N′-tetramethyl-6-carboxyhydroamine (Tamra) at the 3′-end. For human IL-1β mRNA detection, the forward and reverse primers were 5′-ACGACTGCAGCTGACGGTACGTC313-3′ and 5′-TCTGTTGTTTTTCTGGTC3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′- (FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively. The internal probe was 5′-(FAM)-AGCTGCAGCTGACGGTACGTC313-3′, respectively.
amplified by RT-PCR using the following primers: sense, 5'-GGGAGGCAGGACTTACAC-3'; and antisense, 5'-TCCTCGGAAATCTCGGAC-3'. A 350-bp of human Bcl-xL cDNA fragment was amplified by RT-PCR using the following primers: sense, 5'-GCTCGGATCCCTCGGAC-3'; and antisense, 5'-CCTAGAAGCATTGCCTGACG-3', resulting in a 285-bp band.

Single-strand cDNA synthesis and PCR were carried out using the manufacturer’s protocol (Invitrogen) with SuperScript one-step RT-PCR kit. Thirty-five cycles of PCR amplification for Bcl-xL, monoamine oxidase B, and β-actin were performed as follows: denaturation at 95°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 1 min. The final extension was carried out for 8 min. The PCR products were characterized using 1% agarose gel electrophoresis.

Electrophoretic mobility shift assays. To obtain nuclear extracts from neutrophils, we suspended cells in lysis buffer containing 10 mM Tris·HCl (pH 7.5), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1 mM Na3VO4, and 0.1% Triton X-100, and the samples were incubated on ice for 20 min. After cytoplasm was removed from the nuclei by 15 passages through a 25-gauge needle, the nuclei were collected by centrifugation at 5,000 g for 10 min at 4°C. The pellets were suspended in extraction buffer containing 20 mM Tris·HCl (pH 7.5), 420 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 25% glycerol, 0.5 mM EDTA, 0.5 mM PMSF, and 0.1 mM Na3VO4. After a 30-min incubation on ice, the suspension was centrifuged at 14,000 g for 20 min at 4°C and the supernatants were collected. The protein concentration in the supernatants was determined using Coomassie Plus protein assay reagent (Pierce). Nuclear extracts (5 μg) were incubated at room temperature for 15 min in 20 μl of reaction buffer containing 10 mM Tris·HCl (pH 7.5) and 100 ng/ml LPS, heat-inactivated HMGB1 protein (1 μg/ml), or LPS (100 ng/ml). Levels of monoamine oxidase B and Bcl-xL mRNA were detected by RT-PCR. Heat inactivation of HMGB1 protein was performed at 95°C for 30 min. The results shown are representative of 3 experiments.

Fig. 1. A: hierarchical cluster images showing gene expression patterns among the 12,626 human genes examined (Affymetrix GeneChip Human Genome U95Av2 array) after neutrophil (5 × 10⁶/ml) stimulation with high mobility group box 1 (HMGB1) protein (1 μg/ml) or LPS (100 ng/ml) for 1 h. Genes that showed >2-fold changes in control vs. HMGB1 or control vs. LPS were subjected to clustering analysis (Gene Spring 4.07 program; Silicon Genetics, Redwood City, CA) The fold changes were based on comparison with internal standards, including β-actin. Each sample was loaded on separate chips and was run separately with its own control. These data represent the average of 2 separate experiments from the same donors. B: HMGB1 increases the expression of monoamine oxidase B and Bcl-xL mRNA in human neutrophils. Neutrophils (5 × 10⁶ cells/ml) were treated with HMGB1 (1 μg/ml), heat-inactivated HMGB1 (1 μg/ml), or LPS (100 ng/ml) for 1 h. Levels of monoamine oxidase B and Bcl-xL mRNA were detected by RT-PCR. Heat inactivation of HMGB1 protein was performed at 95°C for 30 min. The results shown are representative of 3 experiments.
supershift studies, a 200-fold excess before the addition of labeled probe. For the NF-κB or mutant NF-κB oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the samples at pH 8.0–borate-EDTA buffer at 10 V/cm. Dried gels were formed after X-ray cation was performed by image analysis using densitometry (Chemidoc system; Bio-Rad, Hercules, CA).

