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*J Immunol* 2014; 193:6114-6123; Prepublished online 12 November 2014; doi: 10.4049/jimmunol.1400359

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PARP-1 Mediates LPS-Induced HMGB1 Release by Macrophages through Regulation of HMGB1 Acetylation

Zhiyong Yang, Li Li, Liujuan Chen, Weiwei Yuan, Liming Dong, Yushun Zhang, Heshui Wu, and Chunyou Wang

The high-mobility group box protein 1 (HMGB1) is increasingly recognized as an important inflammatory mediator. In some cases, the release of HMGB1 is regulated by poly(ADP-ribose) polymerase-1 (PARP-1), but the mechanism is still unclear. In this study, we report that PARP-1 activation contributes to LPS-induced PARylation of HMGB1, but the PARylation of HMGB1 is insufficient to direct its migration from the nucleus to the cytoplasm; PARP-1 regulates the translocation of HMGB1 to the cytoplasm through upregulating the acetylation of HMGB1. In mouse bone marrow–derived macrophages, genetic and pharmacological inhibition of PARP-1 suppressed LPS-induced translocation and release of HMGB1. Increased PARylation was accompanied with the nucleusto-cytoplasm translocation and release of HMGB1 upon LPS exposure, but PARylated HMGB1 was located at the nucleus, unlike acetylated HMGB1 localized at the cytoplasm in an import assay. PARP inhibitor and PARP-1 depletion decreased the activity ratio of histone acetyltransferases to histone deacetylases that elevated after LPS stimulation and impaired LPS-induced acetylation of HMGB1. In addition, PARylation of HMGB1 facilitates its acetylation in an in vitro enzymatic reaction. Furthermore, reactive oxygen species scavenger (N-acetyl-L-cysteine) and the ERK inhibitor (FR180204) impaired LPS-induced PAR activation and HMGB1 release. Our findings suggest that PARP-1 regulates LPS-induced acetylation of HMGB1 in two ways: PARylating HMGB1 to facilitate the latter acetylation and increasing the activity ratio of histone acetyltransferases to histone deacetylases. These studies revealed a new mechanism of PARP-1 in regulating the inflammatory response to endotoxin. The Journal of Immunology, 2014, 193: 6114–6123.
transfer of the ADP-ribosyl group in NAD\(^+\) to the carboxyl groups in the side chain of glutamic acid residues, resulting in O-linked poly(ADP-ribose) (14). Posttranslational modification is an important mechanism of PARP-1 action (14). Some proteins, such as PARP-1 itself, histone proteins, HMG proteins, DNA helicase, and several transcription factors, are substrates of PARP-1 (13). It has been reported that PARP-1 also participates in inflammation, because the suppression of PARP-1 activity can reduce the expression of inflammatory cytokines in animal models of endotoxemia (15).

The release of HMGB1 by LPS-challenged macrophages and mouse embryonic fibroblasts is also dependent on the activation of PARP-1 (16). However, the mechanism through which HMGB1 is released after PARP-1 activation remains unclear. In the current study, we report that LPS-induced HMGB1 release by macrophages is mediated by the reactive oxygen species (ROS)/ERK/PARP-1 signaling pathway, and the migration of HMGB1 to the cytoplasm as well as the subsequent release is accompanied by increased PARylation of HMGB1. However, the PARylation of HMGB1 is not sufficient to direct its translocation. PARP-1 regulates the translocation of HMGB1 to the cytoplasm through upregulating the acetylation of HMGB1 in two ways: PARylating HMGB1 to facilitate its acetylation and elevating the activity ratio of acetylases to deacetylases, which can catalyze acetylation and deacetylation of HMGB1.

