HHS Public Access

Author manuscript

J Thromb Haemost. Author manuscript; available in PMC 2020 May 01.

Published in final edited form as:

J Thromb Haemost. 2019 May ; 17(5): 803–817. doi:10.1111/jth.14425.

Activated protein C inhibits LPS-mediated acetylation and secretion of HMGB1 in endothelial cells

Xiaofeng Cai[*,1], **Indranil Biswas**[*,1], **Sumith R. Panicker**[1], **Hemant Giri**[1], and **Alireza R. Rezaie**[1,2]

[1]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation

[2]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Summary.

Background: Activated protein C (APC) inhibits high mobility group box 1 (HMGB1) signaling and its LPS-mediated release by EPCR-dependent activation of PAR1 in endothelial cells. Post-translational acetylation is known to modulate the subcellular localization of HMGB1 and its hyperacetylated form is translocated to the cytoplasm of innate immune cells before secretion to extracellular space.

Objective: The objective of this study was to determine whether APC inhibits LPS-mediated HMGB1 secretion from endothelial cells by modulating its acetylation status.

Methods: The subcellular localization of HMGB1 in LPS-treated endothelial cells was monitored in the absence and presence of APC by Western-blot analysis of fractionated cell lysates and confocal immunofluorescence microscopy.

Results: Both Western-blot and immunofluorescence data indicated that APC effectively inhibits LPS-mediated translocation of HMGB1 from the nucleus to the cytoplasm by EPCR- and PAR1-dependent mechanisms. When EPCR was ligated by Gla-domain of protein C/APC, thrombin also inhibited LPS-mediated HMGB1 translocation. Further studies revealed that APC inhibits the translocation of HMGB1 from the nucleus to the cytoplasm by inhibiting LPS-mediated hyperacetylation of HMGB1 by (de)acetylating enzymes. Furthermore, the translocated HMGB1 was found to be associated with LAMP1 in LPS-treated endothelial cells. The in vivo relevance of these findings was investigated in the mouse cremaster muscle demonstrating that both wild-type

Address of corresponding author: Alireza R. Rezaie, Ph.D. Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, Tel: (405) 271-4711 Ray-Rezaie@omrf.org.

*Both authors made equal contributions to this study

Addendum

X.C. and I.B. designed experiments, performed research and analyzed data; S.R.P designed and performed the mouse cremaster muscle experiments and analyzed data; H.G. performed research; A.R.R. designed experiments, analyzed data and wrote the manuscript. All authors approved the final version of the manuscript.

Disclosure of Conflict of Interests

The authors declare no conflict of interests.

Supplementary Materials

1. Supplementary figures 1S–8S for LPS-mediated HMGB1 and HDAC translocation.

and a signaling-selective mutant of APC inhibit LPS-mediated HMGB1 expression and translocation in CD31-positive endothelial cells.

Conclusion: These results suggest that APC inhibits LPS-mediated cytoplasmic translocation and secretion of HMGB1 in endothelial cells by epigenetic mechanisms.

Keywords

activated protein C; endothelial protein C receptor; protease-activated receptor 1; HMGB1; acetylation

Introduction

Activated protein C (APC) exerts its cytoprotective and antiinflammatory signaling function through endothelial protein C receptor (EPCR)-dependent activation of protease-activated receptor 1 (PAR1) on endothelial and other cell types (1–6). This direct cellular signaling function of APC inhibits activation of NF-κB, expression of proinflammatory cytokines (IL-1, IL-6 and TNF-α) and cell adhesion molecules in cytokine-stimulated cells (3–7). Moreover, EPCR- and PAR1-dependent signaling activity of APC enhances the barrier-protective function of endothelial cells in response to bacterial lipopolysaccharide (LPS), high mobility group box 1 (HMGB1) and other proinflammatory stimuli that promote cytoskeletal rearrangement and barrier disruption in the microvasculature (8–11). In a recent study, we demonstrated that APC inhibits LPS-mediated extracellular secretion of HMGB1 and downregulates cell surface expression of the receptor for advanced glycation end-products (RAGE) and Toll-like receptors (TLR) 2 and 4 through which HMGB1 initiates its intracellular signal effects (8). HMGB1 is a chromatin-associated protein that may be actively secreted into the extracellular space by cytokine-stimulated cells or passively released by damaged tissues and necrotic cells in response to bacterial endotoxin and/or trauma-related injury (12–15). High levels of extravascular HMGB1, secreted/released with delayed kinetics, have been observed in the plasma of patients with severe sepsis and in animal models of LPS-induced endotoxemia (16). A high concentration of plasma HMGB1 has been found to correlate with poor prognosis and high mortality rates in severe sepsis patients and its inhibition by neutralizing antibodies and/or specific antagonists has been shown to improve survival in animal models of endotoxemia (16,17).

