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Hemorrhagic Shock Induces NAD(P)H Oxidase Activation in Neutrophils: Role of HMGB1-TLR4 Signaling

Jie Fan,*† Yuehua Li,* Ryan M. Levy,* Janet J. Fan,‡ David J. Hackam,* Yoram Vodovotz,* Huan Yang,§ Kevin J. Tracey,§ Timothy R. Billiar,* and Mark A. Wilson*†

Hemorrhagic shock/resuscitation (HS/R)-induced generation of reactive oxygen species (ROS) plays an important role in post-hemorrhage inflammation and tissue injury. We have recently reported that HS/R-activated neutrophils (PMN), through release of ROS, serve an important signaling function in mediating alveolar macrophage priming and lung inflammation. PMN NAD(P)H oxidase has been thought to be an important source of ROS following HS/R. TLR4 sits at the interface of microbial and sterile inflammation by mediating responses to both bacterial endotoxin and multiple endogenous ligands, including high-mobility group box 1 (HMGB1). Recent studies have implicated HMGB1 as an early mediator of inflammation after HS/R and organ ischemia/reperfusion. In the present study, we tested the hypothesis that HS/R activates NAD(P)H oxidase in PMN through HMGB1/TLR4 signaling. We demonstrated that HS/R induced PMN NAD(P)H oxidase activation, in the form of phosphorylation of p47^{phox} subunit of NAD(P)H oxidase, in wild-type mice; this induction was significantly diminished in TLR4-mutant C3H/HeJ mice. HMGB1 levels in lungs, liver, and serum were increased as early as 2 h after HS/R. Neutralizing Ab to HMGB1 prevented HS/R-induced phosphorylation of p47^{phox} in PMN. In addition, in vitro stimulation of PMN with recombinant HMGB1 caused TLR4-dependent activation of NAD(P)H oxidase as well as increased ROS production through both MyD88-IRAK4-p38 MAPK and MyD88-IRAK4-Akt signaling pathways. Thus, PMN NAD(P)H oxidase activation, induced by HS/R and as mediated by HMGB1/TLR4 signaling, is an important mechanism responsible for PMN-mediated inflammation and organ injury after hemorrhage. The Journal of Immunology, 2007, 178: 6573–6580.

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Hemorrhagic shock promotes the development of multiple organ dysfunction by priming the innate immune system for an exaggerated inflammatory response through as yet unclear mechanisms. Studies have suggested that ischemia/reperfusion primes circulating neutrophils (PMN) for increased reactive oxygen species (ROS)\(^5\) production, thereby augmenting neutrophil-mediated lung injury once the PMN are sequestered in the lung (1, 2). We have recently reported that oxidants derived from PMN activated by hemorrhagic shock plus resuscitation (HS/R) play an important role in regulating TLR2 expression in alveolar macrophages (AM\(\phi\)). We demonstrated that shock-activated PMN initially sequestered in alveoli could lead AM\(\phi\) to up-regulate TLR2 in response to LPS/TLR4 signaling, thereby sensitizing AM\(\phi\) to TLR2 ligands and promoting enhanced lung inflammation (3). Taken together, HS/R-induced generation of ROS in PMN serves an important signaling function in mediating shock-primed inflammation and organ injury.

Recent studies have suggested that NAD(P)H oxidase is an important source of ROS mediating organ injury after hemorrhagic shock (4–6). NAD(P)H oxidase is a highly regulated membrane-bound enzyme complex that catalyzes the production of superoxide by the one-electron reduction of oxygen using NAD(P)H as the electron donor. The core enzyme is comprised of both membrane-bound (i.e., gp91^{phox} and p22^{phox}) and cytosolic (i.e., p40^{phox}, p47^{phox}, p67^{phox}, and rac-1/2) components (7, 8). Upon stimulation, receptor-mediated activation of the oxidase complex involves activation of secondary signaling intermediates, culminating in the phosphorylation and recruitment of the cytosolic components to the membrane-bound components to assemble the active oxidase (7–9).