Statistical analysis. The data are shown as means ± SE and represent information from three separate experiments. Statistical significance was determined by analysis of variance after the normality of the data was verified, followed by the Tukey-Kramer multiple comparisons test. A P value <0.05 was considered significant.

RESULTS

HMGB1 and LPS induce distinct patterns of gene expression in neutrophils. The hierarchical clustering method was used to identify gene expression patterns in neutrophils stimulated by either HMGB1 or LPS for 1 h, and the results were compared with patterns obtained from experiments using unstimulated neutrophils. As shown in Fig. 1, a total of 470 genes showed at least twofold increase in expression among neutrophils cultured with either HMGB1 or LPS. Of these 470 genes, the expression of 95 genes was increased by both LPS and HMGB1. The expression of 140 genes was upregulated at least twofold by HMGB1 but not LPS, whereas the expression of 235 genes was increased by LPS but not HMGB1 (Fig. 1A).

LPS signal transduction in neutrophils occurs in part through activation of ERK1/2, PI 3-K, and p38 MAPK, and pathways involving these kinases play important roles in modulating expression of inflammatory gene products (4, 20, 21, 31, 32, 33, 38, 48, 50). As expected, LPS exposure increased the expression of neutrophil genes regulated by p38 MAPK, PI 3-K, or ERK1/2 (Table 1) (5, 9, 10, 12, 18, 19, 22, 24, 30, 39, 42, 45). Increased expression of genes under the regulatory control of these kinases was also observed after exposure of neutrophils to HMGB1 (Table 1). To validate the gene array results, we used RT-PCR to confirm that expression of monoamine oxidase B and Bcl-xL was increased in neutrophils by HMGB1 but not by LPS or heat-inactivated HMGB1 (Fig. 1B).

Effects of HMGB1 on proinflammatory cytokine and chemokine expression in human neutrophils. To determine the molecular basis for HMGB1-mediated neutrophil activation, we examined mRNA levels of IL-1β, IL-8, and TNF-α in neutrophils cultured with LPS or

Table 1. Increased expression of genes following exposure of neutrophils to HMGB1 or LPS

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold Changes</th>
<th>Signaling Pathways Involved in Expression</th>
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<tbody>
<tr>
<td>Serpin plasminogen activator inhibitor-1</td>
<td>ND</td>
<td>p38 MAPK, ERK1/2</td>
</tr>
<tr>
<td>Monocyte chemoattractant protein 1 receptor</td>
<td>ND</td>
<td>ERK1/2</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>100</td>
<td>p38 MAPK, PI 3-K</td>
</tr>
<tr>
<td>Monoamine oxidase B</td>
<td>12.4</td>
<td>p38 MAPK</td>
</tr>
<tr>
<td>Basic helix-loop-helix-PAS (HIF)</td>
<td>7.2</td>
<td>PI 3-K</td>
</tr>
<tr>
<td>IL-1 receptor α</td>
<td>7.1</td>
<td>ERK1/2</td>
</tr>
<tr>
<td>Burkitt’s lymphoma receptor</td>
<td>ND</td>
<td>p38 MAPK</td>
</tr>
<tr>
<td>G protein γ 7</td>
<td>5.5</td>
<td>ERK1/2, p38, PI 3-K</td>
</tr>
<tr>
<td>CC chemokine receptor 3</td>
<td>ND</td>
<td>ERK1/2, p38, PI 3-K</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2</td>
<td>4.1</td>
<td>p38 MAPK</td>
</tr>
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Genes listed demonstrated ≥4-fold increase in expression after 1-h exposure of neutrophils to high mobility group box 1 (HMGB1) protein or LPS. ND; not detected. Results represent the mean increase from 2 separate experiments using the same donors.

