Hemorrhagic Shock Activates Lung Endothelial Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Via Neutrophil NADPH Oxidase

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The vascular endothelium plays an important role in the regulation of inflammatory responses after trauma and hemorrhage. Interactions of neutrophils with endothelial cells (ECs) contribute to the activation of specific EC responses involved in innate immunity. We have previously reported that oxidants derived from the neutrophil reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a critical regulator to EC activation. Our objective was to test the role of neutrophil NADPH oxidase–derived oxidants in mediating and enhancing hemorrhagic shock (HS)–induced activation of lung endothelial NADPH oxidase. Mice were subjected to HS and neutrophil depletion. The mice were also replenished with the neutrophil from NADPH oxidase–deficient mice. The resultant activation of lung NADPH oxidase was analyzed. The *in vivo* studies were also recapitulated with *in vitro* neutrophil–EC coculture system. HS induces NADPH oxidase activation in neutrophils and lung through high-mobility group box 1/Toll-like receptor 4–dependent signaling. In neutropenic mice, shock-induced NADPH oxidase activation in the lung was reduced significantly, but was restored upon repletion with neutrophils obtained from wild-type mice subjected to shock, but not with neutrophils from shock mice lacking the gp91^{phox} subunit of NADPH oxidase. The findings were recapitulated in mouse lung vascular ECs cocultured with neutrophils. The data further demonstrate that neutrophil-derived oxidants are key factors mediating augmented High mobility group box 1 (HMGB1)-induced endothelial NADPH oxidase activation through a Rac1-dependent, but p38 mitogen-activated protein kinase–independent, pathway. Oxidant signaling by neutrophil NADPH oxidase is an important determinant of activation of endothelial NADPH oxidase after HS.

Keywords: High mobility group box 1 (HMGB1); inflammation; lung; oxidants; Toll-like receptor

Hemorrhagic shock (HS), as a result of major trauma, promotes the development of systemic inflammatory response syndrome by activating and priming the innate immune system for an exaggerated inflammatory response through as yet unclear mechanisms. The vascular endothelium plays important roles in inflammatory responses and development of organ injury (1, 2). Reactive oxygen species (ROS) decisively contribute to cellular signaling, affecting gene expression, proliferation, cell death, migration, and inflammation. In the vasculature, several enzyme systems contribute to ROS formation, including nitric oxide synthase, enzymes of the respiratory chain, cytochrome P450 monoxygenases, xanthine oxidase, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Evidence has accumulated that an initial generation of ROS by NADPH oxidase triggers the release of ROS through the other enzyme systems (3). Recent studies have also revealed that ROS derived from NADPH oxidase play an important role in mediating organ injury after HS (4, 5).

CLINICAL RELEVANCE

Hemorrhagic shock (HS), as a result of major trauma, promotes the development of systemic inflammatory response syndrome, including acute lung injury (ALI) by activating and priming the innate immune system for an exaggerated inflammatory response through as yet unclear mechanisms. The vascular endothelium plays important roles in inflammatory responses and development of ALI. NADPH oxidase as a major source of reactive oxygen species decisively contributes to endothelial signaling and activation. The present study explores a novel mechanism underlying HS activation of lung endothelial NADPH oxidase and, thus, presents a new therapeutic target for ALI after hemorrhage and trauma.

NADPH oxidases are a group of multimeric enzymes, the activity of which results in the production of O_2^-. Neutrophil (polymorphonuclear leukocyte [PMN]) NADPH oxidase is the best-characterized enzyme (6), which consists of p40^{phox}, p47^{phox}, and p67^{phox}, which exist in cytosol as a complex, and p22^{phox} and gp91^{phox}, both of which are located in membranes of secretory vesicles and specific granules of PMNs. In addition, two GTP-binding proteins, Rap1A and Rac1/2, are involved in the activation of NADPH oxidase. Upon stimulation, p47^{phox} is phosphorylated, and the entire cytosolic complex migrates to the membrane, where it associates with cytochrome b_{558} to assemble the active oxidase (6). In nonphagocytic cells, including vascular endothelial cells (ECs), the homologs of the NADPH oxidase were also found (7). Although the phagocytic oxidase reduces molecular oxygen to O_2^- using NADPH as the electron donor, the nonphagocytic enzyme uses either NADH or NADPH as electron donor.

We have previously reported that oxidant signaling derived from the PMN NADPH oxidase complex is a critical regulatory factor to TNF-α–induced NF-κB activation, and the resultant inter-cellular adhesion molecule 1 (ICAM-1) expression in ECs through PMN–EC interaction (8). Furthermore, we implicated PMN NADPH oxidase in the mechanism of Toll-like receptor (TLR) 2 up-regulation induced by LPS–TLR4 signaling in ECs (9). These findings point to an important regulatory role of the

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extracellular release of ROS from PMN NADPH oxidase in modulating the activation of ECs.

Within the EC redox network, NADPH oxidase is situated downstream to many important modulators of EC physiology, including vascular endothelial growth factor (10), angiotensin II (11), transforming growth factor–β1 (12), and mechanical stimuli (13). However, the mechanism of NADPH oxidase activation in lung vascular ECs after HS is unclear. Based on our previous findings, we hypothesized that ROS derived from PMN NADPH oxidase might be important in mediating or enhancing HS-induced activation of lung endothelial NADPH oxidase.