**Materials and Methods**

**Reagents**

LPS (Escherichia coli 0111:B4), SB202190, FR180204, and SP600125 was purchased from Calbiochem. Anti-HMGB1, E1A-associated protein p300 (p300/CREB-binding protein (CBP))-associated factor (PCAF), CBP, p300, histone deacetylase (HDAC) 1, and HDAC4 Abs were obtained from Abcam. Full-length recombinant proteins of PCAF, HDAC1, and HDAC4 were from Abcam. Cell Science, and Origene. Normal rabbit IgG, protein A+G Agarose beads, HRP-conjugated secondary Abs, FITC-conjugated secondary Abs, anti–PARP-1, and GAPDH Abs were from Santa Cruz Biotechnology. \(^{13}C\)Acetate CoA was from AppliChem. DMEM, FBS, and heat-inactivated FBS were from Hyclone. DMEM/F12 and nonenzymatic cell dissociation solution were from Cellgro. Trypsin, penicillin, and streptomycin were from Life Technologies. Centrifugal filters (UFC510008; Millipore) and were from Cell Science Technology. PARP-1 small interfering RNA (siRNA), scrambled siRNA (sc siRNA), and DharmaFect 1 transfection reagent were obtained from Thermo Scientific. N-acetyl-l-cysteine (NAC), DMSO, and digitonin were from Sigma-Aldrich. Normal rabbit IgG and 20 \(\mu\)l protein A+G Agarose beads for 2 h at 4°C. After centrifugation at 10,000 \(\times\) g for 5 min, supernatants were transferred to new tubes and incubated with 40 \(\mu\)l protein A+G Agarose beads and rabbit anti-HMGB1 antibody and washed three times with PBS. In immunoblotting, samples were loaded equally for PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBST, probed with primary Abs for 2 h at room temperature or overnight at 4°C, and then incubated with HRP-conjugated secondary Abs at room temperature for 1 h. In certain experiments, HRP-conjugated Ab (Anti-biotin) was used instead of the native rabbit IgG. The bands were determined using ECL reagent (Pierce) and quantified using ImageJ Software (National Institutes of Health) from scanned films.

**ELISA**

HMGB1 in culture supernatants was detected with ELISA kits (Shino test) according to the manufacturer’s instructions.

**Immunofluorescence**

Cells plated on coverslips were fixed with 2% paraformaldehyde for 15 min and washed three times with 100 mM glycine in HBSS for 10 min followed by 100 mM Na2VO4, and protease inhibitor mixture. Lysates were sonicated and centrifuged at 10,000 \(\times\) g for 5 min. Supernatants were transferred to new tubes and incubated with 40 \(\mu\)l protein A+G Agarose beads and rabbit anti-HMGB1 antibody and washed three times with PBS. In immunoblotting, samples were loaded equally for PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBST, probed with primary Abs for 2 h at room temperature or overnight at 4°C, and then incubated with HRP-conjugated secondary Abs at room temperature for 1 h. In certain experiments, HRP-conjugated Ab (TrueBlot) was used instead of the native rabbit IgG. The bands were determined using ECL reagent (Pierce) and quantified using ImageJ Software (National Institutes of Health) from scanned films.

**PARP-1 depletion**

A total of 2 \(\times\) 10\(^6\) BMDMs were planted in six-well plates and cultured overnight. A pool of three target-specific 20–25 nt siRNA was used to knockdown PARP-1 at a concentration of 50 nmol/l. The transfection procedure followed the protocol of Dharmicon. Forty-eight hours after transfection, the depletion of PARP-1 was confirmed by Western blotting, and cells were used in subsequent experiments.

**PARP activity assay**

Intracellular PARP activity was measured by cell ELISA as Bakondi et al. (18) described. A total of 5 \(\times\) 10\(^4\) BMDMs were planted in 96-well plates. After incubation in 100 \(\mu\)l PARP buffer (56 mM HEPES, 28 mM KCl, 28 mM NaCl, and 2 mM MgCl\(_2\)) with 0.01% digitonin and 10 \(\mu\)M biotin-NAD\(^+\) for 30 min at 37°C, cells were treated with 200 \(\mu\)l 95% ethanol precooled at -20°C for 10 min. Then, cells were washed once with PBS followed by blocking in 1% BSA for 30 min. After that, cells were incubated in 50 \(\mu\)l streptavidin-HRP (1:500) at 37°C for 30 min. Three washes with PBS were followed by incubation in 100 \(\mu\)l TACS-Sapohire for 15 min at room temperature. The reaction was stopped by 1 M H\(_2\)SO\(_4\). The absorbance was detected at 450 nm with a microplate reader (Model 550; Bio-Rad).

