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Hemorrhagic Shock Activation of NLRP3 Inflammasome in Lung Endothelial Cells

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Hemorrhagic shock (HS) due to major trauma and surgery predisposes the host to the development of systemic inflammatory response syndrome (SIRS), including acute lung injury (ALI), through activating and exaggerating the innate immune response. IL-1β is a crucial proinflammatory cytokine that contributes to the development of SIRS and ALI. Lung endothelial cells (EC) are one important source of IL-1β, and the production of active IL-1β is controlled by the inflammasome. In this study, we addressed the mechanism underlying HS activation of the inflammasome in lung EC. We show that high mobility group box 1 acting through TLR4, and a synergistic collaboration with TLR2 and receptor for advanced glycation end products signaling, mediates HS-induced activation of EC NAD(P)H oxidase. In turn, reactive oxygen species derived from NAD(P)H oxidase promote the association of thioredoxin-interacting protein with the nucleotide-binding oligomerization domain-like receptor protein NLRP3 and subsequently induce inflammasome activation and IL-1β secretion from the EC. We also show that neutrophil-derived reactive oxygen species play a role in enhancing EC NAD(P)H oxidase activation and therefore an amplified inflammasome activation in response to HS. The present study explores a novel mechanism underlying HS activation of EC inflammasome and thus presents a potential therapeutic target for SIRS and ALI induced after HS. *The Journal of Immunology, 2011, 187: 000–000.

Systemic inflammatory response syndrome (SIRS) and multiorgan failure are common complication following trauma, severe surgery, or hemorrhagic shock (HS) and result in high mortality and morbidity (1, 2). Acute lung injury (ALI) is an important component of multiorgan failure and often serves as a direct cause of death in these patients (3). HS promotes the development of ALI by inducing the immune system to produce an exaggerated inflammatory response to endogenous and exogenous danger signals (4–6). The lung vascular endothelium is an active organ that critically contributes to the pathogenesis of ALI following trauma, sepsis, and shock by affecting pulmonary and systemic homeostasis, including secretion of cytokines, chemokines, and adhesion molecules (7, 8). There is a significant gap in our knowledge concerning the mechanisms of HS regulation of lung endothelial cell (EC) activation and subsequent promotion of lung inflammation.

The cytokine IL-1β is a key proinflammatory mediator involved in the development of posttrauma SIRS (9–14). EC are one important source of IL-1β and conversely are also a target of IL-1β, which causes them to release a range of inflammatory molecules in response to IL-1β stimulation (7, 13, 15). Thus, EC are postulated to be an amplifier of inflammation through the sensing and release of IL-1β. The production of active IL-1β is tightly controlled by the formation and activation of the inflammasome, which is comprised of nucleotide-binding oligomerization domain-like receptors, caspase-1 (or caspase-5), and apoptosis-associated specklike protein containing a caspase activation recruiting domain (ASC) (16, 17). IL-1β is synthesized initially as an inactive precursor molecule (pro–IL-1β p35), which must be cleaved by caspase-1 at aa position 116 to produce the actively mature IL-1β (p17) that is then secreted in response to stimulating signals. Caspase-1 is also synthesized as an inactive 45-kDa protein (procaspase-1) that undergoes autocatalytic processing after assembly of the inflammasome in response to an appropriate stimulus (18). However, it is not clear how HS induces inflammasome activation and IL-1β maturation in the lung.

Reactive oxygen species (ROS) have been implicated as regulators of inflammasome activation (19). The major source of ROS within pulmonary EC is the nonphagocytic NAD(P)H oxidase (20), which is composed of membrane-bound gp91phox and p22phox, as well as cytosolic subunits such as p47phox, p67phox, and the small GTPase Rac. Endothelial NAD(P)H oxidase can be activated in many ways, including by growth factors, cytokines, shear stress, and hypoxia (21). We have recently reported that HS activates lung endothelial NAD(P)H oxidase through high mobility group box 1 (HMGB1)–TLR4 signaling (22). HMGB1 can be secreted by innate immune cells in response to microbial products or other inflammatory stimuli (23, 24), as well as be released by injured cells, and is a prototypical damage-associated molecular pattern molecule (25–27). HMGB1 has been identified as an inflammatory cytokine that mediates lethality in sepsis (23, 24), development of ALI after trauma/hemorrhage (28–

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HMGB1 knockout (NLRP3 Richard Flavell (Yale University) and bred in Dr. Billiar's laboratory; and mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

In the current study, we investigated the mechanism of HS regulation of the inflammasome in lung EC. We demonstrate that HS, through HMGB1, activates endothelial NAD(P)H oxidase, and the created oxidants cause thioredoxin (TRX)-interacting protein (TXNIP) to associate with NLRP3, leading to inflammasome activation and IL-1β secretion. We also show that HS-activated PMN and PMN NAD(P)H oxidase are required for augmented activation of the endothelial inflammasome through enhanced ROS production in lung EC. These findings reveal a pathway that links the global insult of HS to activation of the inflammasome molecules in lung EC.