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HMGB1. In the gene array experiments shown in Fig. 1, the expression of IL-1β and IL-8 was increased approximately twofold by LPS and HMGB1, and the expression of TNF-α was increased sevenfold by LPS and fivefold by HMGB1. Real-time RT-PCR analysis revealed induction of IL-1β, IL-8, and TNF-α mRNA as early as 30 min after addition of HMGB1 to the cells. Cytokine gene expression was HMGB1 concentration dependent (Fig. 2, A–C). Maximum expression levels of IL-1β and TNF-α were found in neutrophils 60 min after exposure to HMGB1 (Fig. 2, A and B), whereas maximum levels of IL-8 mRNA were present 30 min after culture with HMGB1. LPS stimulation of neutrophils produced alterations in the magnitude and timing of IL-8 and IL-1β mRNA expression that were similar to those observed after exposure to HMGB1 (Fig. 2, A and C), whereas expression of TNF-α mRNA in neutrophils appeared to reach maximal levels at later time points after culture with LPS compared with HMGB1 (Fig. 2B).

Fig. 2. Effect of HMGB1 on proinflammatory cytokine and chemokine gene expression in human neutrophils. Neutrophils (5 × 10^6/ml) were incubated in the presence or absence of recombinant HMGB1 (0–1,000 ng/ml) or LPS (100 ng/ml) for the indicated time (30, 60, or 240 min). Total RNA was then isolated and processed for real-time RT-PCR to determine the expression of the proinflammatory cytokines IL-1β (A), TNF-α (B), and IL-8 (C). Values represent means ± SE from 3 consecutive experiments. *P < 0.05; †P < 0.01; and ‡P < 0.001 vs. control.
HMGB1 induced kinase activation in human neutrophils. LPS can activate multiple signaling pathways including PI 3-K/Akt, ERK1/2, and p38 MAPK in fibroblasts, macrophages, and neutrophils (4, 20, 21, 31, 32, 33, 38, 48, 50). To determine whether HMGB1 activates similar pathways in neutrophils, we cultured human neutrophils with HMGB1 and directly examined the extent of activation of Akt, ERK1/2, and p38 MAPK. As shown in Fig. 3, HMGB1 increased the activation of p38 MAPK by approximately fourfold in neutrophils and had lesser effects on Akt and ERK1/2.

To determine the importance of HMGB1 induced activation of pathways involving p38 MAPK, PI 3-K, or ERK1/2, we examined proinflammatory cytokine expression in neutrophils stimulated with HMGB1 in the presence of specific inhibitors of each of these kinase pathways. Preincubation of neutrophils with a specific inhibitor of p38 (SB-202190), effectively decreased HMGB1-induced activation of p38 (Fig. 3). Similarly, addition of inhibitors of PI 3-K (LY-294002) and MEK1/2 (U0126) prevented the minimal activation of Akt and ERK1/2 produced by exposure of neutrophils to HMGB1.

Inhibition of p38, MEK1/2, or PI 3-K reduced HMGB1-induced increases in IL-1β, TNF-α, and IL-8 gene expression (Fig. 4, A–C). Of note, inhibition of p38 MAPK had a greater effect on HMGB1-induced cytokine expression than did blockade of MEK1/2 or PI 3-K, suggesting a relatively greater role for p38 MAPK in HMGB1-initiated inflammatory processes.

HMGB1 increases nuclear translocation of NF-κB in human neutrophils. Stimulation of neutrophils with LPS increases nuclear translocation of NF-κB and NF-κB-dependent gene expression (26, 28, 44, 49). Figure 5A shows that HMGB1 also leads to increased nuclear translocation of NF-κB in human neutrophils. In these experiments, specificity of NF-κB binding was confirmed by demonstrating that inclusion of 200-fold mo-
lar excess of unlabeled competitor probe prevented the appearance of the specific NF-κB band. In contrast, no competition was observed with a NF-κB mutant oligonucleotide. In supershift experiments, addition of antisera to p50 decreased the intensity of the NF-κB band, whereas with antisera specific for the p65 subunit of NF-κB, generation of a slow-migrating protein-DNA complex was observed, showing that the binding proteins induced by HMGB1 included the heterodimeric p65-containing NF-κB complex. Amounts of p65 protein were also increased in the nucleus of neutrophils after exposure to HMGB1 (Fig. 5B).