**Preparation of cytoplasmic and nuclear extracts**

Cytoplasmic and nuclear extracts were prepared using the Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer’s instructions (Beyotime Institute of Biotechnology). Briefly, cells were scraped off, washed in ice-cold PBS, and then resuspended in 200 \(\mu\)l ice-cold cytoplasmic extraction buffer A with 1 mM PMSF, 1 mM Na2VO4, and protease inhibitor mixture. After incubation with cytoplasmic extraction buffer B for 1 min in an ice bath and following vortex for 5 s, cell lysates were centrifuged at 12,000 \(\times\) g for 5 min at 4°C. Supernatants were aliquoted and stored at -70°C. Nuclear pellets were resuspended in 50 \(\mu\)l PARP buffer (56 mM HEPES, 28 mM KCl, 28 mM NaCl, and 2 mM MgCl\(_2\)) for 10 min and then resuspended in 100 \(\mu\)l PARP buffer for 1 min in an ice bath followed by vortex for 5 s, cell lysates were extracted and stored at -70°C. Nuclear pellets were resuspended in 50 \(\mu\)l PARP buffer for 1 min in an ice bath followed by vortex for 5 s, cell lysates were centrifuged at 12,000 \(\times\) g for 5 min at 4°C. Supernatants were aliquoted and stored at -70°C until use.

**Immunoblotting and immunoprecipitation**

Cells were lysed in RIPA buffer supplemented with 1 mM PMSF, 1 mM Na2VO4, and protease inhibitor mixture. Lysates were sonicated and centrifuged at 10,000 \(\times\) g for 10 min at 4°C. The serum and medium were first ultracentrifuged with Centrifugal Filters (UCFS01024; Millipore) and then concentrated 30-fold with Centrifugal Filters (UCFS01008; Millipore). The concentration of proteins was measured with BCA kits. In immunoprecipitation, cell lysates were precleared with 1 \(\mu\)g normal rabbit IgG and 20 \(\mu\)l protein A+G Agarose beads for 2 h at 4°C. After centrifugation at 1000 \(\times\) g for 5 min, supernatants were transferred to new tubes and incubated with 40 \(\mu\)l protein A+G Agarose beads and rabbit anti-HMGB1 antibody overnight at 4°C. Cells were washed once with PBS followed by centrifugation and washed three times with PBS. In immunoblotting, samples were loaded equally for PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBST, probed with primary Abs for 2 h at room temperature or overnight at 4°C, and then incubated with HRP-conjugated secondary Abs at room temperature for 1 h. In certain experiments, HRP-conjugated Ab (TrueBlot) was used instead of the native rabbit IgG. The bands were determined using ECL reagent (Pierce) and quantified using ImageJ Software (National Institutes of Health) from scanned films.

**Expression of recombinant proteins**

The full-length HMGB1 (HMGB1-F1) was generated using forward primer 5'-TCTACTGCAGATCATGGCGAACAGGAGATCATC-3' and reverse primer 5'-CAATGCACAGATCATGGCGAACAGGAGATCATC-3'.
treated with the restriction enzymes EcoRI and Xhol, cloned in pET28a+ (NdeI). For the recombinant HMGB1-EGFP protein, the gene encoding human HMGB1 was cloned upstream of EGFP in pEGFP-N1 (BD Clontech), and then a SacI/NotI fragment from pHMGB1-EGFP was subcloned into pET28a+. Proteins were expressed in E. coli BL21. His-tagged proteins were purified on a HIS-Select NF Nickel Affinity gel (Sigma-Aldrich). The purity of all protein preparations was confirmed by SDS-PAGE.

PARylation and acetylation of proteins in vitro

The PARylation was done using biontin-NAD instead of [*]NAD (10). The final reaction was performed in a 50-μl mix buffer containing 50 mM Tris-HCl (pH 8), 25 mM MgCl₂, with 5 μg PAR-activated DNA (R&D Systems), 20 μM biontin-NAD (R&D Systems), and 0.3 μg each purified recombinant proteins. PARP (1 μl; R&D Systems) was added to each reaction and then incubated at room temperature for 30 min. The acetylation (19) were performed in 30 μl HAT buffer including 50 mM Tris-HCl (pH 8), 10% glycero, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 1 μM trichostatin A, and 30 μM trichostatin A (Sigma-Aldrich). The reaction was started by adding 1 μl recombinant PCAF, P300, and CBP catalytic domain (all from Enzo Life Sciences) and then incubated at room temperature for 30 min. For successive PARylation and acetylation, the probes of the PARylation were precipitated with 20% final concentration of TCA at −20°C for 16 h. After spin at 15,000 g for 20 min, and 100,000 g for 15 min, the pellets were washed three times with cold acetone and dissolved in 5 μl TE buffer. The reaction was started by adding 1 μl recombinant PARP, PCAF, P300, or CBP catalytic domain and then incubated at room temperature for 30 min. Heat-inactivated enzyme was used as control. To stop the reaction, loading buffer was added, and samples were boiled for 5 min prior to loading. The samples were analyzed by SDS-PAGE, stained with Coomassie R250 (Sigma-Aldrich), and the absorbance was detected at 405 or 440 nm with a microplate reader (Model 550; Bio-Rad).