Materials and Methods

Recombinant HMGB1 was purchased from R&D Systems (Minneapolis, MN). Stimulating activity of recombinant HMGB1 was confirmed in mouse macrophages by assay of TNF release, with an ED50 of 3–12 μg/ml. Polyclonal neutralizing Ab against HMGB1, prepared as described previously (24), was provided by Dr. K.J. Tracey (Feinstein Institute for Medical Research, North Shore-Long Island Jewish Health System, Manhasset, NY). Nonimmune rabbit IgG (catalog number 9006) and all other chemicals were obtained from Sigma–Aldrich, except where noted.

Mouse model of HS and resuscitation

Male C57BL/6 wild-type (WT) mice and ggP knockouts (ggP / ) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TL4 knockout (TL4 / ) mice, TL4 knockout (TLR2 / ) mice, and receptor for advanced glycation endproducts (RAGE) knockout (RAGE / ) mice were bred in Dr. Biliari’s laboratory at the University of Pittsburgh; caspase-1 knockout (caspase-1 / ) mice were originally provided by Dr. Richard Flavell (Yale University) and bred in Dr. Biliari’s laboratory; and NLRP3 knockout (NLRP3 / ) mice were obtained from Millennium Pharmaceuticals (Cambridge, MA). All mice used are on a C57BL/6 background. All experimental protocols involving animals were approved by Institutional Animal Care and Use Committee of Veterans Affairs Pittsburgh Healthcare System and University of Pittsburgh. Mice were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine i.p. into the mice 10 min before hemorrhage. The animals were kept against HMGB1 (600 μg/ml) for 0–6 h, washed with HBSS three times, and harvested for further analysis.

PMN–MLVEC coinoculation

PMN–MLVEC coinoculation was performed using Transwell plates (Corning Life Sciences, Acton, MA). The preparation of MLVEC and PMN was performed as described method (38) that was modified in our laboratory as follows: briefly, mice were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine i.p. The chest cavity was opened, and the right ventricle was cannulated. PBS was infused to remove blood from lungs. Peripheral lung tissue slices in a size ~1 mm3 were prepared and cultured in a 60-mm culture dish in growth medium (MEM D-Val medium [Invitrogen Gibco] containing 2 mM glutamine, 10% FBS, 5% human serum, 50 μg/ml penicillin/streptomycin, 5 μg/ml heparin, 1 mM hydrocortisone, 80 μg/ml endothelial cell growth supplement from bovine brain, 5 μg/ml amphotericin, and 5 μg/ml mycophenolic acid) at 37°C in 5% CO2. The adherent cells were continued in culture for 3 d after removal of the tissue slices, followed by purification using bioin-conjugated rat anti-mouse CD31 (PECAM-1) mAb and BD IMag streptavidin particles PLUS, and the immunomagnetic separation system (BD Pharmingen) following the manufacturer’s instructions. The cells were allowed to grow for 3 to 4 d after purification. The cells were characterized by their cobblestone morphology, uptake of 5% DUC-LDL (Biomolecular Technologies, Stoughton, MA), and staining for factor VIII-related Ag (Sigma Chemical, St. Louis, MO). MLVEC passed between three and five times were used in experiments in which cells were treated with HMGB1 (0.5 μg/ml) for 0–6 h, washed with HBSS three times, and harvested for further analysis.

Caspase-1 depletion in MLVEC

MLVEC (104 to 105 cells) were seeded onto the chamber slide in a 35-mm petri dish and cultured in the EC culture media for 12 h at 37°C. MLVEC were then washed twice with serum-free DMEM and incubated in serum-free DMEM for 30 min. The MLVEC were incubated with 0.5 μg/ml HMGB1 for 0–6 h followed by staining of the cells with 1 × cell permeable Caspase-1 carboxyfluorescein-labeled fluorochrome inhibitor of caspase-1 (ImmunoChemistry Technologies, Bloomingon, IN), which binds to activated caspase-1, in serum-free DMEM for 1 h. The cells were then washed three times with PBS, fixed with 4% parafomaldehyde in PBS for 15 min at room temperature and further washed with ice-cold PBS three times. The nuclei of the cells was stained with 1 μg/ml Hoechst for 15 min at room temperature in the dark. The green fluorescence of caspase-1–positive cells was imaged by confocal microscope with excitation at 480 nm, and Hoechst-stained nuclei were observed with excitation at 365 nm.