In the present study, we demonstrate that HS-induced lung EC NADPH oxidase activation depends on high mobility group box 1 (HMGB1)/TLR4; that functional impairment of PMN NADPH oxidase or deletion of PMNs in mice markedly decreases HS-induced activation of lung EC NADPH oxidase; and that replenishment of NADPH oxidase–deficient mice with PMNs obtained from wild-type (WT) mice subjected to HS results in restored activation of lung EC NADPH oxidase. The latter findings were recapitulated in mouse lung vascular ECs (MLVECs) cocultured with PMNs isolated from WT HS mice. Thus, oxidant signaling by PMN NADPH oxidase is an important determinant of activation of endothelial NADPH oxidase after HS.

MATERIALS AND METHODS

HS and Resuscitation

Male C57BL/6 WT mice, gp91^{phox} knockout (gp91$^{phox-/-}$) mice, TLR4-mutant C3H/HeJ mice, and TLR4 WT C3H/HeOuJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of VA Pittsburgh Healthcare System. HS was initiated by blood withdrawal from femoral artery and reduction of the mean arterial pressure to 30 mm Hg. After a hypotensive period of 2 hours, animals were resuscitated with the shed blood and an equal volume of Ringer's lactate. Sham animals underwent the same surgical procedures without hemorrhage and resuscitation. In some experiments, mice were injected intraperitoneally with neutralizing antibodies against HMGB1 (600 μg per mouse) or nonimmune control IgG 10 minutes before hemorrhage. At 0–6 hours after resuscitation, before lung tissue harvesting, leukocytes in the alveoli and pulmonary circulation were removed by bronchoalveolar lavage and flushing of the lung vasculature through the right ventricle using PBS (*see* the online supplement for details).

In Vivo Neutrophil Depletion and Repletion

At 16 hours before HS, 10 μg of monoclonal anti-mouse Ly-6G/Gr1 antibody (eBioscience, San Diego, CA) (14) or control rabbit anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) was administered intraperitoneally to mice to deplete circulating PMNs (9). PMN repletion in neutropenic mice was performed by tail vein injection of PMNs ($\sim 2 \times 10^6$ cells) isolated from WT or gp91$^{phox-/-}$ mice that were subjected to either HS or sham operation. PMNs were isolated by the immunomagnetic method. Please see the online supplement for details.

MLVEC Isolation and Characterization

MLVECs were isolated using a previously described method (15). Please see the online supplement for details.

PMN–MLVEC Coincubation

PMNs and MLVECs were coincubated in Transwell plates and stimulated with HMGB1 (0.5μg/ml) for up to 4 hours at a PMN concentration of 1×10^6 cells/ml medium (*see* the online supplement for details).

Immunoprecipitation and Detection of Phosphorylated p47^{phox} and p47^{phox}- gp91^{phox} Binding

Mouse lung tissue or MLVECs were homogenized or lysed ($\sim 1 \times 10^6$ cells/ml) in lysis buffer. The same amount of total protein ($\sim$ 600 μg) for each sample was then immunoprecipitated with anti-p47^{phox} antibody (16). The immunoprecipitated proteins were then subjected to immunoblotting analysis using anti-phosphoserine antibody (Invitrogen, South San Francisco, CA) (17) and anti-gp91^{phox} antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), respectively (please *see* the online Supplement for details).

Measurement of Superoxide Generation in Live MLVECs

Live MLVECs were stained with the cell-permeable ROS detection reagent, H2DFFDA (Invitrogen Molecular Probes, Carlsbad, CA) as described previously (15). (See "Online Supplemental Material" for the details).

Measurement of Myeloperoxidase in the Lung

The myeloperoxidase (MPO) concentration in sonicated whole-lung homogenates was measured by ELISA (Cell Sciences, Canton, MA) according to the manufacturer's directions.

Data Presentation and Statistical Analysis

The data are presented as means (±SEM) of the indicated number of experiments. Statistical significance among group means was assessed by ANOVA. Student-Neuman-Keuls *post hoc* test was performed. Differences were considered significant at a P value less than 0.01.

RESULTS

HS Induction of Phosphorylation of p47^{phox} in the PMNs and Lung

Phosphorylation of p47^{phox} subunit of NADPH oxidase is an initial step of the enzyme activation, and, consequently, the entire cytosolic complex migrates to the membrane, where it associates with cytochrome b_{558} to assemble the active oxidase (6). Thus, either phosphorylation of p47^{phox} or binding of p47^{phox}-gp91 ^{phox} represents an activation of NADPH oxidase in cells. We first examined the phosphorylation of p47^{phox} in circulating PMNs and lung 1–6 hours after HS. As shown in Figure 1A, p47^{phox} phosphorylation in the PMNs was induced by HS at 1 hour, and further increased at 4 and 6 hours. However, p47^{phox} phosphorylation in the lung exhibited a delayed induction, starting at 2 hours after HS.

Our previous studies have shown that HMGB1 mediates HS-induced activation of PMN NADPH oxidase through the TLR4 signaling pathway (18). To determine if HMGB1 is also involved in the HS-induced phosphorylation of p47^{phox} in the lung, neutralizing antibody to HMGB1 was administered to mice before hemorrhage and resuscitation. Treatment with anti-HMGB1 antibody caused a reduction of p47^{phox} phosphorylation in the lung at 4 hours after resuscitation compared with that seen in animals treated with nonspecific IgG (Figure 1B). No differences in p47^{phox} phosphorylation were observed in animals subjected to sham operation, and this phosphorylation was unaffected by treatment with anti-HMGB1 antibody.