Increasing evidence suggests that translocation of HMGB1 from the nucleus to the cytoplasm in activated innate immune cells is mediated by acetylation of HMGB1 (18), a reversible post-translational modification process that is epigenetically regulated by coordinated activities of histone modifying enzymes including histone acetyltransferases (HATs) and histone deacetylases (HDACs) (19). LPS-mediated acetylation of distinct lysine residues of HMGB1 by specific acetyltransferases can destabilize the interaction of HMGB1 with the chromatin, thus facilitating its translocation from the nucleus to the cytoplasm followed by storage in specific secretory vesicles (20,21). In activated monocytes/macrophages, acetylated HMGB1 has been shown to accumulate in secretory lysosomal vesicles and that its subsequent extracellular secretion can occur as the result of exocytosis of these vesicles in response to proinflammatory signaling cues initiated by LPS and other cytokines (i.e., TNF-α and IL-1β) (18,21). We previously demonstrated that when EPCR is

occupied by Gla-domain of protein C/APC, cleavage of PAR1 by either APC or thrombin elicits a cytoprotective signaling response that inhibits LPS-mediated extracellular secretion of HMGB1 by endothelial cells (6,8). The mechanism by which EPCR-dependent cleavage of PAR1 by APC or thrombin inhibits secretion of HMGB1 in LPS-treated endothelial cells remains unknown. We addressed this question in this study and investigated whether LPS-mediated acetylation of HMGB1 modulates subcellular localization of the nuclear protein in endothelial cells and if APC inhibits LPS-mediated translocation and secretion of HMGB1 by endothelial cells through regulation of the acetylation status of the nuclear protein by epigenetic mechanisms.

Materials and Methods

Reagents

Recombinant proteins including activated protein C (APC), the signaling-selective mutant of APC (APC-2Cys), the catalytically inactive protein C-S195A (PC-S195A), thrombin, meizothrombin and PC-Gla/meizothrombin (meizothrombin derivative containing Gla-domain of protein C) were prepared as described previously (22,23). LPS (Lipopolysaccharide, L4391), prepared from *Escherichia coli* O111:B4, and Trichostatin A (TSA) were purchased from Sigma-Aldrich Chemical Co (St Louis, MO). Protein A/G PLUS-Agarose (#2003) was purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). Human HMGB1 ELISA kit (Colorimetric) (#NBP2–62766) was from Novus Biologicals (Centennial, CO). Thrombin receptor agonist peptide (TRAP) was from Bachem Bioscience (Torrance, CA). Anti-EPCR (clone JRK1535), anti-PAR1 (clone ATAP2, #MABF244) and anti-Mac-1 function-blocking antibodies were from Millipore (Burlington, MA). Anti-HMGB1 (High mobility group protein B1, Rabbit, #6893), anti-CBP (CREB-binding protein, #7389), anti-PARP1 (poly (ADP-ribose) polymerase 1, #9541), anti-HDAC1 (histone deacetylase 1, #5356), anti-HDAC2 (#5113), anti-HDAC4 (#7628), anti-acetylated-lysine (#9441) and anti-β-actin (#4967) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-HMGB1 (mouse, #651401), anti-CD31 (#102501) and anti-LAMP1 (lysosome-associated membrane protein 1, #328601) antibodies were from BioLegend (San Diego, CA). Anti-HMGB1 (mouse, #MAB1690) antibody was from R&D Systems (Minneapolis, MN). Biotin-conjugated anti-HMGB1 (Rabbit, #PA5–22719) antibody was from Thermo Fisher Scientific (Waltham, MA). Anti-acetyl-HMGB1 (Lys12) (Rabbit, #OASG03545) antibody was from Aviva Systems Biology (San Diego, CA), anti-CBP (#369), anti-GAPDH (glyceraldehyde-3-phoshate dehydrogenase, #47724), the non-blocking anti-EPCR (FL-238) and anti-PAR1 (S-19) antibodies were from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA); Cy3-conjugated streptavidin (#016–160-084), Cy3-conjugated goat anti-rabbit IgG (#111–166-144) and Cy3-conjugated donkey anti-mouse IgG (#715–165-150) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 488-conjugated donkey anti-rat IgG (#A21208), Alexa Fluor 488-conjugated goat anti-mouse IgG (#A11029) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (#A11008) were from Invitrogen Corporation (Carlsbad, CA).

Cell lines and culture

Primary human umbilical vein endothelial cells (HUVECs, Invitrogen), transformed HUVECs (EA.hy926 cells, ATCC) and murine macrophage cell line J774A.1 (ATCC® TIB67™) was maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine, and cultured at 37°C in a humidified incubator with 5% CO2 as described (8,10).

