Hemorrhagic shock augments lung endothelial cell activation: role of temporal alterations of TLR4 and TLR2

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Li Y, Xiang M, Yuan Y, Xiao G, Zhang J, Jiang Y, Vodovoz Y, Billiar TR, Wilson MA, Fan J. Hemorrhagic shock augments lung endothelial cell activation: role of temporal alterations of TLR4 and TLR2. Am J Physiol Regul Integr Comp Physiol 297: R1670–R1680, 2009. First published October 14, 2009; doi:10.1152/ajpregu.00445.2009.—Hemorrhagic shock (HS) due to major trauma predisposes the host to the development of acute lung inflammation and injury. The lung vascular endothelium is an active organ that plays a central role in the development of acute lung injury through generating reactive oxygen species and synthesizing and releasing of a number of inflammatory mediators, including leukocyte adhesion molecules that regulate neutrophils emigration. Previous study from our laboratory has demonstrated that in a setting of sepsis, Toll-like receptor-4 (TLR4) signaling can induce TLR2 expression in endothelial cells (ECs), thereby increasing the cells‘ response to TLR2 ligands. The present study tested the hypothesis that TLR4 activation by HS and the resultant increased TLR2 surface expression in ECs might contribute to the mechanism underlying HS-augmented activation of lung ECs. The results show that high-mobility group box 1 (HMGB1) through TLR4 signaling mediates HS-induced surface expression of TLR2 in the lung and mouse lung vascular endothelial cells (MLVECs). Furthermore, the results demonstrate that HMGB1 induces activation of NAD(P)H oxidase and expression of ICAM-1 in the lung, and MLVECs sequentially depend on TLR4 in the early phase and on TLR2 in the late phase following HS. Finally, the data indicate an important role of the increased TLR2 surface expression in enhancing the activation of MLVECs and augmenting pulmonary neutrophil infiltration in response to TLR2 agonist peptidoglycan. Thus, induction of TLR2 surface expression in lung ECs, induced by HS and mediated by HMGB1/TLR4 signaling, is an important mechanism responsible for endothelial cell-mediated inflammation and organ injury following trauma and hemorrhage.

acute lung injury; neutrophil; Toll-like receptors

GLOBAL ISCHEMIA/REPERFUSION related to resuscitation from hemorrhagic shock (HS) is a major cause of multiorgan failure, in which acute lung injury (ALI) is an important component and often serves as a direct cause of death (18). Resuscitated HS is believed to promote the development of lung injury by priming the immune system for an exaggerated inflammatory response to a second, often trivial stimulus, the so-called “two-hit hypothesis” (41). Previous studies have demonstrated that a period of sustained shock followed by resuscitation leads to augmented lung neutrophil sequestration and lung injury in response to a small dose of intratracheal bacterial cell wall constituent, such as LPS (11, 16). This effect is due, in part, to the increased LPS-stimulated release of cytokines and chemokines from alveolar macrophages (11, 16). From another aspect, the lung vascular endothelium is a multifunctional cell monolayer that plays important roles in the regulation of vascular tone, coagulation, and fibrinolysis, as well as immune and inflammatory responses (6, 25, 37). Therefore, lung endothelial cell (EC) activation is critically involved in the development of ALI. However, the mechanism underlying HS initiating and enhancing lung EC activation presents a significant gap in our knowledge.

Reactive oxygen species (ROS) have been implicated as important in the pathogenesis of ALI through regulating the expression of a number of inflammatory mediators and activation of signaling pathways (19). The major source of ROS within ECs is the nonphagocytic NAD(P)H oxidase (3), which is composed of membrane-bound gp91phox and p22phox, as well as cytosolic subunits such as p47phox, p67phox, and small GTPase Rac. In addition to these components, ECs also express homologues of gp91phox (Nox2) including Nox1, Nox4, and Nox5. Endothelial NAD(P)H oxidase is activated by many factors including growth factors, cytokines, shear stress, hypoxia, and G protein-coupled receptor agonists (21). It has been reported that HS-induced P-selectin expression in vascular tissue depends on functional NAD(P)H oxidase (1), suggesting that HS is an initial factor for NAD(P)H oxidase, although direct activation of endothelial NAD(P)H oxidase by HS has not been reported.