Additionally, in the lung harvested from TLR4-mutant mice, HS-induced p47^{phox} phosphorylation was markedly attenuated (15% of the phosphorylation evident in TLR4 WT lung [Figure 1B]). We observed no statistically significant difference in the extent of p47^{phox} phosphorylation between C3H/HeOuJ (the wild-type strain corresponding to C3H/HeJ) and C57BL/6 mice in response to HS (data not shown).

PMN NADPH Oxidase Contributes to Augmented Activation of EC NADPH Oxidase after HS

We have shown previously that PMNs and PMN-derived oxidants play an important role in mediating the up-regulation of TLR2 in lung ECs that is induced subsequent to TLR4

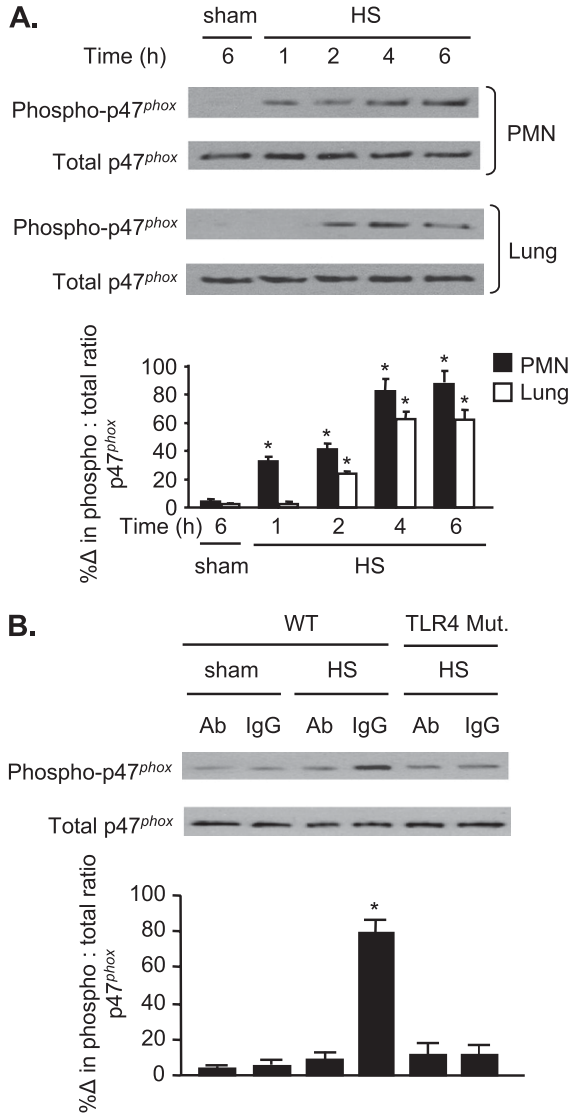

Figure 1. Hemorrhagic shock (HS) induction of phosphorylation of p47^{phox} in the polymorphonuclear leukocyte (PMN) and lung. (*A*) Wild-type (WT; C57BL/6) mice were subjected to HS or sham operation. PMN and lung tissue were obtained 1, 2, 4, and 6 hours after HS, or 6 hours after sham operation. Total and phospho-p47^{phox} in the PMN and lung were detected using immunoprecipitation and immunoblotting, as described in MATERIALS AND METHODS. The *graph* depicts the mean and SEM of the changes in ratio of phospho:total p47^{phox} from five mice. *$P < 0.01$ compared with the groups labeled with no *asterisk*. (*B*) high mobility group box 1 (HMGB1) mediates HS-induced p47^{phox} phosphorylation in the lung. WT mice and Toll-like receptor (TLR) 4–mutant mice received anti-HMGB1 antibody (600 μg per mouse) or nonimmune control IgG by intraperitoneal injection 10 minutes before hemorrhage or sham operation. Lung tissue was then collected from the mice at 4 hours after HS or sham operation for detection of total and phospho-p47^{phox} using immunoprecipitation and immunoblotting. The *images* are representative of four independent studies. The *graph* depicts the mean and SEM of the changes in ratio of phospho: total p47^{phox} from four mice. *$P < 0.01$ compared with all other groups.

signaling (9). Accordingly, we hypothesized that HS-activated PMNs might contribute to the augmented activation of NADPH oxidase in lung ECs through PMN–EC interaction. To test this hypothesis, we first evaluated the changes in HS-induced phosphorylation of p47^{phox} in the lung after depletion of circulating PMNs. In some cases, we replenished the PMNs in the mice made neutropenic during the resuscitation phase after hemorrhage, to validate the causal role of PMNs in mediating this phenomenon. As shown in Figure 2A, at 4 hours after HS, neutropenia induced in mice subject to HS was associated with an approximately 71% reduction in phosphorylation of p47^{phox} in the lung (Figure 2A, lane 8) as compared with mice subjected to HS with no PMN depletion (Figure 2A, lane 7). In contrast, depletion of PMNs did not alter the low levels of p47^{phox} phosphorylation in the lung of sham animals (Figure 2A, lane 2). Repletion with WT PMNs isolated from animals subjected to HS restored the p47^{phox} phosphorylation in the lung in response to HS (Figure 2A, lane 11). However, repletion with WT PMNs derived from sham-operated animals failed to restore the p47^{phox} phosphorylation (Figure 2A, lane 12).