The TLRs are a family of pattern recognition receptors through which the innate immune system senses the invasion of pathogenic microorganisms or sterile tissue damage. TLRs are capable of recognizing specific molecular patterns that are present in microbial products (pathogen-associated molecular pattern molecules) or endogenous molecules released by damaged tissues (danger signals) (10, 11). TLR4 sits at the interface of microbial and sterile inflammation by responding to both bacterial endotoxin and multiple other endogenous ligands, including hyaluronic acid (12), heparan sulfate (13), fibrinogen (14), high-mobility group box 1 (HMGB1) (15, 16), and heat shock proteins (17). Both inflammation and injury responses in organs subjected to ischemia/reperfusion partially depend on TLR4 (15, 18, 19).

HMGB1 was originally identified as a nuclear protein that functions to stabilize nucleosome formation and also acts as a transcription factor that regulates the expression of several genes (20).
HMGB1 can be secreted by innate immune cells in response to microbial products or other inflammatory stimuli (21, 22). HMGB1 is also released by injured cells and known as one of the main prototypes of the emerging damage-associated molecular pattern molecules (DAMPs) (10, 23, 24). HMGB1 was initially identified as an inflammatory cytokine that is a late mediator of lethality in sepsis (21, 22). However, recent studies suggest that HMGB1 acts as an early mediator of inflammation contributing to the development of acute lung injury after hemorrhage (25), and hepatic injury after liver ischemia/reperfusion (15).

In the present study, we addressed the role of HMGB1/TLR4 signaling in mediating HS/R-induced PMN NAD(P)/H oxidase activation. Here we demonstrate that HS/R induces PMN NAD(P)/H oxidase activation in wild-type (WT) mice, and that this induction is diminished in TLR4-mutant C3H/HeJ mice. Neutralizing Ab to HMGB1 prevents HS/R-induced activation of PMN NAD(P)/H oxidase. In addition, in vitro stimulation of PMN with recombinant HMGB1 causes TLR4-dependent activation of NAD(P)/H oxidase as well as increased ROS production through both MyD88-IRAK4-p38 MAPK and MyD88-IRAK4-Akt signaling pathways. Thus, PMN NAD(P)/H oxidase activation, induced by HS/R and mediated by HMGB1/TLR4 signaling, is an important mechanism responsible for PMN-mediated inflammation and organ injury after HS/R.

Materials and Methods

Recombinant HMGB1 was purchased from R&D Systems. Staining activity of the recombinant HMGB1 was confirmed in mouse macrophages by assay of TNF release, with an ED50 of 3 to 12 µg/ml. Polyclonal neutralizing Ab against HMGB1 was prepared as described previously (22). The neutralizing Ab against HMGB1 was raised by rabbits, and titers were determined by immunoblotting. Nonimmune rabbit IgG (item 5006) was purchased from Sigma-Aldrich. Polyclonal anti-HMGB1 Ab for Western blotting and kinase assay kits for IRAK4, p38 MAPK, and Akt were purchased from Cell Signaling Technology. Anti-p47phox Ab and anti-gp91phox Ab were obtained from Santa Cruz Biotechnology, and rabbit anti-phosphoserine Ab and anti-phosphoserine inhibitor (O-phospho-l-serine) were purchased from Invitrogen Life Technologies. Polyclonal rabbit anti-IRAK4 Ab and anti-phosphoribosylation inhibitor peptide set were purchased from eBioscience. Akt inhibitor was purchased from BioVision, and p38 MAPK inhibitor SB-202190 was obtained from EMD Biosciences.

Hemorrhagic shock and resuscitation

Male C3H/HeJ mice, which are not responsive to LPS because of a point mutation of tlr4 affecting the TIR domain (26, 27) and control WT C3H/HeOuJ mice were purchased from The Jackson Laboratory. All experimental protocols involving animals were approved by Institutional Animal Care and Use Committee of Veterans Affairs Pittsburgh Healthcare System. Mice were 12–14 wk of age at the time of experiments. Animals were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine via i.p. administration. Femoral arteries were cannulated for monitoring of mean arterial pressure, blood withdrawal, and resuscitation. Hemorrhagic shock was initiated by blood withdrawal and reduction of the mean arterial pressure to 40 mm Hg within 15 min. Blood was collected into a 1-ml syringe and heparinized to prevent clotting. After a hypotensive period of 2 h, the catheters were then removed, the femoral artery was ligated, and the incisions were closed. Sham animals underwent the same surgical procedures without hemorrhage and resuscitation. In some experiments, mice received anti-HMGB1 Ab (600 µg per mouse) or nonimmune control IgG by i.p. injection 10 min before hemorrhage. At various time points after resuscitation, peripheral blood was collected through cardiac puncture and PMN were isolated from the blood using the immunomagnetic separation system (BD Biosciences) (28). Viability of the isolated PMN was >95%, and PMN population was >95% as assessed by trypan blue exclusion (29) and Wright-Giemsa staining, respectively. NAD(P)/H oxidase activation and ROS release were then assessed as described below.