The accumulation of polymorphonuclear neutrophils (PMN) in the lung vasculature, interstitium, and alveolar space is considered a critical event in ALI and has been the target of various preventative strategies. The lung EC-derived intercellular adhesion molecule-1 (ICAM-1), a counter receptor for the leukocyte β2-integrins LFA-1 and Mac-1 (CD11a/CD18 and CD11b/CD18) (8, 24), plays an important role in the regulation of PMN sequestration. The interaction of ICAM-1 with CD11/CD18 integrins enables PMN to adhere firmly to the vascular endothelium and thereby migrate across the microvascular barrier (53). Studies have shown that HS can activate ECs and induce ICAM-1 expression (20, 38, 54, 63). However, the mechanisms underlying this process have not been fully elucidated.

Toll-like receptors (TLRs), a family of pattern recognition receptors, are now defined as the receptors for recognizing pathogen-associated molecular pattern molecules as well as endogenous molecules released by damaged tissues (“danger
signals”) (2, 39). TLR4 and TLR2 sit at the interface of microbial and sterile inflammation by selectively responding to both bacterial products and multiple other endogenous ligands, including hyaluronic acid (56), heparan sulfate (28), fibrinogen (52), heat shock proteins (62), and high-mobility group box 1 (HMG1) (43, 60, 61). Both inflammation and injury responses in organs subjected to ischemia/reperfusion partially depend on TLR4 (46, 60, 61, 69). Previous studies from both our group and others have demonstrated that ECs express a low level of TLR2, which can be upregulated by TLR4 signaling (9, 26). These studies suggest a mechanism of inducible cellular sensitivity to both exogenous and endogenous stimuli.

HMG1 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 (36). HMG1 can be secreted by innate immune cells in response to microbial products or other inflammatory stimuli (64, 66), be released by injured cells, and is known as one of the main prototypes of the emerging damage-associated molecular pattern molecules (39, 50, 68). HMG1 was initially identified as an inflammatory cytokine that is a late mediator of lethality in sepsis (64, 66). However, recent studies suggest that HMG1 also acts as an early mediator of inflammation contributing to the development of ALI after trauma/hemorrhage (30, 44, 67) and hepatic injury after liver ischemia-reperfusion (60).

The present study aimed to test the hypothesis that TLR4 activation by HS and the resultant increased TLR2 expression in ECs might contribute to the mechanism underlying the HS-augmented activation of lung ECs. The role of HMG1-TLR4-TLR2 signaling in HS/resuscitation (HS/R)-augmented activation of lung ECs was addressed. The study shows that HMG1/TLR4 signaling mediates the HS-induced increase in TLR2 surface expression and decrease in TLR4 surface expression in the lung as well as in mouse lung endothelial cells (MLVECs). These alterations in TLR4 and TLR2 surface expression result in HMG1-mediated activation of NAD(P)H oxidase and expression of ICAM-1 in MLVECs that is TLR4-dependent in the early phase and switches to being TLR2-dependent in the late phase following HS. More importantly, the HS-induced surface expression of TLR2 contributes to an enhanced activation of MLVECs and augmented pulmonary PMN infiltration in response to the TLR2 agonist peptidoglycan (PGN). Thus, the present study demonstrates a novel mechanism underlying HS-augmented lung inflammation, namely that induction of increased TLR2 surface expression in lung ECs, which is induced by HS/R and mediated by HMG1 activation of TLR4 signaling, is an important mechanism responsible for EC-mediated inflammation and organ injury following HS/R.

MATERIALS AND METHODS

Materials. Recombinant HMG1 was purchased from R&D Systems (Minneapolis, MN). Stimulation activity of the recombinant HMG1 was confirmed in mouse macrophages by assay of TNF release, with an ED50 of 3–12 μg/ml. Polyclonal neutralizing antibody against HMG1 prepared as described previously (66) was provided by Dr. K. J. Tracey (Feinstein Institute for Medical Research, North Shore-Long Island Jewish Health System, Manhasset, NY). Polyclonal anti-HMG1 antibody for Western blot analysis, and kinase assay kits for IRAK4 were purchased from Cell Signaling Technology (Danvers, MA). Polyclonal rabbit anti-IRAK4 antibody and MyD88 homodimerization inhibitory peptide set were purchased from Invitrogen (San Diego, CA). Nonimmune rabbit IgG (cat. no. 15006), diphenylethionodionium, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), except where noted.