In vivo neutrophil depletion and repletion

PMN depletion was induced using RB6-8C5 mAb (Ly-6G/Gr-1–specific) (eBioscience, San Diego, CA) (35). At ~16 h before performing shock or sham operation, 10 μg anti-mouse Ly-6G/Gr-1 Ab or control Ab (rabbit anti-mouse IgG) was administered i.p. to mice in PBS (35). Our previous studies have shown that during the period of 16–24 h after injection of anti-mouse Ly-6G/Gr-1 Ab, the circulating PMN count in the Ab-treated group was decreased to 0.08 ± 0.02% of total WBCs versus 22.2 ± 1.9% in the control group (36). There were no statistically significant differences in the number of peripheral lymphocytes, atypical lymphocytes, monocytes, or eosinophils between the Ab-treated and control groups (36).

To determine the role of PMN NAD(P)H oxidase in the mechanism of HS-induced activation of lung EC inflammasome, PMN depletion in neutropenic mice was performed by tail vein injection of PMN (~2 × 106 cells) isolated from WT or ggP / mice that were subjected to either HS or sham operation. An immunomagnetic separation system (BD Pharmingen, San Diego, CA) (37) was used to isolate PMN. Viability of the isolated PMN was >95% and PMN purity was assessed by trypan blue exclusion and Wright–Giemsa staining, respectively.

Mouse lung vascular EC isolation and characterization

Mouse lung vascular EC (MLVEC) were isolated using a previously described method (38) that was modified in our laboratory as follows: briefly, mice were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine i.p. The chest cavity was opened, and the right ventricle was cannulated. PBS was infused to remove blood from lungs. Peripheral lung tissue slices in a size ~1 mm3 were prepared and cultured in a 60-mm culture dish in growth medium (MEM D-Val medium [Invitrogen Gibco] containing 2 mM glutamine, 10% FBS, 5% human serum, 50 μg/ml penicillin/streptomycin, 5 μg/ml heparin, 1 mM hydrocortisone, 80 μg/ml endothelial cell growth supplement from bovine brain, 5 μg/ml amphotericin, and 5 μg/ml mycophenolic acid) at 37°C in 5% CO2.

The adherent cells were continued in culture for 3 d after removal of the tissue slices, followed by purification using bioin-conjugated rat anti-mouse CD31 (PECAM-1) mAb and BD IMag streptavidin particles PLUS, and the immunomagnetic separation system (BD Pharmingen) following the manufacturer’s instructions. The cells were allowed to grow for 3 to 4 d after purification. The cells were characterized by their cobblestone morphology, uptake of 5% DUC-LDL (Biomolecular Technologies, Stoughton, MA), and staining for factor VIII-related Ag (Sigma Chemical, St. Louis, MO). MLVEC passed between three and five times were used in experiments in which cells were treated with HMGB1 (0.5 μg/ml) for 0–6 h, washed with HBSS three times, and harvested for further analysis.

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Measurement of IL-1β

IL-1β in BAL fluid and cell-culture media was measured using the ELISA Ready-Set-Go kit for mouse IL-1β (eBioscience) following the manufacturer’s instructions.

Measurement of superoxide generation in live MLVEC

Live MLVEC cultured in 12-well cell-culture plates were stained with the cell-permeable ROS detection reagent H2DFFDA (Invitrogen Molecular Probes, Carlsbad, CA) at the concentration of 10 μM for 10 min. Cells were then washed with HBSS three times followed by incubation in the growth medium in the presence or absence of HMGB1 (0.5 μg/ml) for 0.5–2 h. ROS production was then detected by fluorescence microscopy at different time points.

Transfection of small interfering RNA in MLVEC

TXNIP small interfering RNA (siRNA), control siRNA, and transfection kit were purchased from Santa Cruz Biotechnologies. MLVEC (2 × 10^5 cells) were seeded in a six-well tissue-culture plate and incubated at 37°C in a CO2 incubator until the cells were 80% confluent. The cells were then transfected with TXNIP siRNA or control siRNA using the siRNA transfection kit following the manufacturer’s instructions. At 24–72 h after the transfection, TXNIP expression in the transfected cells was analyzed by Western blot. Because we observed a confirmed knockdown of TXNIP in the EC at 48 h after siRNA transfection, we set this time point as time 0 for the experiments using HMGB1 treatment.