Immuno precipitation and detection of phosphorylated p47phox and binding of p47phox-gp91phox

PMN isolated from the mice subjected to HS/R or recovered from in vitro incubation treated with recombinant HMGB1 were lysed (1 × 10^6 cells/ml) in lysis buffer (10 mM Tris (pH 7.4) 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM Na3VO4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 20 mM PMSF). PMN lysates were then immunoprecipitated with anti-p47phox Ab as described (30). The immunoprecipitated proteins were separated on a 10% SDS-PAGE gel and were then electrophoresed onto PVDF membrane and blocked for 1 h at room temperature with Tris-buffered saline containing 3% nonfat dried milk. The membranes were probed with anti-phosphoserine Ab (31–33) at 1/500 dilution and detected with an HRP-conjugated secondary Ab at a dilution of 1/3000 using the Western blotting detection system (Amersham Biosciences). The specific binding of anti-phosphoserine Ab was tested using anti-phosphoserine inhibitor (O-phospho-l-serine) added in the membrane incubation solution with primary anti-phosphoserine Ab in a concentration of 20 mM (34). Blots were then stripped and reprobed with either anti-gp91phox Ab to detect p47phox–gp91phox binding, or anti-ph47phox Ab to detect total amount of p47phox.

Measurement of superoxide generation

PMN superoxide production was measured with the fluorogenic substrate lucigenin (35). PMN isolated from the mice subjected to HS/R or sham operation were resuspended in HBSS followed by adding 10^-5 M lucigenin. The luminescence was then measured with Veritas microplate luminometer (Turner BioSystems). In vitro experiments, PMN isolated from either WT or C3H/HeJ mice were resuspended in HBSS with 5% FCS and 10 mM PMSF. The PMN lysates were then incubated at 25°C for 30 min in a final volume of 37.5 µl of kinase buffer in the presence of biotinylated ezrin/radixin/moesin peptide as a substrate (1.5 µM sample) and 200 µM ATP, both of which were provided in the HTSscan IRAK4 kinase assay kit (Cell Signaling Technologies). After adding 50 µl/sample stop buffer (50 mM EDTA, pH 8), 25 µl of each reaction and 75 µl of 0.002% H2O2 were transferred to 96-well streptavidin-coated plate (PerkinElmer Life Sciences) and incubated at room temperature for 60 min. IRAK4 activity was then measured following the manufacturer’s instructions for the kit using primary anti-phospho-epitope (radixin/moesin) Ab (Cell Signaling Technologies) and secondary europium-labeled anti-rabbit Ab (PerkinElmer Life Sciences). Fluorescence emission at 615 nm was detected with SpectraMax M2 multi detection reader (Molecular Devices).

Akt kinase assay

Akt kinase activity in PMN was measured with the nonradioactive Akt kinase assay kit (Cell Signaling Technology) (36). PMN lysates were pre-cleared by centrifugation and preabsorbed with protein A-antibody G (1:1) agarose slurry. Immunoprecipitation was conducted using the immobilized anti-Akt1G1 mAb cross-linked to agarose. Immunoprecipitates were washed three times with lysis buffer and twice with Akt kinase buffer (20 mM HEPES (pH 7.4) 10 mM MgCl2, 10 mM MnCl2). Kinase assays were performed for 30 min at 30°C under continuous agitation in kinase buffer containing 200 µM ATP, 1 µM of GSK-3 fusion protein, according to the manufacturer’s instructions. Phosphorylation of GSK-3 was analyzed by Western blot analysis using anti-phospho-GSK-3αβ Ab.