Nuclear import assay

Nuclear import assays were performed as in Cassany and Gerace (20) by minor modification. Firstly, the cytosol of BMDMs was prepared. After washing twice in ice-cold PBS and once in washing buffer [10 mM HEPES (pH 7.3), 110 mM KOAc, 2 mM Mg(OAc)₂, and 2 mM DTT], BMDMs were homogenized with hypotonic lysis buffer [5 mM HEPES (pH 7.3), 10 mM KOAc, 2 mM Mg(OAc)₂, 2 mM DTT, 1 mM PMSF, and 1 μg/ml each leupeptin, pepstatin, and aproitin]. After centrifugation at 1500 × g for 15 min, at 100,000 × g for 20 min, and 100,000 × g for 1 h at 4°C, the final supernatants were collected and dialyzed at 4°C against transport buffer (TB; 20 mM HEPES (pH 7.3), 110 mM KOAc, 2 mM Mg(OAc)₂, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, and 1 μg/ml each leupeptin, pepstatin, and aproitin). The cytosol was then lyophilized, stored at −80°C. HMGB1-GFP was PARylated or acetylated in vitro as mentioned above. The reactions were precipitated with 20% final concentration of TCA at −20°C for 16 h. After spin at 15,000 × g for 15 min, the pellet was washed three times with cold acetone and dissolved in TE buffer (pH 8). For the assays, BMDMs were permeabilized for 5 min on ice in TB containing 40 μg/ml digitonin. After rinsing for 10 min with TB, cells were incubated in transport buffer for 15 min. The transport buffer contained a cocktail of BMDM cytosol (2 mg/ml, preincubated for 30 min at room temperature with ATP-regenerating system) with 50 μM g-lactosamin and 30 μg/ml PARylated HMGB1-GFP or 10 nM trichostatin A (Sigma-Aldrich) and 30 μg/ml acetylated HMGB1-GFP. ATP-regenerating system obtained 1 mM ATP, 3 mM creatine phosphate, 20 U/ml creatine phosphokinase, and 0.5 mM GTP. Finally, cells were fixed in 2% paraformaldehyde for 15 min and immediately examined by fluorescent microscopy (Olympus BX51; Olympus).

HDAC and HAT activity assay

HDAC and HAT activity was measured with HDAC and HAT Activity Colorimetric Assay Kits according to the manufacturer’s instructions. The absorbance was detected at 405 or 440 nm with a microplate reader (Model 550; Bio-Rad).

Animal experiments

C57BL/6 mice weighing 25–30 g were obtained from Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Mice were housed under specific-pathogen-free conditions with free access to water and standard mouse chow. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using the Student t test. Differences were considered significant when p < 0.05. Kaplan–Meier survival curves were compared using a log-rank test to determine significance.

Results

PARP-1 regulates LPS-induced HMGB1 migration from the nucleus to the cytoplasm in macrophages