Data presentation and statistical analysis

The data are presented as mean ± SEM of the indicated number of experiments. Statistical significance among group means was assessed by ANOVA. The Student Neuman–Keuls post hoc test was performed. Differences were considered significant at p < 0.05.

Results

HS activates NLRP3 inflammasome in the lung

The assembly of inflammasome components including NLRP3, ASC, and procaspase-1 is an initial step of inflammasome activation and subsequent procaspase-1 cleavage to an active form, leading to pro–IL-1β cleavage to produce mature IL-1β (39). Thus, the association of NLRP3 and ASC, as well as caspase-1 cleavage, represents intracellular activation of inflammasome. We first determined the ability of HS to induce inflammasome activation in the lung using the mouse model of HS/R as described in the Materials and Methods. Lung tissue and BAL fluid were recovered 1, 2, 4, and 8 h after HS/R. As shown in Fig. 1A, the association of NLRP3 and ASC in WT lung tissue was induced at 1 h after HS/R and further increased between 2 and 8 h. Caspase-1 cleavage was detected starting at 2 h, reached a peak at 4 h, and was maintained up to 8 h after HS/R. IL-1β concentration in BAL fluid, which represents the released IL-1β from pulmonary cells, was elevated at 2 h after HS/R and sustained at a high level for at least 8 h. However, the HS-induced release of IL-1β was absent in NLRP3−/− mice and caspase-1−/− mice (Fig. 1B). Additionally, lack of caspase-1 also prevented the association of NLRP3 and ASC (Fig. 1A). Altogether, these data demonstrate NLRP3 inflammasome-dependent and caspase-1–dependent mechanisms of IL-1β release at an early stage following HS/R.

HMGB1 is responsible for HS-induced lung NLRP3 inflammasome activation

We have previously reported that HS/R causes a significant increase of HMGB1 in the serum, lung, and liver at 2 h after HS/R (31). To determine if endogenous HMGB1 contributes to HS/R-induced inflammasome activation in the lung, neutralizing Ab to HMGB1 was administered to mice 15 min before HS/R. Treatment with anti-HMGB1 Ab resulted in a significant decrease in the HS/R-induced NLRP3–ASC association in the lung and IL-1β concentration in BAL fluid at 4 h after HS/R as compared with nonspecific IgG-treated animals (Fig. 2A, 2B).

Receptors include TLR4, TLR2, and RAGE. We thus further defined the role of HMGB1 and its receptors by using TLR4−/−, TLR2−/−, and RAGE-deficient mice. The results show that knockout of TLR4 prevented the HS/R-induced inflammasome activation and IL-1β secretion in the lungs. WT (C57BL/6) mice, NLRP3−/− mice, and caspases-1−/− mice were subjected to HS/R (HS) or sham operation (SM). Lung tissue and BAL fluid were recovered 1, 2, 4, and 8 h after HS/R or 1 and 8 h after sham operation. A, The association of NLRP3 and ASC was detected using immunoprecipitation with anti-ASC Ab followed by immunoblotting for NLRP3 and ASC, as well as for caspase-1 cleavage product p10 fragments. Western blotting as described in Materials and Methods. The images are representatives of five independent experiments. B, IL-1β in BAL fluid was measured with ELISA. The graph shows the mean and SEM from five mice. *p < 0.01 compared with the groups labeled with no asterisk, **p < 0.01 compared with all other groups.

HS activates PMN augmentation of inflammasome in lung EC

We have previously shown that PMN and PMN-derived oxidants play an important role in mediating an augmented activation of
isolated from WT, TLR4 C–E interactions. To test this hypothesis, we depleted circulating PMN activation of the inflammasome in lung EC through PMN–EC that HS-activated PMN might also contribute to an enhanced ac-

HMGB1 acting through TLR4 mediates HS/R-induced HEMORRHAGIC SHOCK ACTIVATES ENDOTHELIAL INFLAMMASOME