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 (45, 47) and control wild-type (WT) C3H/HeOuj mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TLR2−/− mice were obtained from Dr. Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) (55), and C57BL/6 mice that are WT control for TLR2−/− mice were purchased from the Jackson Laboratory. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of Veterans Affairs Pittsburgh Healthcare System. Mice were 12–14 wk of age at the time of experiments and were maintained on standard rodent chow and water ad libitum. The mice were not fasted before experiments. Animals were anesthetized with 50 mg/kg of ketamine and 5 mg/kg of xylazine via intraperitoneal administration. Femoral arteries were cannulated for monitoring of mean arterial pressure, blood withdrawal and resuscitation. HS was initiated by blood withdrawal and reduction of the mean arterial pressure to 30 mmHg within 20 min. Blood was collected into a 1-ml syringe and heparinized to prevent clotting. To exclude the effect of heparin on immune processes, equal amounts of heparin (10 units) were injected into sham animals through the cannulated femoral artery during the sham operation. After a hypotensive period of 2 h, animals were further resuscitated by transfusion of the shed blood and Ringer’s lactate in a volume equal to that of shed blood over a period of 60 min. 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, neutralizing antibodies against HMG1 (600 μg per mouse) or nonimmune control IgG was injected intraperitoneally into the mice 10 min before hemorrhage, respectively. The animals were kept anesthetized during the whole experiment period by xylazine and ketamine. At various time points after resuscitation (0 to 8 h) whole lung tissue was harvested for Western blot and RT-PCR analysis.

MLVEC isolation and characterization. MLVECs were isolated by using a previously described method (23, 57) but modified in our laboratory. Briefly, mice were anesthetized with 50 mg/kg of ketamine and 5 mg/kg of xylazine ip. The chest cavity was opened, and the right ventricle was cannulated. PBS was infused to remove blood from lungs. Peripheral lung tissue diced into a size about 1 mm3 were prepared and cultured in a 60-mm culture dish in growth medium (MEM D-Val medium containing 2 mM glutamine, 10% FBS, 5% human serum, 50 μg/ml penicillin/streptomycin, 5 μg/ml heparin, 1 μg/ml hydrocortisone, 80 μg/ml endothelial cell growth supplement from bovine brain, 5 μg/ml amphotericin, and 5 μg/ml mycoplasma removal agent) at 37°C with 5% CO2 for 60 h. The adherent cells were continued to culture for 3 days after removal of the tissue dices, followed by a purification procedure with biotin-conjugated rat anti-mouse CD31 (PECAM-1) monoclonal antibody and BD IMag streptavidin particles plus-DM, and the immunomagnetic separation system (BD Biosciences Pharmingen, San Diego, CA), following the manufacturer’s instruction. The cells were allowed to grow for 3 to 4 days after purification. The cells were characterized by their cobblestone morphology, uptake of Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA), and staining for factor VIII-related antigen (Sigma, St. Louis, MO). The MLVEC passed between 3 and 5 times were used in experiments in which cells were treated with HMG1 (0.5 μg/ml) for 30 min, and washed with HBSS three times. At various time points (0 to 8 h) after HMG1 treatment, the cells were harvested for further analysis. In experiments carried out to confirm the role of MyD88 and NF-κB in the mechanism of HMG1-induced TLR2 upregulation, NAD(P)H oxidase activation, and ICAM-1 expression, the MLVECs were preincubated with either MyD88 inhibitory pep-
tide (100 μM; Imgenex, San Diego, CA) (35) or NF-κB inhibitor IKK-NBD (100 μM; Biomol, Plymouth Meeting, PA) (40) for 2 h before HMGB1 treatment.

**Immunoprecipitation and detection of phosphorylated p47phox**. Mouse lung tissue or MLVECs were homogenized or 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). The supernatants were then immunoprecipitated with anti-p47phox antibody as described (42). The immunoprecipitated proteins were separated on a 10% SDS-PAGE gel and were then electroblotted 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 antibody (Invitrogen, South San Francisco, CA) (17, 29) at 1:500 dilution and detected with an horseradish peroxidase-conjugated secondary antibody.