To further define the role of PMN NADPH oxidase in the activation of EC NADPH oxidase, we replenished neutropenic WT HS mice with gp91$^{phox-/-}$ PMNs. As shown in Figure 2A, PMNs collected from gp91$^{phox-/-}$ mice that were subjected to either sham or shock failed to restore p47^{phox} phosphorylation in the lung (Figure 2A, lanes 9 and 10).

The lung samples that were used for determining p47^{phox} phosphorylation were flushed through bronchoalveolar lavage and received a right ventricular injection of PBS to remove PMNs and other myeloid cells before tissue harvesting. However, to confirm whether residual phagocytes in the lungs accounted for the observed increase in p47^{phox} phosphorylation in the lungs, MPO concentration was measured in the lungs that were either flushed or unflushed. As shown in Figure 2B, the MPO level in unflushed lungs increased in a time-dependent manner after HS. However, in the flushed lungs, the MPO activities in HS groups were the same as that in the sham animals, suggesting that the increase in p47^{phox} phosphorylation in the lungs after HS is not originated from recruited PMNs.

To further confirm this observation, PMNs in gp91$^{phox-/-}$ mice were replaced with WT PMNs using PMN depletion and repletion techniques, followed by being subjected to HS. Binding of p47^{phox} and gp91^{phox} in the lungs at 4 hours after HS was then detected after lung flushing. Figure 2C shows that WT PMNs did not alter the p47^{phox}-gp91^{phox} binding in the gp91$^{phox-/-}$ lungs, further suggesting that the observed increase in p47^{phox} phosphorylation in the lungs originated from lung parenchymal cells, but not from PMNs.

The role of PMN NADPH oxidase in enhancing EC NADPH oxidase activation was further addressed by using a PMN–EC coculture system. ECs from WT mice were cocultured with PMNs that were isolated from either WT or gp91$^{phox-/-}$ mice in the presence of HMGB1 for 2 hours. In ECs, p47^{phox} is confirmed to be critical in modulating enzymatic activity by interacting with catalytic unit of gp91^{phox} (19). Thus, the alterations in p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation in the ECs were measured by immunoprecipitation–immunoblotting. As shown in Figure 3, a constitutive binding of p47^{phox} and gp91^{phox} in the MLVECs could be detected in the absence of stimulation with HMGB1. HMGB1 induced a slight increase in the p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation in the MLVECs incubated without PMNs. However, the coculture of MLVECs with WT PMNs, but not gp91$^{phox-/-}$ PMNs, caused an enhanced p47^{phox}-gp91^{phox} binding in the MLVECs in response to HMGB1. In TLR4-mutant MLVECs, coincubation with WT PMNs and stimulation with HMGB1 did not result in a significant induction of the binding of p47^{phox}-gp91^{phox} and p47^{phox} phosphorylation, which further confirmed the role of TLR4 in mediating HS activation of EC NADPH oxidase.