p38 MAPK assay

The activity of p38 MAPK in PMN was analyzed by using a p38 MAPK assay kit (Cell Signaling Technology) (37). Phosphorylated p38 was immunoprecipitated with a p38-phospho-specific Ab from 200 µl of lysate. This Ab specifically recognized phosphorylated p38 and did not cross-react with phosphorylated JNK or ERK1/2. The immune complex was washed thoroughly and resuspended in kinase buffer containing ATP and 1 µM of
recombinant activating factor-2 (ATF-2) as a p38 MAPK substrate. The reaction was incubated at 30°C for 45 min and terminated by adding SDS sample buffer. The kinase reaction was analyzed by Western blotting with a phospho-specific anti-ATF-2 Ab.

Statistics

The data are presented as mean ± SEM of n determinations as indicated in the figures. Data were analyzed by one-way ANOVA; post hoc testing was performed using the Bonferroni modification of the t test. When individual studies are demonstrated, these are representative of at least three independent studies.

Results

TLR4 is involved in HS/R-induced NAD(P)H oxidase activation

We first examined the phosphorylation of the p47\textsuperscript{phox} subunit of NAD(P)H oxidase in circulating PMN that were recovered from TLR4 WT (C3H/HeOuJ) and TLR4-mutant (C3H/HeJ) mice 1 to 4 h after HS/R. As shown in Fig. 1A, p47\textsuperscript{phox} phosphorylation was induced by HS/R at 1 h, and further increased at 4 h. However, in PMN obtained from TLR4 mutant mice, the HS/R-induced p47\textsuperscript{phox} phosphorylation was markedly attenuated. At 4 h, p47\textsuperscript{phox} phosphorylation in TLR4 mutant PMN was 20% of that in TLR4 WT PMN. To test the specificity of anti-phosphoserine Ab binding in the Western blotting, anti-phosphoserine inhibitor (O-phospho-L-serine) was applied (34). Anti-phosphoserine inhibitor specifically blocks the binding of phosphoserine and anti-phosphoserine Ab, thereby the band of phosphoserine protein visualized by HRP-conjugated second Ab and the ECL detection system is faded (Fig. 1B).

Binding of p47\textsuperscript{phox} and gp91\textsuperscript{phox} is thought to be a marker of NAD(P)H oxidase assembly and activation. The binding was detected as shown in Fig. 1A. The changes in the binding of p47\textsuperscript{phox} and gp91\textsuperscript{phox} are parallel to the changes in p47\textsuperscript{phox} phosphorylation.

Furthermore, we measured ROS release in the PMN that were isolated from TLR4 WT and TLR4-mutant mice 4 h after HS/R. HS/R induced a marked increase in ROS release in WT PMN, which was 4.5-fold greater than that in TLR4-mutant PMN (Fig. 1C).

Taken together, these data indicate an important role for TLR4 in mediating HS/R-induced NAD(P)H oxidase activation.

HMGB1 is responsible for HS/R-induced NAD(P)H oxidase activation

TLR4 recognizes a variety of endogenous ligands including HMGB1 (20, 38). Because HMGB1 plays a role in inflammation in the lung after HS/R (25) and in the liver following ischemia/reperfusion (15), we sought to determine whether HMGB1, as a TLR4 ligand, mediates HS/R-induced NAD(P)H oxidase activation. HMGB1 protein levels in serum and tissues were measured by Western blotting (WB) as described in Materials and Methods. The graph depicts the mean and SEM of the changes in ratio of phosphototal p47\textsuperscript{phox} from three mice. **, p < 0.01 compared with all other groups; *, p < 0.01 compared with the groups labeled with no asterisk. B. To test the specificity of anti-phosphoserine Ab, p47\textsuperscript{phox} in PMN isolated from shocked mice was immunoprecipitated with anti-p47\textsuperscript{phox} Ab, and blotted on PVDF membrane. The duplicated membranes were then incubated with primary anti-phosphoserine Ab in the presence or absence of anti-phosphoserine inhibitor (O-phospho-L-serine; 20 mM), and then detected with an HRP-conjugated second Ab using the ECL Western blotting detection system. The specific binding of anti-phosphoserine Ab and phosphoserine was blocked by the inhibitor. C. ROS production in PMN in response to HS/R. The PMN were isolated from WT or TLR4 mutant mice 4 h after HS/R or sham operation, and ROS production was measured with the fluorogenic substrate lucigenin. The graph depicts the mean and SEM of changes in ROS production compared with the WT PMN isolated from sham operated mice (as 100%). The data are from three independent experiments. **, p < 0.01 compared with other groups.