**Measurement of superoxide generation in live MLVECs**. Live MLVECs that were cultured in 12-well cell culture plate were stained with the cell-permeable ROS detection reagent H2DFFDA (Invitrogen, Carlsbad, CA) at the concentration of 10 μM for 10 min. The cells were then washed with HBSS for three times followed by incubation in the growth medium in the presence or absence of HMGB1 (0.5 μg/ml) for 8 h. The ROS production was then detected by fluorescence microscopy at different time points.

**RT-PCR**. Total RNA from the lung tissue and MLVECs was isolated using TRI-reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer’s instruction. Total RNA was then reverse-transcribed using a SuperScript Preamplification kit (Invitrogen, Carlsbad, CA). Primer pairs for mouse TLR2, TLR4, and GAPDH amplification were purchased from R&D Systems. The reverse-transcribed using a SuperScript Preamplification kit (Invitrogen, Carlsbad, CA) and secondary Europium labeled anti-rabbit antibody (Cell Signaling Technologies) and secondary Europium labeled anti-rabbit antibody with Delfia enhancement solution (PerkinElmer Life Sciences, Waltham, MA) and incubated at room temperature for 60 min. IRAK4 activity was then measured following the manufacturer’s instructions for the kit by using primary anti-phospho-ezrin/radixin/moesin antibody (Cell Signaling Technologies) and secondary Europium labeled anti-rabbit antibody with Delfia enhancement solution (PerkinElmer Life Sciences). Fluorescence emission at 615 nm was detected with SpectraMax M2 Multidetection reader (Molecular Devices, Sunnyvale, CA).

**Statistics**. The data are presented as means ± SE 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 P < 0.01.

**RESULTS**

**HS/R modulates TLR2 and TLR4 expression in the lung and MLVECs through HMGB1-TLR4 signaling**. HS/R caused an increase in TLR2 mRNA and protein expression in the lung at 2 h and a more marked increase at 4 and 6 h, compared with the sham group (Fig. 1). By contrast, HS/R induced a decrease in TLR4 mRNA and protein expression starting at 4 h following HS/R (Fig. 1).

Our previous studies have shown that TLR4 signaling plays an important role in mediating HS/R-induced activation of innate immunity (10, 14, 46). To address the role of TLR4 in HS/R-induced TLR2 expression, TLR4-mutant C3H/HeJ mice were subjected to HS/R, and TLR2 expression in the lung was detected. As shown in Fig. 2, HS/R failed to induce the TLR2 upregulation in TLR4-mutant mice at 4 h after resuscitation, indicating an essential role for functional TLR4 in HS/R-induced TLR2 upregulation. TLR4 recognizes a variety of endogenous ligands including HMGB1 (36, 51). A previous report has shown that HS/R causes a significant increase of HMGB1 in the serum, lung, and liver at 2 h after HS/R (13). To determine whether endogenous HMGB1 contributes to HS/R-induced TLR2 expression in the lung, neutralizing antibody to HMGB1 was administered to mice 10 min before HS/R. Treatment with anti-HMGB1 antibody prevented the HS/R-induced increase in TLR2 mRNA and protein and decrease in TLR4 protein in the lung compared...
with nonspecific IgG-treated animals (Fig. 2). Furthermore, while TLR4 mutation attenuated the HS/R-induced TLR2 expression, TLR2 deficiency could not prevent HS/R-induced TLR4 downregulation (Fig. 2). Compared with the WT sham/IgG group, no changes in the TLR2 and TLR4 expression were found in the WT sham/HMGB1 Ab, TLR4-mutant sham/IgG, and TLR2+/−/IgG groups (data not shown). These results suggest that HMGB1 regulates TLR2 and TLR4 expression through TLR4 but not TLR2.

Lung vascular ECs are one of the major pulmonary cell populations and the most important source of lung ICAM-1. Since in vivo experiments have shown a role of HMGB1 in mediating HS/R-induced increase in TLR2 expression in the lung, we next isolated the MLVECs to directly address the role of HMGB1 activation of TLR4 signaling in inducing TLR2 surface expression in lung ECs. MLVECs were isolated from WT (C3H/HeOuJ) and TLR4-mutant mice, treated with HMGB1 for 0 to 6 h, and assessed for TLR2 surface expression. HMGB1 increased TLR2 surface expression in WT MLVECs at as early as 1 h, and reached a peak at 4 h (Fig. 3A). However, HMGB1 failed to induce an increase in TLR2 surface expression in TLR4-mutant MLVECs.