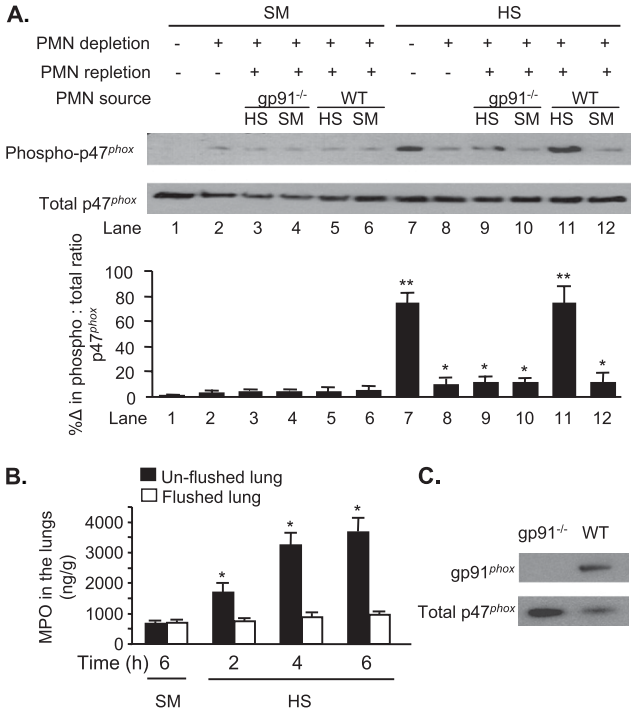

Figure 2. PMN reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase signals HS-induced activation of endothelial cell (EC) NADPH oxidase. (*A*) Effect of PMN depletion and repletion on the HS-induced p47^{phox} phosphorylation in the lung. PMN depletion was performed as described in the MATERIALS AND METHODS 16 hours before hemorrhage or sham (SM) operation. PMN repletion was performed in neutropenic mice during resuscitation using tail vein injection of PMNs that were isolated from blood of WT or gp91$^{phox-/-}$ mice subjected to sham or HS. The lungs were harvested from the mice 4 hours after resuscitation or sham operation for the detection of p47^{phox} phosphorylation by immunoprecipitation and immunoblotting. The *graph* depicts the mean and SEM of the changes in ratio of phospho:total p47^{phox} from four mice. **$P < 0.01$ compared with all other groups; *$P < 0.01$ compared with the groups labeled with no *asterisk*. (*B*) Myeloperoxidase (MPO) concentration in the unflushed and flushed lungs after HS. MPO concentration in the lungs, which were flushed through bronchoalveolar lavage (BAL) and received a right ventricular injection of PBS to remove PMNs and other myeloid cells before the tissue harvesting, was measured by ELISA, and compared with that in the lungs that were not flushed (unflushed lung). The lungs were collected at 2, 4, and 6 hours after HS, or 6 hours after sham operation (SM). The *graph* depicts the mean and SEM of the changes in the lung MPO concentration from four mice. *$P < 0.01$ compared with the groups labeled with no *asterisk*. (*C*) PMN-derived p47^{phox}-gp91^{phox} binding was undetectable in the flushed lung. PMNs in gp91$^{phox-/-}$ mice were replaced with WT PMNs using PMN depletion and repletion techniques before the mice were subjected to HS. At 4 hours after HS, the lungs were flushed through BAL and received a right ventricular injection of PBS, and the p47^{phox}-gp91^{phox} binding in the lungs was then measured by immunoprecipitation and immunoblotting. The gel as such is a representative of three independent experiments.

To address the role of PMN-derived oxidants in the HMGB1-induced activation of NADPH oxidase in ECs, the membrane-permeable antioxidant, PEG catalase (1,000 U/ml), was applied to the coculture system. As shown in Figure 3, polyethylene glycol (PEG) catalase attenuated the enhancement in NADPH oxidase activation in the MLVECs stimulated by WT PMNs. Taken together, these results demonstrate a critical role of PMN NADPH oxidase in activating and augmenting p47^{phox} phosphorylation in lung ECs.

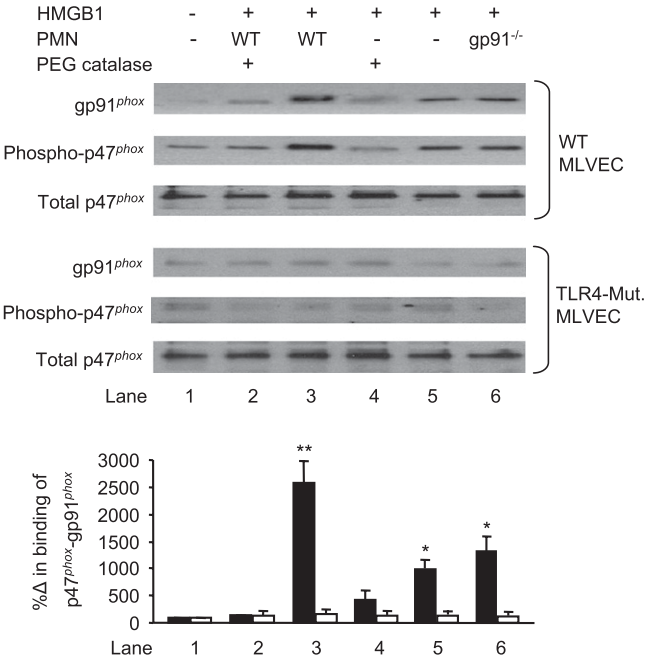

Figure 3. Effect of oxidants derived from PMN NADPH oxidase on HMGB1-induced binding of p47^{phox}-gp91^{phox} and p47^{phox} phosphorylation in mouse lung vascular ECs (MLVECs). WT and TLR4-mutant MLVECs were coincubated with PMNs that were isolated from the blood of WT or gp91$^{phox-/-}$ (gp91$^{-/-}$) mice in the presence or absence of HMGB1 (0.5 μg/ml) for 2 hours. Membrane-permeable polyethylene glycol (PEG) catalase (1,000 U/ml) was applied to the coculture system as indicated. The MLVECs were then recovered for the detection of p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation using immunoprecipitation and immunoblotting. The *graph* depicts the mean and SEM of the percentage changes in the p47^{phox}-gp91^{phox} binding in the MLVECs isolated from WT (*solid bars*) and TLR4 mutant (*open bars*), which were normalized by the density of total p47^{phox}, from four experiments. **$P < 0.01$ compared with all other groups; *$P < 0.01$ compared with the groups labeled with no *asterisk*.

ROS Mediate the Interaction of PMN and EC NADPH Oxidases

Because we found that an antioxidant prevented the augmented activation of EC NADPH oxidase induced by HS-activated PMNs, we next tested the role of ROS in mediating the activation of EC NADPH oxidase. MLVECs were treated with HMGB1 for 1 hour in the presence or absence of H_2O_2 to mimic HS-activated PMNs. As shown in Figure 4A, H_2O_2 enhanced the p47^{phox}-gp91^{phox} binding in response to HMGB1.

To determine if the activation of NADPH oxidase in ECs is functionally associated with an increase in intracellular ROS production, the ROS in live MLVECs were directly detected using fluorescence microscopy. The cells were observed for 2 hours after treatment with HMGB1 and/or H_2O_2. Figure 4B shows that the changes in ROS production in the MLVECs were consistent with the alterations in the p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation shown in Figure 4A. To define the source of the ROS, the NADPH oxidase inhibitor, diphenyleneiodonium (100 μM), was added to the MLVECs immediately before the treatment of HMGB1 and H_2O_2. Diphenyleneiodonium markedly diminished the ROS production in the MLVECs in response to HMGB1 and H_2O_2 (Figure 4B), suggesting that the observed ROS production is derived mainly from NADPH oxidase.