PARP activity in BMDMs increased significantly, peaking at 4 h after LPS stimulation and lasting for 12 h (Fig. 1A). The HMGB1 level in the medium of the BMDMs increased at 4 h after LPS exposure (Fig. 1B), a finding that is consistent with previous research on this topic (21). To demonstrate the roles of PARP activation in LPS-induced HMGB1 release by macrophages, we used 3-AB to suppress the activity of PARP. We found that 3-AB significantly impaired the release of HMGB1 by BMDMs (Fig. 1B). Because PARP-1 is the main member of the PARP family (11), we knocked down PARP-1 to determine the influence of PARP-1 on LPS-induced HMGB1 release (Fig. 1C). We observed a sharp decline in LPS-induced HMGB1 release after PARP-1 knockdown (Fig. 1D). Therefore, we deduced that LPS-induced HMGB1 release by macrophages is dependent on PARP-1 activation. In the resting state, HMGB1 is mainly located in the nucleus, and its migration from the nucleus to the cytoplasm is a prerequisite for its release into the extracellular space. Using immunoblotting and indirect immunofluorescence labeling of HMGB1, we found that PARP-1 knockdown remarkably suppressed the LPS-induced translocation of HMGB1 to the cytoplasm in BMDMs (Fig. 1E, 1F). These results indicated that the LPS-induced migration of HMGB1 from the nucleus to the cytoplasm, and its release was dependent on the activation of PARP-1 in macrophages.

The PARylation of HMGB1 companies, but not directs, its relocalization to the cytoplasm in macrophages

PARP-1 catalyzes the PARylation of HMGB1 in vitro (10, 16). To elucidate the role of PARP-1 on intracellular PARylation of HMGB1, we knocked down PARP-1 in BMDMs and found that LPS-induced PARylation of HMGB1 was significantly suppressed (Fig. 2A). To further determine whether PARP-1 regulates the relocalization of HMGB1 through PARylation of HMGB1, we first measured the PARylation level of HMGB1 in the nucleus, cytoplasm, and cell-culture supernatant, respectively. The PARylation of HMGB1 in the cytoplasm and cell-culture supernatant was prominently upregulated after BMDMs were exposed to LPS (Fig. 2B). It indicated that the LPS-induced PARylation of HMGB1 is catalyzed by PARP-1, and this modification is accompanied by the migration of HMGB1 from the nucleus to the cytoplasm of macrophages and subsequent release into the extracellular space. To further elucidate whether PARylation of HMGB1 directs its migration, HMGB1 was PARylated in an in vitro enzyme reaction and then subjected to an import assay. As a control, GST-GFP was located in the cytoplasm. HMGB1-GFP and PARylated HMGB1-GFP were located in the nucleus; however, acetylated HMGB1-GFP was located in the cytoplasm (Fig. 2D). In addition, anacardic acid (AA), an inhibitor of acetylation, showed no influence on LPS-induced PARP activation (Fig. 2E) and PARylation of HMGB1 (Fig. 2F), but obviously suppressed the migration of HMGB1 to the cytoplasm (Fig. 2G). These data demonstrated that PARylation, not like acetylation, is insufficient for the translocation of HMGB1 to the cytoplasm.
PARP-1 regulates LPS-induced HMGB1 acetylation in macrophages

The migration of HMGB1 into the cytoplasm is dependent on the acetylation of lysine residues in monocytes or macrophages (8).

To clarify whether PARP-1 regulates the nucleus-to-cytoplasm migration of HMGB1, we suppressed the activity of PARP using 3-AB and found that LPS-induced HMGB1 acetylation was remarkably reduced (Fig. 3A). A similar phenomenon was observed through the knockdown of PARP-1 by siRNA (Fig. 3B). These data demonstrate that PARP-1 may regulate the nucleus-to-cytoplasm migration of HMGB1 by influencing HMGB1 acetylation.

**PARylation of HMGB1 facilitates its acetylation**

PARP can catalyze the transfer of the ADP-ribosyl group from NAD⁺ to the carboxyl groups of the glutamic acid residues, resulting in O-linked PAR (14). To elucidate the relationship between PARP-1 activation and HMGB1 acetylation, we investigated the influence of HMGB1 PARylation on HMGB1 acetylation. HMGB1-FL and HMGB1ΔC were PARylated through an in vitro enzymatic reaction and subjected to acetylation by
PCAF, CBP, or P300. PARylation reinforced the acetylation of HMGB1-FL, but not of HMGB1ΔC, by these acetylases (Fig. 4A, 4B). Glutamic acid residues are abundant in the C-terminal of HMGB1. We found that the PARylation level of HMGB1-FL was higher than that of HMGB1ΔC (data not shown), a finding that is consistent with previous research (10). It suggests the C-tail of HMGB1 may be PARylated by PARP. These data indicated that the PARylation of C-tail might influence the acetylation of HMGB1.