Previous studies have shown that activation of TLR4 can signal through both MyD88-dependent and MyD88-independent pathways (22). To determine whether TLR2 expression induced by HMGB1 activation of TLR4 in the MLVECs is a MyD88-dependent event, the MyD88 inhibitor homodimerization inhibitory peptide (35) was applied. MLVECs isolated from TLR4 WT mice were preincubated with MyD88 inhibitory peptide (100 μM) for 2 h and subsequently treated with HMGB1 for 6 h. As shown in Fig. 3A MyD88 inhibitor significantly attenuated the effect of HMGB1 on TLR2 induc-

Fig. 1. Heat shock resuscitation (HS/R) regulation of expression of Toll-like receptor-2 (TLR2) and TLR4 in the lung. Wild-type (WT; C3H/HeOuJ) mice were subjected to HS/R or sham operation. Lung tissue was harvested 1 to 6 h after resuscitation or sham operation, and TLR2 and TLR4 mRNA (A) and protein (B) expression in the lung were detected by RT-PCR and Western blot analysis, respectively. The blots are representative of 3 independent studies; n = 3 mice/group.

Fig. 2. Role of high-mobility group box 1 (HMGB1) in mediating HS/R regulation of TLR2 and TLR4 expression in the lung. A: pretreatment with neutralizing antibody to HMGB1 or TLR4 mutation (mut.) prevents HS/R-induced TLR2 upregulation in the lung. Anti-HMGB1 antibody (Ab; 600 μg per mouse) or nonspecific control IgG was given intraperitoneally to WT (C3H/HeOuJ) or TLR4-mutant mice 10 min before HS or sham operation, as described in A. Lung tissue was then collected from the mice at 4 h after HS/R or sham operation for detection of TLR2 expression using Western blot analysis. The blots are representative of 3 independent studies. The graph depicts the means ± SE of the %changes in TLR2 expression from 3 mice. *P < 0.01 compared with sham group.

B: pretreatment with neutralizing antibody to HMGB1 prevents HS/R-induced TLR4 downregulation in the lung. Anti-HMGB1 antibody or nonspecific control IgG was given to WT (C57BL/6) or TLR4-mutant mice 10 min before hemorrhage or sham operation. Lung tissue was then collected from the mice at 4 h after HS/R or sham operation for detection of TLR4 expression using Western blot analysis. The blots are representative of 3 independent studies. The graph depicts the means ± SE of the fold changes in TLR4 protein expression. *P < 0.01 compared with other groups.
To confirm that the HS/R-induced ICAM-1 expression is mediated by HMGB1, WT (C3H/HeOuJ) mice were pretreated with neutralizing antibody against HMGB1 before HS/R. As shown in Fig. 5, neutralizing antibody against HMGB1 markedly attenuated HS/R-induced ICAM-1 expression in the lung at an 8-h time point compared with that in WT shock mice treated with nonspecific IgG. In the experiments shown in Figs. 4 and 5, only C3H/HeOuJ mice were used to reduce the usage of WT control mice based on our finding that there was no statistical significance in the changes of multiple parameters, including those shown in Fig. 4, between C3H/HeOuJ and C57BL/6 mice in response to HS/R (data not shown).

The role of TLR2 and TLR4 in the activation of MLVECs by HMGB1 was also studied in vitro. Figure 6A shows that WT

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Fig. 3. Dynamic changes in TLR2 and TLR4 in mouse lung vascular endothelial cells (MLVECs) in response to HMGB1. MLVECs were isolated from TLR4-mutant, TLR4 WT (C3H/HeOuJ), TLR2−/−, and TLR2 WT (C57BL/6) mice and incubated with recombinant HMGB1 (0.5 μg/ml) for up to 6 h; TLR2 and TLR4 expression in the MLVECs were then assessed using Western blot analysis. In some experiments, MyD88 inhibitory peptide (100 μM) or NF-κB inhibitor IKK-NBD (100 μM) was added to WT MLVECs 2 h prior to HMGB1. The images are representative of 3 independent studies.