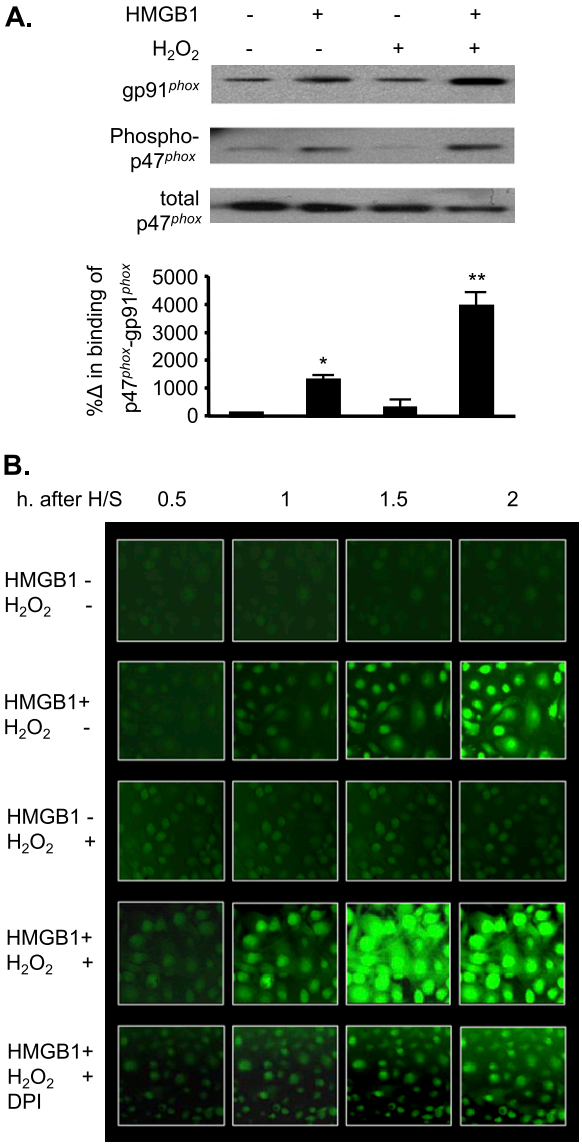

Figure 4. Role of H_2O_2 in enhancing NADPH oxidase activation and reactive oxygen species (ROS) production in MLVECs. WT MLVECs were treated with HMGB1 (0.5 μg/ml) in the presence or absence of H_2O_2 (250 μM) for 1 hour. The MLVECs were then recovered for the detection of p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation using immunoprecipitation and immunoblotting. H_2O_2 enhanced the p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation in response to HMGB1. The *graph* depicts the mean and SEM of the percent changes in the p47^{phox}-gp91^{phox} binding, which were normalized by the density of total p47^{phox}, from four experiments. $^{**}P < 0.01$ compared with all other groups; $^{*}P < 0.01$ compared with the groups labeled with no *asterisk*. (*B*) ROS production in live MLVECs. WT MLVECs that were cultured in 12-well cell culture plates were stained with the cell-permeable ROS detection reagent, H2DFFDA, in the concentration of 10 μM for 10 minutes. The cells were then washed three times with Hanks' balanced salt solution, followed by incubation in the growth medium in the presence or absence of HMGB1 (0.5 μg/ml) and/or H_2O_2 (250 μM) for 2 hours. The ROS production was detected by fluorescence microscopy at different time points, as indicated. To define the source of the ROS, the NADPH oxidase–specific inhibitor, diphenyleneiodonium (DPI; 100 μM), was added to the MLVECs immediately before the treatment of HMGB1 and H_2O_2. The *images* are representative of four independent studies.

Rac1 and p38 Mitogen-Activated Protein Kinase Contribute Differently to the HMGB1-Induced Activation of EC NADPH Oxidase

Rac1, Akt, and p38 mitogen-activated protein kinase (MAPK) have been reported to be involved in p47^{phox} phosphorylation in cells in response to different mediators (20). We therefore tested the role of Rac1, Akt, and p38 MAPK in HMGB1-induced NADPH oxidase activation in MLVECs. Inhibitors of Rac1 (NSC23766, 100 μM; EMD Biosciences, Inc., Darmstadt, Germany), Akt (4 μM; BioVision, Mountain View, CA), and p38 MAPK (SB202190, 2 μM; EMD Biosciences) were added to MLVECs. The cells were then treated with HMGB1 and/or H_2O_2 for 1 hour. As shown in Figure 5, inhibitors of Rac1 and p38 MAPK, but not of Akt, markedly attenuated the p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation in response to HMGB1 alone. However, although the inhibitor of Rac1 decreased the binding of p47^{phox}-gp91^{phox} and p47^{phox} phosphorylation in the ECs in response to HMGB1 and H_2O_2, inhibitors of p38 MAPK and Akt failed to exhibit any effect. These results suggest that a different signaling pathway mediates HMGB1-activated and oxidant-enhanced NADPH oxidase activation in ECs.