received anti-HMGB1 Ab (600 µg/mouse) by i.p. injection 10 min before HS or sham operation. Lung tissue and BAL fluid were then recovered at 4 h after HS/R or sham operation. In the lung tissue, the association of NLRP3 and ASC was detected using immunoprecipitation with anti-ASC Ab and immunoblotting with anti-ASC and NLRP3, as well as caspase-1 p10 fragments, all detected by Western blotting (A), and IL-1β in BAL fluid was measured with ELISA (B). The images are representative of five independent experiments. The graph shows the mean and SEM from five mice. *p < 0.01 compared with all other groups, **p < 0.01 compared with all other groups, *p < 0.05 compared with the groups labeled with no asterisk. C–E. In vitro stimulation of MLVEC with HMGB1. MLVEC isolated from WT, TLR4−/−, TLR2−/−, and RAGE−/− mice were treated with HMGB1 (0.5 µg/ml) for the time as indicated, and the association of NLRP3 and ASC and production of caspase-1 p10 fragments were detected (C). The activated caspase-1 in WT and TLR4−/− was visualized with caspase-1 fluorochrome inhibitor of caspase-1 reagent and observed under confocal microscope (D). IL-1β in the cell-culture media was measured with ELISA (E). The images are representative of three independent experiments. The graph shows the mean and SEM from five independent experiments. *p < 0.01 compared with all other groups, **p < 0.01 compared with all other groups, *p < 0.05 compared with the groups labeled with no asterisk.

lung EC following HS/R (22, 36). Accordingly, we hypothesized that HS-activated PMN might also contribute to an enhanced activation of the inflammasome in lung EC through PMN–EC interactions. To test this hypothesis, we depleted circulating PMN prior to HS/R, and, in some cases, we replenished the neutropenic mice during the resuscitation phase with PMN that were isolated from either sham operated or HS mice at 2 h after HS/R. As shown in Fig. 3A, at 4 h after HS/R, neutropenia induced in mice subjected to HS was associated with a significant reduction in the association of NLRP3–ASC and cleavage of caspase-1 in the lung, as well as a lower concentration of IL-1β in the BAL fluid, as compared with the mice subjected to HS/R with no PMN depletion. Repletion with WT PMN that were isolated from HS animals at 2 h after resuscitation, but not from sham animals, restored the NLRP3–ASC association in the lung and IL-1β level in the BAL fluid in response to HS/R (Fig. 3A). These in vivo observations were further recapitulated in vitro using a PMN–MLVEC coculture system. MLVEC that were stimulated with HMGB1 and cocultured with HS-activated PMN demonstrated an enhanced intracellular NLRP3–ASC association and caspase-1 cleavage, as well as a higher concentration of IL-1β in the medium as compared with those cocultured with no PMN or with the PMN isolated from sham animals (Fig. 3B). Taken together, these data indicate an important role of HS-activated PMN in augmenting HS/R-induced endothelial inflammasome.

**NAD(P)H oxidase is required for HS-induced activation of inflammasome**

To further define the role of PMN NAD(P)H oxidase in the activation of EC inflammasome, we replenished neutropenic WT HS mice with PMN isolated from gp91phox−/− mice, in which the gp91 subunit of NAD(P)H oxidase was genetically deleted and therefore a dysfunction of NAD(P)H oxidase. As shown in Fig. 4A, PMN collected from gp91phox−/− mice, which were subjected to

FIGURE 2. HMGB1 acting through TLR4 mediates HS/R-induced NLRP3 inflammasome activation and IL-1β secretion. A and B, WT (C57BL/6) mice, TLR4−/− mice, TLR2−/− mice, and RAGE−/− mice were subjected to HS/R (HS) or sham operation (SM). Some WT mice received anti-HMGB1 Ab (600 µg/mouse) by i.p. injection 10 min before HS or sham operation. Lung tissue and BAL fluid were then recovered at 4 h after HS/R or sham operation. In the lung tissue, the association of NLRP3 and ASC was detected using immunoprecipitation with anti-ASC Ab and immunoblotting with anti-ASC and NLRP3, as well as caspase-1 p10 fragments, all detected by Western blotting (A), and IL-1β in BAL fluid was measured with ELISA (B). The images are representative of five independent experiments. The graph shows the mean and SEM from five mice. *p < 0.01 compared with all other groups, **p < 0.01 compared with all other groups, *p < 0.05 compared with the groups labeled with no asterisk. C–E. In vitro stimulation of MLVEC with HMGB1. MLVEC isolated from WT, TLR4−/−, TLR2−/−, and RAGE−/− mice were treated with HMGB1 (0.5 µg/ml) for the time as indicated, and the association of NLRP3 and ASC and production of caspase-1 p10 fragments were detected (C). The activated caspase-1 in WT and TLR4−/− was visualized with caspase-1 fluorochrome inhibitor of caspase-1 reagent and observed under confocal microscope (D). IL-1β in the cell-culture media was measured with ELISA (E). The images are representative of three independent experiments. The graph shows the mean and SEM from five independent experiments. *p < 0.01 compared with all other groups, **p < 0.01 compared with all other groups, *p < 0.05 compared with the groups labeled with no asterisk.