HMGB1 acts through TLR4-MyD88-IRAK4 signaling

To determine whether HMGB1 acts through TLR4 to induce NAD(P)H oxidase activation, PMN isolated from TLR4 WT and TLR4 mutant mice were incubated with recombinant HMGB1 for 30 min, and levels of p47\textsuperscript{phox} phosphorylation were examined. As shown in Fig. 4A, TLR4 mutant PMN exhibited an approximately 79% decrease in p47\textsuperscript{phox} phosphorylation in response to HMGB1 compared with that of TLR4 WT PMN. The alterations in ROS...
released from HMGB1-treated PMN paralleled the changes in p47\textsuperscript{phox} phosphorylation in the PMN obtained from both TLR4 WT and TLR4 mutant mice (Fig. 4B). These results suggested a predominant role for TLR4 in mediating the HMGB1-induced activation of NAD(P)H oxidase in PMN.

Previous studies have shown that TLR4 can signal through both MyD88-dependent and MyD88-independent pathways (39). To address the role of MyD88 in HMGB1/TLR4-induced NAD(P)H oxidase activation, the MyD88 inhibitor homodimerization inhibitory peptide was applied (40). PMN recovered from TLR4 WT mice were preincubated with either MyD88 inhibitory peptide or control peptide (100 μM) for 4 h (40) and subsequently treated with HMGB1 for 30 min. In MyD88 inhibitor-treated PMN, HMGB1-induced p47\textsuperscript{phox} phosphorylation was reduced by approximately 77% compared with that in the PMN treated with control peptide (Fig. 5A). This demonstrates a critical role for MyD88-dependent signaling in HMGB1/TLR4-induced NAD(P)H oxidase activation.

Next, the role of IRAK4 in MyD88 signaling was examined by evaluating IRAK4 activity in the PMN. We found that HMGB1 induced a 6-fold increase in IRAK4 activity in TLR4 WT PMN compared with the non-HMGB1-treated WT PMN (p < 0.01; Fig. 5B). Pretreatment with MyD88 inhibitor decreased IRAK4 activity by 91% in WT PMN (Fig. 5B). An impaired IRAK4 activation was observed with TLR4 mutant PMN, in which the HMGB1-induced IRAK4 activity was attenuated by 64% compared with that in TLR4 WT PMN (Fig. 5B). However, MyD88 inhibitor further reduced IRAK4 activity by 36% in TLR4 mutant PMN (Fig. 5B), suggesting that receptors other than TLR4 may mediate the effects of HMGB1 in this experimental setting. These results demonstrate a significant role for TLR4-MyD88-IRAK4 signaling in mediating HMGB1-induced PMN NAD(P)H oxidase activation.

Akt and p38 MAPK contribute to the HMGB1-induced NAD(P)H oxidase activation

Both Akt and p38 MAPK have been reported to be involved in p47\textsuperscript{phox} phosphorylation in cells in response to different mediators (41–43). In this study, we tested the role of Akt and p38 MAPK in HMGB1-induced NAD(P)H oxidase activation. We detected Akt and p38 MAPK activation in PMN in response to HMGB1 using kinase assays. As shown in Fig. 6, A and B, HMGB1 induced...
activation of both Akt and p38 MAPK in TLR4 WT PMN as early as 15 min, detected as the phosphorylation of GSK-3 and ATF-2, respectively. Pretreatment of PMN with MyD88 inhibitor prevented HMGB1-induced activation of both Akt and p38 MAPK (Fig. 6, C and D), indicating that the activation of both Akt and p38 MAPK are downstream events that depend on MyD88 activation. To further confirm the role of Akt and p38 MAPK in signaling the HMGB1-induced NAD(P)H oxidase activation, Akt inhibitor (4 μM) and p38 MAPK inhibitor SB202190 (2 μM) were added to PMN, which were then stimulated with HMGB1. Levels of p47<sup>phox</sup> phosphorylation in the PMN were measured at 30 min. As shown in Fig. 7A, SB202190 and Akt inhibitor partially prevented p47<sup>phox</sup> phosphorylation by approximately 47 and 55%, respectively, whereas the combination of SB202190 and Akt inhibitor reduced p47<sup>phox</sup> phosphorylation by approximately 95%. The alterations in ROS released from the PMN paralleled the changes in p47<sup>phox</sup> phosphorylation in the PMN (Fig. 7B). Thus, both Akt and p38 MAPK signaling are involved in HMGB1-induced NAD(P)H oxidase activation.