DISCUSSION

The cell–cell interactions underlying post-trauma acute lung inflammation and injury are complex (8, 18, 21–25). In the present study, we show that oxidant signaling by the PMN

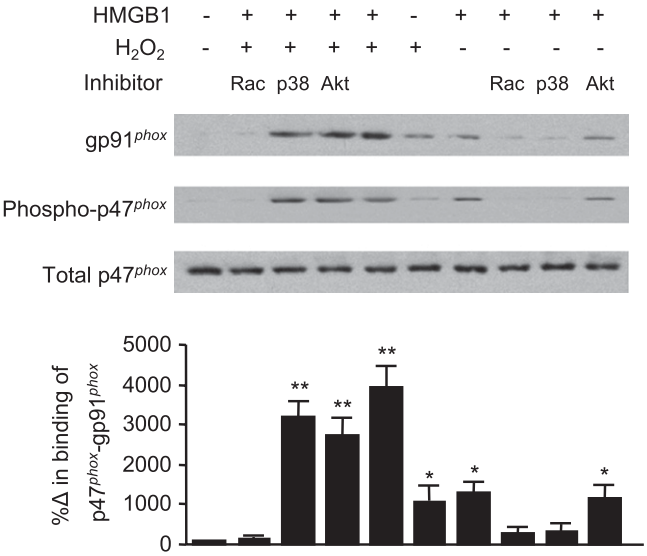

Figure 5. Effect of inhibitors on HMGB1 and/or H_2O_2 induction of NADPH oxidase activation in MLVECs. Inhibitors of Rac1 (NSC23766, 200 μM), Akt (4 μM), and p38 mitogen-activated protein kinase (MAPK) (SB202190, 4 μM) were added to WT MLVECs, which were then treated with HMGB1 and/or H_2O_2 for 1 hour. Inhibitors of Rac1 and p38 MAPK, but not inhibitor of Akt, markedly attenuated the p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation in response to HMGB1 alone. In the groups treated with HMGB1 and H_2O_2, the inhibitor of Rac1 decreased the p47^{phox}-gp91^{phox} binding and p47^{phox} phosphorylation, whereas inhibitors of p38 MAPK and Akt failed to exhibit an effect in the MLVECs. The *graph* depicts the mean and SEM of the percent changes in the p47^{phox}-gp91^{phox} binding, which were normalized by the density of total p47^{phox}, from four experiments. $^{**}P < 0.01$ compared with all other groups; $^{*}P < 0.01$ compared with the groups labeled with no *asterisk*.

NADPH oxidase plays an important role in mediating amplified HS-induced activation of lung EC NADPH oxidase. Our findings suggest that ROS may enhance the activation of EC NADPH oxidase in response to HS through a signaling pathway involving HMGB1, TLR4, and Rac1, but independent of p38 MAPK. These findings are also in keeping with the emerging role of NADPH oxidase in various signaling processes (26).

ROS produced in ECs function as signaling molecules to mediate various biological responses, such as gene expression, cell proliferation, migration, angiogenesis, apoptosis, and senescence (27). Excessive production of ROS contributes to various pathophysiological responses, including endothelial dysfunction, atherosclerosis, hypertension, diabetes, and acute respiratory distress syndrome (ARDS) (27). We have reported recently that HS augments lung EC activation in a form of increasing EC adhesion molecule inter-cellular adhesion molecule 1 (ICAM-1) expression and ROS production through sensitization of the responses of ECs to HMGB1 (15).

Despite those findings, the question of how HS leads to the activation of the NADPH oxidase in lung ECs remained open. We have previously reported a mechanism of PMN NADPH oxidase activation after HS (18). We showed that HMGB1/TLR4 signaling mediates HS-induced PMN NADPH oxidase activation, which involves both myeloid differentiation primary response gene 88 (MyD88)-interleukin-1 receptor-associate kinase (IRAK) 4–p38 MAPK and MyD88–IRAK4–Akt signaling pathways. In the present study, the role of PMN NADPH oxidase in mediating augmented activation of EC NADPH oxidase was delineated using *in vivo* and *in vitro* approaches. The results demonstrate that, in neutropenic mice, the HS-induced phosphorylation of $p47^{phox}$ in the lung was significantly reduced. This response was restored upon repletion with PMNs obtained from WT mice subjected to HS, but not with PMNs from either WT sham-operated mice or HS mice lacking the $gp91^{phox}$ subunit of NADPH oxidase. Noteworthy, HS-induced NADPH oxidase activation mainly through regulating the enzyme's intrinsic activity rather than up-regulating the expression of the enzyme's components. As shown in Figure 1, HS induced phosphorylation of $p47^{phox}$, but did not alter the level of total $p47^{phox}$. In addition, the changes of MPO activities in the unflushed lungs, as shown in Figure 2B, provide evidence showing that PMNs physically exist in the lungs. The findings were recapitulated in MLVECs cocultured with PMNs isolated from WT mice, suggesting that PMN NADPH oxidase is an important determinant in mediating PMN–EC interaction and the resultant activation of EC NADPH oxidase. Although ROS derived from PMN NADPH oxidase are important, the ROS from other enzymes in PMNs may also play a certain role in inducing NADPH oxidase activation in lung ECs. As shown in Figure 3, HMGB1 can also induce NADPH oxidase activation in the MLVECs that were cocultured with $gp91^{phox-/-}$ PMNs, although the induction is lower than that in the MLVECs cocultured with WT PMNs.