FIGURE 3. HS-activated PMN are required for augmented activation of inflammasome. A, In vivo studies show that HS-activated PMN enhance HS/R-induced coupling of NLRP3 and ASC as well as caspase-1 cleavage in the lungs and IL-1β release in BAL fluid. PMN depletion was performed as described in Materials and Methods 16 h before HS/R. PMN repletion was performed in neutropenic mice during resuscitation using tail vein injection of PMN that were isolated from blood of WT mice subjected to sham (SM) or HS at 2 h after resuscitation. The lung tissue and BAL fluid were collected at 4 h after resuscitation. The images are representative of five independent experiments. The graph shows the mean and SE from five mice. *p < 0.01 compared with all other groups, **p < 0.01 compared with all other groups, #p < 0.01 compared with all other groups, **p < 0.01 compared with all other groups, *p < 0.01 compared with the groups labeled with no asterisk. B, In vitro studies show that HS-activated PMN augment HMGB1-induced binding association of NLRP3 and ASC and caspase-1 cleavage in MLVEC with IL-1β release in the cell-culture media. PMN (5 × 10⁵ cells) were isolated from blood of WT mice subjected to SM or HS at 2 h after resuscitation and cocultured with MLVEC in the presence or absence of HMGB1 (0.5 µg/ml) for 0, 2, and 6 h. The images are representative of five independent experiments. The graph shows the mean and SEM from five independent studies. *p < 0.01 compared with all other groups, **p < 0.01 compared with all other groups, *p < 0.01 compared with the groups labeled with no asterisk.
either sham or shock, failed to restore HS/R-induced NLRP3–ASC association and caspase-1 cleavage in the lung and IL-1β release into the BAL fluid (Fig. 4A, lanes 3 and 4). To test the role of EC endogenous NAD(P)H oxidase in mediating HS/R-induced inflammasome activation, PMN in gp91<sub>phox</sub>-/− mice were depleted, and, following HS procedure, the gp91<sub>phox</sub>-/− mice were replenished with WT PMN during the resuscitation phase. Fig. 4A shows that in NAD(P)H oxidase-deficient mice the HS/R-induced inflammasome activation in the lung and IL-1β release were significantly diminished, even though the mice were replenished with WT PMN (Fig. 4A, lanes 7 and 8). This result suggested a critical role of endogenous NAD(P)H oxidase in mediating HS/R-induced inflammasome activation in EC.

The role of NAD(P)H oxidase in mediating HMGB1 activation of endothelial inflammasome was further addressed by using a PMN–EC coculture approach. MLVEC from WT or gp91<sub>phox</sub>-/− mice were cocultured with PMN that were isolated from WT or gp91<sub>phox</sub>-/− mice in the presence of HMGB1 for 4 h. As shown in Fig. 4B, deficiency of gp91<sub>phox</sub>-/− in EC markedly attenuated HMGB1-induced inflammasome activation and IL-1β release (lanes 3, 6, and 9) as compared with that in WT EC (lanes 2, 5, and 8); HS-activated WT PMN enhanced HMGB1-induced inflammasome activation and IL-1β release in WT EC (Fig. 4B, lane 5). These results indicate that EC NAD(P)H oxidase is an essential component required for HS/R-induced inflammasome activation, whereas PMN NAD(P)H oxidase importantly contributes to an augmented activation of EC inflammasome following HS/R.

To demonstrate that exogenous ROS, such as PMN NAD(P)H oxidase-derived oxidants, can enhance endothelial NAD(P)H oxidase activation and therefore an increased intra-EC production of ROS, the production of ROS in live EC was visualized using H2DFFDA (Invitrogen Molecular Probes) and directly detected by fluorescence microscopy. The EC were observed for 2 h after treatment with HMGB1 and/or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Fig. 4C shows that HMGB1-induced ROS production in WT MLVEC is accelerated and amplified by adding H<sub>2</sub>O<sub>2</sub>, and the ROS production in the gp91<sub>phox</sub>-deficient MLVEC in response to HMGB1/H<sub>2</sub>O<sub>2</sub> is markedly diminished, indicating that the observed ROS production in the EC is derived mainly from NAD(P)H oxidase and amplified by exogenous ROS.

NAD(P)H oxidase mediates HS-induced TXNIP–NALP3 interaction

A recent report showed that TXNIP is a redox-sensitive component of inflammasome activation, and ROS can cause TXNIP to associate with NLRP3, which leads to inflammasome activation (19). Based on the data presented above, we hypothesized that HS activation of endothelial inflammasome might be mediated by ROS-induced association of TXNIP and NLRP3. To test this hypothesis, we first detected the association of TXNIP and NLRP3 in the lung tissue at 4 h following HS/R. As demonstrated in Fig. 5A, HS/R induced the association of TXNIP and NLRP3 in the lung (lane 1), and this effect of HS/R largely depends on NAD(P)H oxidases, because lack of PMN (lane 2), PMN NAD(P)H oxidase (lane 4), or global NAD(P)H oxidase (lanes 5 and 6) decreased the binding of TXNIP and NLRP3 (Fig. 5A).