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Fig. 4. Time-dependent alteration in TLRs dependency of lung activation following HS/R. WT (C3H/HeOuJ), TLR4-mutant, and TLR2−/− mice were subjected to HS/R or sham operation. Lung tissue was harvested at 2 and 8 h after resuscitation or sham operation, and activities of IRAK4 and nuclear NF-κB (B) as well as phosphorylation of p-47 phox and expression of ICAM-1 (B) in the lung were detected. The graph depicts the means ± SE of the %changes, and the white bars and the black bars represent 2 h and 8 h time points, respectively. The graphs and images are representative of 3 independent studies; n = 3 mice/group. *p < 0.01 compared with sham group; **p < 0.01 compared with other groups.

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To confirm that the HS/R-induced ICAM-1 expression is mediated by HMGB1, WT (C3H/HeOuJ) mice were pretreated with neutralizing antibody against HMGB1 before HS/R. As shown in Fig. 5, neutralizing antibody against HMGB1 markedly attenuated HS/R-induced ICAM-1 expression in the lung at an 8-h time point compared with that in WT shock mice treated with nonspecific IgG. In the experiments shown in Figs. 4 and 5, only C3H/HeOuJ mice were used to reduce the usage of WT control mice based on our finding that there was no statistical significance in the changes of multiple parameters, including those shown in Fig. 4, between C3H/HeOuJ and C57BL/6 mice in response to HS/R (data not shown).

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production, the ROS in live MLVECs was directly detected (Fig. 6A).

induced activation of NAD(P)H oxidase and increased production is mainly derived from NAD(P)H oxidase. in response to HMGB1, indicating that the observed ROS diminution markedly diminished the ROS production in the MLVEC treatment of HMGB1. As shown in Fig. 6B, the small dose of either LPS or PGN alone induced a slight increase in the activities of IRAK4 and NF-κB, respectively, the antecedent HS/R markedly augmented the PGN-induced activation of IRAK4 and NF-κB to a level of ~27-fold and ~56-fold, respectively, compared with sham control (Fig. 7A). By contrast, HS/R did not further increase the activities of IRAK4 and NF-κB in response to LPS in a significant manner. Either TLR4 mutation or TLR2 deficiency diminished the HS/R-augmented activation of IRAK4 and NF-κB in response to PGN (Fig. 7A). HS/R also enhanced p47phox phosphorylation and ICAM-1 expression in the lung in response to PGN in WT mice, but not in TLR4-mutant or TLR2−/− mice, as shown in Fig. 7B.

To address the physiological relevance of the altered p47phox phosphorylation and ICAM-1 expression in the lung, PMN in bronchoalveolar lavage (BAL) fluid were counted 2 h after intratracheal administration of saline, PGN, or LPS. As shown in Fig. 7C, HS/R alone induced a small increase in BAL PMN counts. However, while LPS caused a further slight increase in the BAL PMN in WT and TLR2−/− mice, PGN induced an augmented elevation of BAL PMN following antecedent HS/R in WT mice, but not in TLR4-mutant and TLR2−/− mice.

DISCUSSION

The mechanism underlying HS/R-primed ALI is yet unclear. The development of ALI is a cascading event in which macrophages, PMN, ECs, and epithelial cells work in concert. Most previous studies on HS/R-induced ALI focused on the role of macrophages and PMN. For example, our and other reports have demonstrated that the TLR4 upregulation of TLR2 in alveolar macrophages is critical in the pathogenesis of HS/R-induced ALI (14, 15), and HMGB1 is an important mediator causing PMN activation (13) and organ inflammation following trauma and HS (33, 34). However, the role of HMGB1-TLR4 signaling in HS/R initiation and priming of EC activation and subsequent lung inflammation was not reported. The present study showed that HMGB1-TLR4 signaling mediated HS/R-induced expression of TLR2 in the lung and lung ECs, and therefore resulted in prolonged cell activation in response to HS/R as well as augmented cell sensitivity to TLR2 agonist, and consequently, led to exaggerated ROS production, adhesion molecules expression, and pulmonary PMN infiltration. Thus, the present study explores a novel EC-dependent mechanism underlying HS-augmented lung inflammation.