Effects of protein kinase inhibitors on HMGB1-induced NF-κB translocation. The p38 MAPK, ERK1/2, and PI 3-K pathways have all been shown to be involved in cytokine- or LPS-induced nuclear translocation of NF-κB (20, 21, 31, 32, 33, 38, 50). To examine whether HMGB1-induced NF-κB activation also involves p38, ERK1/2, or PI 3-K signal transduction pathways, we used specific kinase inhibitors (Fig. 6). Inhibition of signaling pathways involving p38 or ERK1/2, but not Akt, prevented HMGB1-induced nuclear translocation of NF-κB. Blockade of p38 MAPK had greater effects on NF-κB activation than did inhibition of ERK1/2. These results are consistent with those shown in Fig. 4, where inhibition of p38 had greater effects on HMGB1-induced expression of cytokines whose transcription is dependent on NF-κB than did blockade of ERK1/2 or Akt activation.

HMGB1 induces p38 MAPK activity in a time-dependent manner. Exposure of neutrophils to LPS results in activation of p38 MAPK (31–33). As shown in Fig. 2, p38 MAPK activity was increased approximately four-fold in HMGB1-stimulated neutrophils. To determine whether HMGB1 induced a pattern of p38 activation in neutrophils that was similar to that seen after LPS stimulation, we performed kinetic experiments in HMGB1- and LPS-stimulated neutrophils (Fig. 7A). Increased activity of p38 MAPK was found at early (0–5 min) and later (30–60 min) time points after exposure of neutrophils to HMGB1. In contrast, p38 MAPK was activated only at the later time points (30 and 60 min) in LPS-stimulated neutrophils, similar to previously published data (31, 32). Only minimal alterations in levels of phosphorylated Akt and p44/42 MAPK were present at the time points examined after exposure to HMGB1 (Fig. 7B).

DISCUSSION

HMGB1 is released in a delayed manner from activated macrophages and monocytes in response to stimulation by LPS, but like other proinflammatory cytokines, HMGB1 itself induces the production of proinflammatory cytokines from macrophages (3). Although HMGB1 has been detected in human neutrophils (36), and high levels of HMGB1 occur in association with neutrophil-mediated diseases (1, 41), the role
of HMGB1 in mediating neutrophil responses has not been previously described. In the present studies, we show that HMGB1 increases nuclear translocation of NF-κB as well as the expression of proinflammatory cytokines among human neutrophils. We also demonstrate that p38 MAPK, Akt, and ERK1/2 are involved in HMGB1-induced neutrophil activation.

In response to LPS, neutrophils demonstrate nuclear accumulation of NF-κB/Rel proteins, increased nuclear NF-κB binding activity, and enhanced expression of mRNA transcripts encoding NF-κB-dependent cytokines and chemokines, such as IL-1β, TNF-α, and IL-8 (28, 36, 44, 49). Signaling pathways involving p38 MAPK, ERK1/2, and PI 3-K are activated in neutrophils cultured with LPS and contribute to enhanced nuclear translocation of NF-κB in this setting (20, 21, 31, 32, 33, 38, 50). Similar findings were found in the present studies when human neutrophils were cultured with HMGB1, suggesting that parallel mechanisms of cellular activation are induced by HMGB1 and LPS. However, differences in the kinetics of cytokine expression and kinase activation were apparent between LPS and HMGB1. For example, peak levels of TNF-α mRNA were present 60 min after stimulation with HMGB1 but occurred after 240 min with LPS. Similarly, activation of p38 occurred within 5 min with HMGB1 but was first seen only 30 min after culture with LPS. Additionally, gene array studies, although showing similarities in the patterns of gene expression by neutrophils cultured with HMGB1 or LPS, also demonstrated differences between the two stimuli, indicating that distinct mechanisms of neutrophil activation were initiated by HMGB1 or LPS.