The origination of the observed increase in NADPH oxidase activation in the lungs was addressed in the study. PMN-originated $p47^{phox}$ phosphorylation in the lungs was excluded by two lines of evidence. First, in the flushed lungs, the level of MPO activity was very low and similar to that in sham animals, suggesting an efficient removal of PMNs from the lung tissue. Second, in the $gp91^{phox-/-}$ mice that were replenished with WT PMNs, the $p47^{phox}$-$gp91^{phox}$ binding in WT PMNs could not be detected in the lungs after lung flushing. Indeed, by identifying specific NADPH oxidases (NOXs) to separate NADPH oxidase in PMNs and ECs would be ideal. However, functions of other newly identified $gp91^{phox}$ homologs (i.e. Nox1, Nox4, and Nox5) remain obscure. A recent study reported that Nox4 is more abundantly expressed in ECs compared with other Nox proteins, and thus represents the major catalytic unit of the endothelial NADPH oxidase that is activated by growth halting (28, 29). Nox1, on the other hand, was up-regulated by oscillatory shear stress, mediating ROS-dependent leukocyte adhesion to endothelium (30). In addition, vascular endothelial growth factor receptor–dependent activation of Nox1 was angiogenic, and responsible for tube formation of ECs (31).

It is noteworthy that the current study addressed a role of exogenous ROS from PMN NADPH oxidase in the early stage of activation of EC NADPH oxidase after HS. However, it is plausible that endogenous ROS from EC NADPH oxidase may also play a role in the subsequent activation of the EC NADPH oxidase itself, as shown in Figure 3, that antioxidants can also attenuate HMGB1 only–induced $p47^{phox}$-$gp91^{phox}$ binding and $p47^{phox}$ phosphorylation. A more extensive study will be needed to clarify this point.

HMGB1 is a mediator of organ injury in animal models of infection and endotoxemia (32–34). Intratracheal administration of HMGB1 alone can induce organ dysfunction in the form of acute lung injury (32). However, no role had been ascribed to HMGB1 in the activation of lung EC NADPH oxidase in a setting of HS, a process that 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 patients with HS (34, 35). Studies from our group have also shown increased HMGB1 levels in serum, lungs, and liver at 2 hours after HS (18), and demonstrated that neutralization of HMGB1 reduced the inflammatory and organ damage responses seen in mice after various forms of sterile injury (35, 36). HMGB1 therefore serves not only as a damage-associated molecular pattern in trauma and HS (37, 38), but also as an endogenous signal for the activation of EC NADPH oxidase. Regarding the source of HMGB1, we have reported that β-adrenergic receptor activation by catecholamine of macrophages mediates the HS-induced release of HMGB1 in this animal model (25). However, it is possible that HMGB1 is released from a number of sources in response to ischemia or other stress (39).

Although the receptor for advanced glycation end products had been originally identified as a receptor for HMGB1 in neurites and malignant cells (40), both TLR4 and TLR2 are important in mediating HMGB1-induced inflammatory responses (41–43). In the present study, TLR4 exhibited a key role in mediating HMGB1-induced NADPH oxidase activation, as demonstrated by greatly diminished binding of $p47^{phox}$-$gp91^{phox}$ in response to both *in vivo* HS and *in vitro* HMGB1 stimulation in TLR4-mutant mice and MLVECs. Because the absence of functional TLR4 signaling was insufficient to eliminate the effect of HS and HMGB1 completely (Figures 1B and 3), we cannot rule out the possibility that HMGB1 may also act through other receptors, such as TLR2 or receptor for advanced glycation end products.

The signaling by which HS culminates in activation of EC NADPH oxidase has not been addressed previously. A direct interaction of TLR4 with Nox4, a protein related to $gp91^{phox}$ in phagocytic cells, has been suggested as a mechanism for LPS-mediated Nox4 activation and ROS production in HEK293T cells (44). However, p38 MAPK, extracellular signal–regulated kinase 1/2, protein kinase C, and Akt have also been suggested as activators of phosphorylation of $p47^{phox}$ and $p67^{phox}$ in response to TNF, formyl-met-leu-phe, and bacteria (45, 46). Our results in this study suggest that ROS are a key factor in mediating the enhanced activation of EC NADPH oxidase. In MLVECs, either a Rac1 inhibitor or a p38 MAPK inhibitor

prevented HMGB1-induced binding of $p47^{phox}$-$gp91^{phox}$ and $p47^{phox}$ phosphorylation, suggesting that both Rac1 and p38 MAPK are in one signaling pathway. However, in the case of H_2O_2-augmented activation of EC NADPH oxidase in response to HMGB1, the results suggest a signaling pathway that is Rac1 dependent, but p38 MAPK independent. It is clear that NADPH oxidase activation consists of multiple steps that could be targeted by different kinases, thus explaining the tight control of NADPH oxidase. It is possible that oxidants may induce conformational changes in the molecules of NADPH oxidase that render the enzyme more accessible to other protein kinases. For example, $p47^{phox}$ phosphorylation *in vitro* can induce conformational changes of the protein and subsequent activation of NADPH oxidase (47, 48). Further studies will be needed to define the precise mechanisms responsible for redox–regulation of NADPH oxidase.

In summary, the present study demonstrates a novel function of PMN NADPH oxidase–derived oxidant signaling in mediating enhanced activation of EC NADPH oxidase in a setting of HS. PMN NADPH oxidase mediates this response, at least in part, through its ability to enhance Rac1 signaling, and thereby augment HMGB1-TLR4–induced activation of NADPH oxidase in lung ECs. These results indicate that the lung EC is an important target for PMN NADPH oxidase signaling activity after HS, resulting in the development of posthemorrhage lung inflammation.

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