The present study demonstrated a temporal change in TLRs-dependency of lung EC activation following HS/R. The constitutive level of TLR2 in lung ECs is low, but can be induced to a high expression by HS/R. The inducible expression of TLR2 suggests an important physiological significance of TLR-TLR cooperativity, namely that as ligand activation of TLR4 signaling wanes, the TLR-mediated cellular response can be maintained over a prolonged period of time (9, 31). More importantly, the induction of TLR2 surface expression suggests a mechanism by which cell sensitivity to a TLR2 ligand can be amplified in a setting of HS/R,

MLVECs treated with HMGB1 for 8 h exhibited a sustained activation of NAD(P)H oxidase and ICAM-1 expression. By contrast, HMGB1 failed to induce a high-level activation of NAD(P)H oxidase and ICAM-1 expression in TLR4-mutant MLVECs. However, in TLR2−/− MLVECs, the HMGB1-induced activation of NAD(P)H oxidase and increased ICAM-1 expression was exhibited in the early phase (1 h) but not in the late phase (8 h) (Fig. 6A).

To address whether the NAD(P)H oxidase activation is functionally associated with an increase in intracellular ROS production, the ROS in live MLVECs was directly detected using fluorescence microscopy. As shown in Fig. 6B, the changes in ROS production in the MLVEC were consistent with the alterations in NAD(P)H oxidase activation as shown in Fig. 6A. To specify that the ROS is derived from NAD(P)H oxidase, the NAD(P)H oxidase-specific inhibitor diphenyleneiodonium (100 μM) was added to the MLVECs that were isolated from WT C57BL/6 mice immediately before the treatment of HMGB1. As shown in Fig. 6B, diphenyleneiodonium markedly diminished the ROS production in the MLVEC in response to HMGB1, indicating that the observed ROS production is mainly derived from NAD(P)H oxidase.

Increased surface expression of TLR2 mediates HS/R-augmented activation of lung ECs. An inducible surface expression of TLR2 may imply inducible cell sensitivity to TLR2 agonist. To elucidate the role of increased surface expression of TLR2 in the HS/R-augmented activation of lung ECs, the effect of a small dose of intratracheal PGN on the lung EC activation was tested. Mice were subjected to HS/R followed by intratracheal PGN (3 μg/kg body wt), LPS (3 μg/kg body wt), or saline at 6 h after resuscitation. Activities of IRAK, NF-κB, and NAD(P)H oxidase as well as ICAM-1 expression in the lung were measured 2 h thereafter. The small dose of either LPS or PGN alone induced a slight increase in the activities of IRAK4 and NF-κB in the sham groups, as shown in Fig. 7A. While HS/R alone caused ~11-fold and ~19-fold increases in the activities of IRAK4 and NF-κB, respectively, the antecedent HS/R markedly augmented the PGN-induced activation of IRAK4 and NF-κB to a level of ~27-fold and ~56-fold, respectively, compared with sham control (Fig. 7A).
as demonstrated in the results where antecedent HS/R dramatically exaggerated activation of pulmonary cells in response to PGN. Taken together, these observations reveal a new mechanism of HS/R-primed lung inflammation, which would be important when proposing therapeutic strategies for posttrauma ALI.

The role of activation of TLR4 in the induction of TLR2 expression was demonstrated as HS/R caused the increase in TLR2 expression in WT mice but not in TLR4-mutant and TLR2-/- MLVECs. The results also demonstrate that the TLR4-dependent induction of TLR2 expression is a MyD88- and NF-κB-dependent event as shown in Fig. 3. The role of endogenous TLR4 ligand HMGB1 was defined in the study as well. HMGB1 is increasingly recognized as the prototypic alarmin (65). Evidence indicates that HMGB1 acts as an early inflammatory mediator in ischemia (60, 61), trauma/hemorrhagic shock (13, 44, 67), and noninfectious hepatitis (48). A previous study has shown that direct stimulation of human umbilical vein ECs with HMGB1 could cause ICAM-1 expression (59). However, no role had been ascribed to HMGB1 in HS/R-enhanced ICAM-1 expression and NAD(P)H oxidase activation. The present study shows an important role of HMGB1 in inducing TLR2 and subsequent augmented activation of endothelial NAD(P)H oxidase and expression of ICAM-1 in lung ECs in a setting of HS/R through a TLR4 signaling-dependent mechanism. This mechanism is supported by the findings that a neutralizing antibody to HMGB1 blocks HS/R-induced TLR2 surface expression and ICAM-1 in vivo (Fig. 2, 4, and 5), and that HMGB1 directly induces TLR2 surface expression in WT MLVECs, but not in TLR4-mutant MLVECs (Fig. 3). In addition, it has already been reported that HMGB1 levels in serum, lungs, and liver were significantly increased within 2 h after HS/R in mice (13). However, the mechanism by which HS induces HMGB1 release has not been fully addressed. In our previous study, it has been demonstrated that macrophage β-adrenergic receptor activation by catecholamines serves as an important mechanism for HS/R-induced HMGB1 secretion (34). That study showed that a β-adrenergic receptor antagonist could prevent HS/R-induced increase in serum HMGB1, and epinephrine could directly increase serum HMGB1 in vivo. The study also demonstrated that in vitro direct treatment of alveolar macrophages with epinephrine caused HMGB1 release from the cells, and this effect was suppressed by a β-adrenergic receptor antagonist (34).