The receptor for advanced glycation end products (RAGE), a multiligand member of the immunoglobulin superfamily of cell surface molecules, interacts with HMGB1 and triggers activation of key cell signaling pathways (37). Binding of HMGB1 to RAGE leads to neurite outgrowth and enhanced expression of tissue-type plasminogen activator by macrophages (16, 17, 34). In these cell populations, engagement of RAGE leads to activation of NF-κB through a redox-dependent pathway involving Ras (23). In addition, RAGE ligation has been shown to activate ERK1/2, p38, and SAPK/JNK kinases (17, 23, 47), as well as the small GTPases, Rac and cdc42 (17). In addition to being present on neurites and macrophages, RAGE also exists on monocytes as well as glia, endothelial, and smooth muscle cells (11, 16, 17, 34, 37). Although RAGE has not been directly demonstrated to be present on neutrophils, the fact that synthetic human S100A12, a member of the s100/calgranulin family and a ligand for RAGE, is chemotactic for neutrophils suggests that such receptors are functional, exist on neutrophils, and are likely to interact with HMGB1 (15, 35). It is plausible that signaling through RAGE by HMGB1 can contribute to the responses observed here, but these results do not exclude the possible contribution of other receptors.

The present studies show that HMGB1 potently activates p38 and, to a lesser extent, ERK1/2 and PI 3-K in neutrophils. These kinases appear to be involved in enhancing nuclear translocation of NF-κB as well as in the expression of proinflammatory cytokines in HMGB1-stimulated neutrophils. As noted above, ligation of RAGE has previously been shown to result in activation of p38 and NF-κB in C6 rat glioma cells (37). In C6 cells, HMGB1-induced activation of NF-κB also was shown to be dependent on Ras, implying a role for downstream kinases, including ERK1/2, in this process. However, in neuroblastoma cells stably transfected with full-length RAGE, PI 3-K did not appear to be involved in HMGB1-induced neurite outgrowth (17), consistent with the minimal role found for the PI 3-K/Akt pathway in the present experiments.

In our studies, incubation of neutrophils with HMGB1 resulted in a fourfold increase in the activation of p38, a substantially greater increase than what
was found for PI 3-K or ERK1/2. Consistent with this effect, inhibition of p38 resulted in a more profound decrease of NF-κB activation and expression of proinflammatory cytokines than did blockade of MEKI1/2 or PI 3-K. An important role for p38 in modulating HMGB1-induced neutrophil responses is not surprising given the importance of the p38 MAPK cascade in regulating many neutrophil responses to proinflammatory stimuli, such as LPS and cytokines. Inhibition of p38 produces significant decreases among neutrophils in adhesion (33), chemotaxis (51), oxidative burst (43), synthesis of TNF-α and IL-8 (33, 46), secretion of secondary and tertiary granules (29), and activation of NF-κB (33). In addition to being directly involved in nuclear translocation of NF-κB (33), p38 may also enhance NF-κB transactivation by phosphorylating TATA-binding protein in the NF-κB-associated transcriptional apparatus (8). Although engagement of toll-like receptor 4 (TLR4) receptors by LPS activates p38 (46), our present results show that culture of neutrophils with HMGB1 also leads to p38 activation. This does not necessarily imply a role for TLR4 in mediating the effects of HMGB1 on neutrophil function, since ligation of RAGE by HMGB1 has been demonstrated to lead to p38 activation (37). Whether overlap exists between downstream events initiated by TLR4 and RAGE ligation, or even if cooperation between TLR4 and RAGE is involved in HMGB1 signaling, is unknown at present but is a focus of ongoing investigations.

In previous studies (1), we demonstrated that HMGB1 could initiate acute neutrophil-dependent inflammatory responses in vivo. However, it was not clear from those experiments whether HMGB1 could directly stimulate neutrophils or whether intermediate cell populations, such as macrophages, were necessary. The present studies show that HMGB1 itself can activate neutrophils to produce proinflammatory mediators, such as IL-1β, IL-8, and TNF-α, that are known to have important roles in inflammation and related diseases. Because HMGB1 is released with delayed kinetics, often over a period of several hours, after exposure of cells to LPS and proinflammatory stimuli, such as TNF-α, it may be capable of potentiating inflammatory processes, such as sepsis or acute lung injury, that are initiated by these stimuli under in vivo conditions (3, 41). Similarly, while HMGB1 itself is an attractive therapeutic target for inflammatory conditions, the present results also suggest that therapies directed at inhibition of intracellular signaling pathways, such as p38, may also be able to attenuate the proinflammatory effects of HMGB1.

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REFERENCES


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