PARP-1 regulates the activities of HATs and HDACs in LPS-challenged macrophages

Acetylation is biregulated by HATs and HDACs. The acetylation of HMGB1 is regulated by several HATs (PCAF, CBP, and P300) and HDACs (HDAC1 and HDAC4) (8, 22). After LPS stimulation, the activity of HATs was increased, whereas that of HDACs was reduced in macrophages, resulting in an increase in the ratio of HAT to HDAC activity (Fig. 5A, 5B). Both 3-AB and PARP-1 knockdown

**FIGURE 2.** The PARylation of HMGB1 accompanies but not directs its relocalization in macrophages. (A) Cytoplasmic and extracellular HMGB1 was highly PARylated after LPS stimulation. BMDMs were exposed to LPS for 8 h. (B) Depletion of PARP-1 impaired the PARylation of HMGB1 in BMDMs. After knockdown of PARP-1, cells were exposed to 100 ng/ml LPS for indicated periods. (C) HMGB1 was successfully PARylated or acetylated in vitro. HMGB1-GFP was PARylated or acetylated as described in Materials and Methods. Reactions were analyzed by immunoblotting (top panel) or autoradiography (bottom panel). (D) PARylated HMGB1 located to the nucleus. HMGB1-GFP was PARylated or acetylated before import assay. GST-GFP was used as control. AA did not influence LPS-induced activation of PARP (E) and PARylation of HMGB1 (F). (G) AA impaired LPS-induced relocalization of HMGB1. BMDMs pretreated with 25 μmol/l AA were exposed to 100 ng/ml LPS for indicated time points (E–G). The activity of PARP was measured by Cell-ELISA (E). The culture supernatants, cytoplasmic (Cyto) and nuclear (Nuc) protein extracts (A), and cell lysates (B and F) were subjected to immunoprecipitation (IP) with anti-HMGB1 Ab. The PARylation of HMGB1 was measured by immunoblot (IB) analysis using Abs against PAR. Intracellular HMGB1 was stained by indirect immunofluorescence (G). Scale bars, 50 μm (D) and 100 μm (G).
could suppress the changes in HAT and HDAC activities in macrophages after LPS stimulation and significantly reduced the ratio of HAT to HDAC activity as compared with that in the sc siRNA group (Fig. 5A, 5B). To further determine which of those HATs and HDACs were regulated by PARP-1, we measured the expression of PCAF, CBP, P300, HDAC1, and HDAC4 in the nucleus. We found, 4 h after LPS exposure, the expression of PCAF, CBP, and P300 was increased by ∼60, 300, and 280%, respectively, and the expression of HDAC1 and HDAC4 was decreased by 20 and 80%, respectively (Fig. 5C). PARP-1 depletion inhibited LPS-induced increasing of the expression of PCAF, CBP, and P300 by ∼50–70% and decreasing of HDAC4 expression by ∼50%, but had no effect on the expression of HDAC1 (Fig. 5C). We also detected whether PARP-1 PARylates those HATs and HDACs and found CBP, P300, and HDAC4 were PARylated by PARP-1 in the enzyme system (Fig. 5D). We may infer from these data that in macrophages stimulated by LPS, PARP-1 can boost the acetylation of HMGB1 by increasing the activity ratio of HATs to HDACs that catalyze acetylation and deacetylation of HMGB1.

**LPS-induced PARP-1 activation and HMGB1 release use ROS and ERK signaling pathway**

It was reported that HMGB1 release induced by liver ischemia was dependent on ROS production (23). ROS are recognized as the main signals leading to intracellular PARP-1 activation (24). Macrophages produce large amounts of ROS after LPS stimulation (25). Thus, we hypothesized that ROS might mediate LPS-induced PARP-1 activation and HMGB1 release. After treatment with NAC, a scavenger of ROS, PARP-1 activation in BMDMs was significantly suppressed (Fig. 6A). Moreover, the migration of HMGB1 to the cytoplasm (Fig. 6B) and the release of HMGB1 into the extracellular space were remarkably reduced (Fig. 6B, 6C). It has been reported that PARP-1 can be activated by MAPKs (26). LPS can activate p38MAPK, JNK, as well as the ERK signaling pathway (27); however, only inhibitors of the ERK pathway inhibited the activation of PARP-1 (Fig. 6D). NAC inhibited the LPS-induced activation of ERK (Fig. 6E). Consistently, the LPS-induced release of HMGB1 was inhibited by FR180204, an ERK inhibitor (Fig. 6F). NAC suppressed LPS-induced activation of PARP-1 by >90% (Fig. 6A), although it just inhibited the activation of ERK by ∼50% at 30 min after LPS treatment (Fig. 6E). It indicated that ROS might use other signal pathways to mediate PARP-1 activation in this model. ROS/DNA injury was known as the classic and strong activator of PARP-1. It was reported that ERK activated PARP-1 through phosphorylation, and it was independent of DNA injury (28). These data indicate that LPS-induced activation of PARP-1 may use ROS/ERK pathway and ROS/DNA injury pathway.