This result has also been recapitulated in the EC–PMN coculture system as shown in Fig. 5B. HMGB1 stimulation for 4 h induced association of TXNIP–NLRP3 in WT MLVEC (lane 1), which was amplified when the EC cocultivated with WT HS-activated PMN (lane 3). However, the HMGB1-induced association of TXNIP and NLRP3 was markedly decreased in gp91<sub>phox</sub>-/− EC (lanes 4 and 6).

The role of HMGB1 in mediating HS/R-induced association of TXNIP–NLRP3 in the lung was further confirmed in vivo using neutralizing Ab against HMGB1. As shown in Fig. 5C, treatment with anti-HMGB1 Ab resulted in a significant decrease in the HS/R-induced TXNIP–NLRP3 association in the lung as compared with nonspecific IgG-treated animals.
TXNIP–NLRP3 interaction mediates HMGB1-induced inflammasome activation

To further elucidate the role of TXNIP in activating endothelial NLRP3 inflammasome, we knocked down TXNIP in MLVEC using siRNA techniques. At 48 h after transfection of TXNIP siRNA into MLVEC, the TXNIP protein could not be detected in the cells (Fig. 6A). Knockdown of TXNIP in MLVEC dramatically reduced HMGB1-induced NLRP3–ASC association and caspase-1 cleavage in the EC, as well as IL-1β release, as compared with that in the MLVEC treated with nonspecific control siRNA (Fig. 6B). The images are representatives of three independent experiments. 

Discussion

Pulmonary EC are activated in a very early stage in lung inflammation to be triggered by sepsis, trauma, or shock (8, 22, 32) and play a critical role in promoting the development of ALI. Lung endothelium is an important source of IL-1β in response to HS. Conversely, lung EC are also targets of IL-1β and release a range of inflammatory molecules in response to IL-1β. Thus, IL-1β, through interaction with lung EC, forms a feedback mechanism to amplify lung inflammation following HS. The present study demonstrates that the global ischemia/reperfusion injury initiated by resuscitated HS activates lung endothelial inflamma-
HMGB1 is a mediator of organ injury in animal models of infection and endotoxemia (23, 45–49). A recent study showed that HMGB1 administration induced rapid PMN recruitment in vivo, which was dependent on the interaction between RAGE and Mac-1 in the PMN (50). In addition, intratracheal administration of HMGB1 alone can induce organ dysfunction in the form of ALI (45), whereas blockade of HMGB1 by administering anti-HMGB1 Abs prevented hemorrhage-induced increases in pulmonary levels of proinflammatory cytokines, including IL-1β, and PMN infiltration in the lung as well as lung permeability (28). However, no role had been ascribed to HMGB1 in the activation of the inflammasome in a setting of HS/R, a process that is not associated with exposure to bacteria or bacterial products. The findings from the current in vivo and in vitro studies support a role for HMGB1 in mediating HS/R-induced EC inflammasome activation. As shown in Figs. 2–6, neutralizing Ab against HMGB1 effectively attenuated HS/R-induced inflammasome activation in the lung and IL-1β secretion in BAL fluid, and direct stimulation of MLVEC with HMGB1 led to activation of the NLRP3 inflammasome and IL-1β release.

RAGE had been originally identified as a receptor for HMGB1 in neurites and malignant cells (51–53). However, recent studies have suggested that both TLR4 and TLR2 are important in mediating HMGB1-induced inflammatory responses (54–56). In the current study, the effect of HMGB1 is mainly mediated through TLR4 signaling, because TLR4 deficiency markedly blocked HMGB1-induced inflammasome activation, caspase-1 cleavage, and IL-1β release in the lung and MLVEC. However, TLR2 and RAGE seem also to be involved in signaling HMGB1 activation of the inflammasome, as in TLR2−/− and RAGE−/− mice, inflammasome activation by HMGB1 is partially attenuated. These results may indicate a synergistic collaboration among signaling through TLR4, TLR2, and RAGE in mediating HS/R activation of inflammasome. The mechanism of the cross talk among these receptor-signaling pathways is not clear yet, and an extended study is needed. The importance of caspase-1 in mediating HMGB1/TLR4 signaling-induced IL-1β processing is evident by the observation shown in Fig. 1. Genetic deletion of caspase-1 prevented HS/R-induced IL-1β release in BAL fluid. More interestingly, the lack of caspase-1 even decreased the association of ASC and NLRP3 in the lung, suggesting a role of caspase-1 in keeping the integrity of inflammasome, although caspase-1 does not directly link the binding of ASC and NLRP3.