Fig. 6. TLR4 and TLR2 sequentially mediate HS/R-induced activation of lung ECs. A: MLVECs were isolated from TLR4-mutant, TLR4 WT (C3H/HeOuJ), TLR2-/-, and TLR2 WT (C57BL/6) mice and incubated with recombinant HMGB1 (0.5 μg/ml) for 0 to 8 h; p47phox phosphorylation and ICAM-1 expression in the MLVECs were then assessed. Treatment with HMGB1 for 8 h exhibited a sustained activation of NAD(P)H oxidase and ICAM-1 expression in the WT MLVECs, but not in TLR4-mutant and TLR2-/- MLVECs. B: reactive oxygen species (ROS) production in live MLVECs. MLVECs that were cultured in 12-well cell culture plate were stained with the cell-permeable ROS detection reagent H2DFFDA in the concentration of 10 μM for 10 min. Cells were then washed with HBSS 3 times followed by incubation in the growth medium in the presence or absence of HMGB1 (0.5 μg/ml) for 8 h. The ROS production was then detected by fluorescence microscopy at different time points as indicated. In some experiments, the NAD(P)H oxidase-specific inhibitor diphenyleneiodonium (DPI) (100 μM) was added to the WT (C57BL/6) MLVECs immediately before the treatment of HMGB1 to specify the source of ROS. Blots are representative of at least 3 independent studies.
HMGB1 endothelial NAD(P)H oxidase is a major source of intracellular ROS, which have an important signaling function (19). ROS in ECs contribute to transcriptional regulation of a number of inflammatory mediators through NF-κB (49), AP-1 (c-Jun and c-Fos) (58), and hypoxia-inducible factor-1α (4), etc. Importantly, ROS mediate stable ICAM-1 expression-dependent endothelial adhesivity, resulting in the arrest of PMN (27). Amplified activation of EC NAD(P)H oxidase and expression of ICAM-1 in response to HS/R and PGN were found to be dependent on increased surface expression of TLR2 (3).
TLR2 that is, in turn, induced by HMGB1-TLR4 signaling (Fig. 7). Thus, the present study explores an important role of shock-induced activation of TLR4 in activating TLR2 signal, which in turn leads to exaggerated lung inflammation.

TLR4 expression in the lung and MLVECs was decreased by HS/R as shown in the results. How HS/R downregulates TLR4 is not clear, but it appears that the downregulation of TLR4 is dependent on HMGB1 but is not mediated by a pathway that involves MyD88, NF-κB, or TLR2 signaling, since suppression of MyD88 and NF-κB activation, as well as TLR2 deficiency, did not prevent the TLR4 decrease in HMGB1-stimulated MLVECs. A previous study from our laboratory has shown that in alveolar macrophages LPS downregulates TLR4 and that HS/R attenuated the effect of LPS (12). This modulation may serve as a mechanism by which the role of TLR4 signaling is prolonged, and the resultant inflammatory response induced by TLR4 signaling is enhanced in HS/R (12). The present study, however, revealed a different mechanism of HS/R regulation of TLR4. Nevertheless, HS either acting as a preconditioning factor to prevent TLR4 decrease in response to LPS or working as a coordinator to transfer the signaling momentum from TLR4 to TLR2 through inducing TLR2 expression in a setting without LPS stimulation is believed to be important in promoting the development of inflammation and organ injury after HS/R.

Perspectives and Significance

The burden of disease related to trauma is considerable. The epidemiology of trauma mortality suggests two major phases. In the early phase, death most commonly results from neurologic injury and/or exsanguination. During the later phase, 60% of patients die of causes associated with pulmonary failure (1). The present study explored an important role of TLR2 in shock-augmented lung EC activation.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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