**NAC, 3-AB, and FR180204 reduces the serum HMGB1 level and mortality rate in a mouse model of endotoxemia**

In a mouse model of endotoxemia, the PARP-1 inhibitor 3-AB, the ROS scavenger NAC, and the ERK inhibitor FR180204 could reduce the HMGB1 level in the serum (Fig. 7A) as well as the mortality rate (Fig. 7B).
FIGURE 5. PARP-1 regulates the activity of HATs and HDACs in LPS-challenged macrophages. 3-AB (A) and PARP-1 depletion (B) downregulated the activity ratio of HATs to HDACs in LPS-challenged BMDMs. BMDMs pretreated with 10 mmol/l 3-AB were exposed to 100 ng/ml LPS for indicated periods (A). After PARP-1 knockdown, BMDMs were exposed to 100 ng/ml LPS for indicated periods (B). Nucleus proteins were extracted, and colorimetric assay kits were used for measuring the activity of HATs and HDACs (A and B). The activity ratio of HATs to HDACs was calculated. (C) The effect of PARP-1 depletion on HAT and HDAC expression in the nucleus of LPS-challenged BMDMs. After PARP-1 knockdown, BMDMs were exposed to 100 ng/ml LPS for 4 h. Nucleus proteins were extracted, and the expression of PCAF, CBP, P300, HDAC1, and HDAC4 was measured by immunoblotting. Quantified data are shown in the right panel. (D) CBP, P300, and HDAC4 were PARylated by PARP-1. PCAF, CBP, P300, HDAC1, and HDAC4 were subjected to PARylation in PARP-1 catalytic system as described in Materials and Methods. Data are mean ± SEM of three independent experiments. *p < 0.05 compared with corresponding sc siRNA group, #p < 0.05 compared with corresponding group without LPS treatment.
Discussion

The active secretion of HMGB1 can be activated by inflammatory mediators and hypoxia. This process is composed of three steps: first, migration from the nucleus to the cytoplasm; second, enter into the organelles in the cytoplasm; and third, release into the extracellular space through exocytosis (2). The passive release of HMGB1 from dead cells is due to its migration from the nucleus to the cytoplasm and subsequent release into the extracellular space owing to increased cell membrane permeability (9). The translocation of HMGB1 from the nucleus to the cytoplasm,
PARylation does not direct the migration of HMGB1 to the cytoplasm, as PAR-modified HMGB1 is located in the nucleus, implying that acetylated HMGB1 is located in the cytoplasm, whereas PARylation of HMGB1 in LPS-stimulated macrophages was significantly upregulated, with higher levels of acetylation in an in vitro enzymatic reaction system, indicating that PARylation of HMGB1 can boost its acetylation. Furthermore, we found that PARylation had no influence on the acetylation of HMGB1, which binds to HMG boxes, contains many glutamic acid residues. Further study is required to determine whether the PARylation of these glutamic acid residues would expose acetylation sites hidden by the C-terminal and facilitate the acetylation of lysine residues in HMG boxes.