Discussion
The production and generation of ROS play an important role in promoting the inflammatory response and multiple organ dysfunction that follows HS/R. PMN NAD(P)H oxidase has been defined as a major source of ROS that mediates organ injury after HS/R (4–6). The present studies demonstrate that the global ischemia/reperfusion injury initiated by resuscitated hemorrhagic shock activates PMN NAD(P)H oxidase through HMGB1/TLR4 signaling. This activation is characterized by significantly increased phosphorylation of p47<sup>phox</sup> and binding of p47<sup>phox</sup>-p67<sup>phox</sup> in TLR4 WT PMN after HS/R, but not in PMN recovered from TLR4 mutant mice or from TLR4 WT mice pretreated with neutralizing Ab to HMGB1. These findings are further supported by in vitro studies of PMN treated with recombinant HMGB1, and functionally associated with changes in oxidant production in the PMN. Taken together, these studies indicate that HMGB1/TLR4 signaling plays an important role in mediating HS/R-induced NAD(P)H oxidase activation in PMN.

HMGB1 is a mediator of organ injury in animal models of infection and endotoxemia (21, 44–48). In addition, intratracheal administration of HMGB1 alone can induce organ dysfunction in the form of acute lung injury (44). However, no role had been ascribed to HMGB1 in the activation of PMN NAD(P)H oxidase in a setting of HS/R, a process which is not associated with exposure to bacteria or bacterial products. Previous studies have demonstrated increased circulating HMGB1 in serum of endotoxin-treated mice and septic and hemorrhagic shock patients, presumably due to release from activated macrophages (21, 49). In the
MHGB1 induces activation of Akt and p38 MAPK through MyD88. A and B, MHGB1 induces activation of Akt and p38 MAPK in WT PMN. WT PMN were incubated with MHGB1 (0.5 μg/ml) for the time as indicated, and Akt and p38 MAPK activity was detected as the phosphorylation of GSK-3 and ATF-2, respectively. C and D, Akt and p38 MAPK are downstream events that depend on MHGB1-induced MyD88 activation. WT PMN were pretreated with MyD88 inhibitory peptide or control peptide (100 μM) for 4 h, and then treated with MHGB1 for 30 min. Akt and p38 MAPK activity was then detected as the phosphorylation of GSK-3 and ATF-2, respectively. The images are representative of three independent studies. The graphs depict the means and SEM from three mice. *, p < 0.01 compared with the groups with no asterisk.

In the present experiments, we observed increased HMGB1 levels in serum, lungs, and liver at 2 h after HS/R. The increased HMGB1 may therefore serve as an endogenous signal to initiate PMN NAD(P)H oxidase activation. However, it is not clear from our studies whether HMGB1 is secreted or passively released from damaged cells after HS/R (50). It is possible that HS/R leads to regulated release or expression of HMGB1 as a danger-signaling event.

The receptor for advanced glycation end products (RAGE) had been originally identified as a receptor for HMGB1 in neurites and malignant cells (51–53). However, incubation of HMGB1 with soluble RAGE or RAGE-blocking Abs decreases, but does not eliminate, the stimulatory effects of HMGB1 on cellular activation (51, 54–56), suggesting that HMGB1 can also act through other receptors. Recent studies have suggested that both TLR4 and TLR2 are important in mediating HMGB1-induced inflammatory responses (15, 16, 57). In the present study, TLR4 exhibited a key role in mediating HMGB1-induced NAD(P)H oxidase activation, as demonstrated by greatly diminished NAD(P)H oxidase activation in response to both in vivo HS/R and in vitro HMGB1 stimulation in TLR4 mutant mice. Because absence of functional TLR4 signaling was insufficient to completely eliminate the effect of HMGB1 (Figs. 4A and 5B), we cannot rule out the possibility that HMGB1 may also act through other receptors, e.g., TLR2 and RAGE.