The present study shows that the endogenous endothelial NAD(P)H oxidase is essential for the HMGB1-induced activation of inflammasome in MLVEC, because gp91phox deficiency was consistently associated with a decreased activation of the inflammasome in both in vivo and in vitro experiments, as evidenced by Figs. 4 and 5. However, it is noticeable that gp91phox deficiency did not completely block the HMGB1-induced inflammasome activation, caspase-1 cleavage, and IL-1β release as shown in Fig. 4B, as well as the association between TXNIP and NLRP3 as shown in Fig. 5. These observations suggest that NAD(P)H oxidase is a major, but not a single, source of ROS in EC. Nevertheless, the importance of NAD(P)H oxidase in the development of ALI has been reported with the evidence that either NAD(P)H oxidase inhibitor or genetic deletion of NAD(P)H oxidase components significantly decreases lung inflammation and damage in a setting of HS or sepsis (5, 31, 57–59). We have recently reported a mechanism of EC NAD(P)H oxidase activation by HS/R (22). We showed that HMGB1 is a key factor mediating HS/R-induced EC NAD(P)H oxidase activation, and both Rac1 and p38 MAPK are involved in the signaling pathway (22). The present study also revealed an important role for PMN NAD(P)H oxidase in enhancing inflammasome activation, which is believed to result from an enhanced activation of EC NAD(P)H oxidase by PMN-derived ROS. In a recent report, we also showed that ROS derived from PMN NAD(P)H oxidase are an important mediator in amplifying EC NAD(P)H oxidase activation, and this process is Rac1-dependent but p38 MAPK-independent (22). Indeed, we observed in the current study that oxidants enhanced ROS production in MLVEC in response to HMGB1, as shown in Fig. 4C. Regarding the mechanism of HS-induced activation of PMN NAD(P)H oxidase, our previously studies have shown that HMGB1 mediates HS/R activation of MyD88–IL-1R–associated kinase–p38 MAPK (p38 MAPK) and MyD88–IL-1R–associated kinase–Akt signaling pathways, and, in turn, both of the signaling pathways contribute to the activation of PMN NAD(P)H oxidase (31).

ROS has also been suggested as an activator of the inflammasome (16, 60, 61). A recent report showed that exogenous ROS (H2O2) are responsible for mediating uric acid crystal-induced interaction between TXNIP and NLRP3 and subsequent activation of inflammasome in a monocyte/macrophage model (19). In the current study, however, we elucidated in pulmonary EC how HS/R-induced endogenous ROS initiate inflammasome activation. We found that the association of TXNIP and NLRP3 is ROS-sensitive and plays an important role in mediating inflammasome activation in lung EC. As shown in Fig. 5, HS/R and HMGB1 induced an increase in the association of TXNIP with NLRP3, and NAD(P)H oxidase-derived ROS are required for this association, because deficiency of gp91 of NAD(P)H oxidase in EC significantly decreased TXNIP and NLRP3 association. Also, the association of TXNIP and NLRP3 seems essential for the subsequent caspase-1 cleavage and IL-1β secretion, because silencing of TXNIP significantly attenuated HMGB1-induced inflammasome assembly, caspase-1 cleavage, and subsequent secretion of IL-1β (Fig. 6). TXNIP is a protein with many functions. Although it was originally defined as a TRX-binding protein that regulates the antioxidant function of TRX, its function is actually more diverse. It has been reported that ROS promote dissociation of TXNIP from TRX, which, therefore, allows TXNIP to associate with NLRP3 (19).

In summary, the current study demonstrates a novel mechanism connecting the insult of HS/R and activation of lung EC NLRP3 inflammasome. HMGB1, acting through TLR4 and a synergistic collaboration from the signaling of TLR2 and RAGE, activates EC NAD(P)H oxidase, and, in turn, the created ROS promote the association of TXNIP to NLRP3 and subsequent activation of inflammasome and IL-1β secretion. PMN-derived ROS exhibit a role in enhancing EC NAD(P)H oxidase activation and therefore an amplified inflammasome activation in response to HS/R (Fig. 7).

Disclosures
The authors have no financial conflicts of interest.

References
HOMERGIC SHOCK ACTIVATES ENDOTHELIAL INFLAMMASOME