Under inflammatory conditions, acetylases such as CBP/p300 in monocytes and macrophages are activated, whereas the activities of HDAC1 and HDAC4 are decreased (22). This results in the acetylation of Lys27, Lys43, and Lys74–178 in HMGB1, which reduces the binding capacity of HMGB1 to DNA and leads to its subsequent migration from the nucleus to the cytoplasm, followed by secretion into the extracellular space through exocytosis (8, 22). We found that after stimulation with LPS, the activity of HATs in BMDMs increased, the activity of HDACs decreased, and acetylated HMGB1 increased significantly. Furthermore, 3-AB treatment and knockdown of PARP-1 suppressed the upregulation of HATs activity and downregulation of HDACs activity induced by LPS. It was further confirmed by measuring the expression of PCAF, CBP, P300, HDAC1, and HDAC4 in the nucleus, which can catalyze acetylation and deacetylation of HMGB1. PARP-1 may regulate the activity of HATs and HDACs through PARylation of those enzymes because CBP, P300, and HDAC4 were PARylated in the PARP-1 catalytic system. Decreased ratio of HAT and HDAC activity was accompanied by a lower level of HMGB1 acetylation, implying that it played important roles in the regulation of HMGB1 acetylation. PARP-1 mediated the LPS-induced activity change of those acetylases and deacetylases and thus regulated the acetylation and nucleus-to-cytoplasm migration of HMGB1.

In addition, this study demonstrated that the ROS scavenger NAC can remarkably inhibit the LPS-induced activation of PARP-1 and nucleus-to-cytoplasm migration and release of HMGB1. Although it has been reported that HMGB1 release from monocytes and macrophages can be induced by H2O2 through the ERK and JNK signaling pathways (31), our data showed that among the inhibitors of the p38 MAPK, JNK, and ERK signaling pathways, only the ERK inhibitor FR180204 inhibited LPS-induced PARP-1 activation and HMGB1 release. NAC suppressed LPS-induced activation of PARP-1 by >90% despite inhibiting the activation of ERK by ~50%. ROS might use other signal pathways to activate PARP-1, such as the classic DNA injury/PARP-1 pathway. These results indicate that LPS-induced activation of PARP-1 may use the ROS/ERK pathway and ROS/DNA injury pathway. Data from animal experiments were consistent with the above conclusion, because a PARP-1 inhibitor, an ROS scavenger, and an ERK inhibitor all reduced the serum HMGB1 level and mortality rate in a mouse model of endotoxia.

In summary, we, for the first time, to our knowledge, demonstrated that LPS induced PARP-1 activation using the ROS/ERK signaling pathway and thus elevated the activity ratio of HATs and HDACs by regulating the expression of PCAF, CBP, P300, and HDAC4 in the nucleus, resulting in the acetylation of HMGB1. In contrast, PARP-1 catalyzed PARylation of HMGB1, which facilitated acetylation of HMGB1. Increased acetylation owing to

**FIGURE 7.** NAC, 3-AB, and FR180204 reduce the serum HMGB1 level and mortality rate in a mouse model of endotoxia. Mice, 60 for survival analysis and 30 for serum collection, were randomly divided into five groups (negative control, LPS+PBS, LPS+3-AB, LPS+NAC, and LPS+FR180204). Endotoxia was established by i.p. injection of LPS (40 mg/kg), and PBS was used for establishing negative control. Except for negative control, mice were pretreated with PBS, 3-AB (10 mg/kg), NAC (200 mg/kg), or FR180204 (2 mg/kg) for 30 min according to groups before model establishment. Sixteen hours later, mice were sacrificed, and the serum was collected for detecting HMGB1 by immunoblotting (A). (B) Kaplan–Meier survival plots for the five groups. The amount of surviving mice was recorded every 8 h. *p < 0.05 compared with LPS+PBS group.
PARP-1 activation directed the migration of HMGB1 into the cytoplasm as well as release into the extracellular space.

In recent years, a growing body of studies revealed that HMGB1 release was dependent on inflammasome assembly and caspase-1 activation (32). Also, dsRNA-dependent protein kinase played a crucial role on inflammasome activation and HMGB1 release (33). However, the downstream signal of inflammasome/caspase-1 that causes HMGB1 release is still unknown. Acetylation, which was modulated by HATs and HDACs, and phosphorylation, catalyzed by classical protein kinase C (34) and calcium/calmodulin-dependent protein kinase IV (35), directs the relocalization and release of HMGB1. In this study, we found LPS-induced HMGB1 release was dependent on PARP-1 activation. Whether PARP-1 regulates phosphorylation of HMGB1 and how inflammasome assembly/caspase-1 activation, protein kinase C activation, calcium/calmodulin-dependent protein kinase IV activation, and PARP-1 activation are related need to be further studied.

Acknowledgments

We thank the Pancreatic Disease Institute staff for support during the study.

Disclosures

The authors have no financial conflicts of interest.

References