MyD88 is a common adaptor that induces inflammatory response in TLR signaling. Recently, a role for MyD88 has been reported in controlling phagocyte NAD(P)H oxidase function as initiated by Gram-negative bacteria (41). We show in the present study that MyD88 plays an important role in mediating NAD(P)H oxidase activation in response to HMGB1. This role was evident by a significant diminution of p47phox phosphorylation in PMN treated with both HMGB1 and MyD88 inhibitor. However, a small amount of MyD88-independent p47phox phosphorylation was also observed, as evidenced by the failure of the MyD88 inhibitor to completely block p47phox phosphorylation and IRAK4 activation in response to HMGB1. This finding suggests a non-TLR-mediated signal, perhaps via RAGE, that contributes to HMGB1-induced activation of NAD(P)H oxidase.

The phylogenetically conserved innate immune signaling pathways activated by TLRs in mammals can induce responses through the activation of IRAKs. In the present study, we observed the activation of IRAK4 in association with increased p47phox phosphorylation in TLR4 WT PMN after stimulation with HMGB1;
this activation of IRAK4 was significantly reduced by the MyD88 inhibitory peptide. Thus, these results demonstrate an essential role for both MyD88 and IRAK4 in the NAD(P)H oxidase activation.

In TRL4 mutant PMN, IRAK4 activation was markedly attenuated after HMGB1 treatment compared with the non-HMGB1-treated PMN. Nonetheless, TRL4 mutant PMN still exhibited a 2-fold increase in IRAK4 activity in response to HMGB1, suggesting a role for a receptor other than TRL4 (possibly TLR2) in mediating HMGB1 activation of NAD(P)H oxidase through an IRAK4-dependent mechanism.

The signaling pathways for NAD(P)H oxidase activation seem complex. A direct interaction of TRL4 with NAD(P)H oxidase 4 (Nox4), a protein related to gp91phox in phagocytic cells, has been suggested as a mechanism for LPS-mediated Nox4 activation and ROS production in HEK293T cells (58). However, p38 MAPK, ERK1/2, protein kinase C, and Akt have also been suggested as activators of p47phox and p67phox phosphorylation in response to TNF, IMLP, and bacteria (41, 42, 59, 60). The results reported here show that in the case of HS/R-induced p47phox phosphorylation, both p38 MAPK inhibitor and Akt inhibitor partially prevented HMGB1-induced phosphorylation of p47phox, and the combination of inhibitors exhibited complete inhibition of p47phox phosphorylation in response to HMGB1 stimulation. This result suggests that both p38 MAPK and Akt signaling pathways are involved in the HMGB1-induced NAD(P)H oxidase activation. It is noteworthy that p47phox is phosphorylated on multiple serine residues that could be targeted by different kinases, thus explaining the tight control of NAD(P)H oxidase. It is possible that p47phox phosphorylation by one kinase induces conformational changes that render the other sites of phosphorylation more accessible to other protein kinases. Indeed, it was recently shown that p47phox phosphorylation in vitro induces conformational changes of the protein (61, 62). Thus p38 MAPK and Akt, as downstream components of TLR4-MyD88-IRAK4 signaling, may work in a coordinate manner.

In summary, the present study identifies a previously unrecognized mechanism of HS/R-induced activation of NAD(P)H oxidase in PMN. In this mechanism, HS/R activates TLR4-MyD88-IRAK4 signaling pathway through HMGB1, and further activates p38 MAPK and Akt pathways to initiate phosphorylation of p47phox. This mechanism may serve an important determinant responsible for PMN-mediated inflammation and organ injury after HS/R.

Disclosures

The authors have no financial conflict of interest.

References


16. The authors have no financial conflict of interest.
HEMORRHAGE INDUCES PMN NAD(P)H OXIDASE ACTIVATION