Confocal imaging

Following treatments, all three cell lines (EA.hy926, primary HUVECs and J774A.1) were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100/PBS, followed by blocking for 1h with normal goat serum. For the analysis of HMGB1 translocation, cells were incubated with mouse or rabbit anti-HMGB1 overnight at 4°C and then incubated with Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit secondary antibody for 1h at room temperature in the dark. For colocalization of HMGB1 in lysosomal vesicles, cells were incubated with rabbit anti-HMGB1 and mouse anti-LAMP1 overnight at 4°C and then incubated with fluorescent Cyanine 3 (Cy3)-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies for 1h at room temperature in the dark. Cells were then washed and stained with DAPI or Hoechst (1 μg/mL), and mounted with anti-fade mounting media (DAKO products, Santa Clara, CA). Photomicrographs were obtained using a Nikon C2 Confocal Microscope. For quantitative analysis of HMGB1 translocation, cells with diffused HMGB1 in the cytoplasm were defined as translocation-positive, which were counted for at least 10 randomly selected fields from 3 independent samples. The translocation efficiency was calculated based on the ratio of translocation-positive cells to total cells in these fields.

Cell fractionation

For the preparation of cytoplasmic and nuclear extracts, 1×10^6 treated cells were washed in ice-cold PBS, and harvested in 100 μL ice cold cytoplasmic extraction buffer (10 mM HEPES pH 7.4, 60 mM KCl, 1 mM EDTA, 0.05% NP40, 1 mM DTT, 1 mM PMSF) supplemented with protease inhibitor cocktail as described (24). After incubation with cytoplasmic extraction buffer for 1min in an ice bath and vortexing for 5s, cell lysates were centrifuged at 10,000 g for 5min at 4°C. Supernatants were collected and stored as cytoplasmic fractions. Nuclear pellets were resuspended in 50 μL nuclear extraction buffer (20 mM Tris-Cl pH 7.8, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM PMSF) supplemented with protease inhibitor cocktail. After 30min incubation on ice with 5 times vortexing, lysates were centrifuged at 12000 g for 10min at 4°C. Nuclear extracts were aliquoted and stored at −80°C until use.

Immunoprecipitation and Western-blotting

Treated cells were lysed with RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholic acid, 5 mM tetrasodium pyrophosphate, 50 mM sodium fluoride, 5 mM EDTA, 150 mM NaCl, 25 mM Tris-HCl (pH 7.5), 5 mM Na_3VO_4 and protease inhibitor cocktail). Cell lysates (500 μg protein) was pre-cleared with 1 μg mouse IgG and 20 μL Protein A/G Plus agarose beads for 30min at 4°C. The beads were then pelleted and supernatant was

incubated with 2 μg mouse anti-HMGB1 antibody overnight at 4°C. Beads were washed twice and the bound proteins were eluted with 1x sample loading buffer and resolved on a 12% polyacrylamide-SDS gel. The resolved proteins were then electro-blotted onto a PVDF membrane, blocked with 5% skim milk (wt/vol) and incubated with primary antibodies against acetylated-lysine or CBP. The immunoblotted PVDF membranes were then incubated with the respective horseradish peroxidase-conjugated secondary antibody for 1h and bands were detected using enhanced chemiluminescence reagent. Protein bands were quantified using NIH Image J software. Similar procedures were used in another set of experiments where rabbit anti-PARP1 antibody was used for immunoprecipitation followed by immunoblotting for HMGB1 with mouse anti-HMGB1 antibody as described (25). Rabbit IgG was used here to pre-clear the lysate.

HMGB1 ELISA

HMGB1 release in supernatant was quantified with a commercially available HMGB1 ELISA kit (Novus Biologicals, NBP2–62766) according to the manufacturer's protocol.

Immunohistochemistry and Western-blotting of mouse cremaster muscles

All animal studies were performed in compliance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma Medical Research Foundation. Eight to twelve weeks old male C57BL/6J mice were injected intrascrotally with either saline or LPS (0.5 mg/kg). In another group of mice, LPS was injected 30min after intrascrotal injection of wild-type APC or the signaling-selective APC-2Cys variant (0.2mg/kg). One hour after LPS injection, cremaster muscle tissues were dissected and used for immunohistochemistry and Western-blotting as described (26). For immunohistochemistry, tissues were fixed in 4% paraformaldehyde overnight at 4°C. Tissue samples were washed in PBS, cryoprotected in 20% sucrose in PBS at 4°C overnight, embedded in 50% tissue freezing medium/50% OCT (Optimal cutting temperature) compound and cryosectioned (5–10 μm sections). Cryosections were incubated with biotin-conjugated rabbit antibody against mouse HMGB1 combined with rat anti-mouse CD31 overnight at 4°C, developed with Cy3-conjugated streptavidin and Alex Fluor 488-conjugated donkey anti-rat antibody, co-stained with DAPI for nucleus, and mounted with Permafluor anti-fade mounting medium. Specimens were analyzed by confocal imaging using a Nikon C2 confocal laser-scanning microscopy. Species-specific, isotype control antibodies were used as negative controls. For Western-blotting, cremaster muscle tissue lysates were prepared using RIPA buffer as described above.

Statistical analysis

Data are presented as mean ± SE from ≥3 independent experiments. Data were analyzed by the Student t-test, and group data were analyzed using ANOVA followed by Bonferroni post hoc test using Graph Pad Prism7 (Graph Pad Prism, CA). A p value of <0.05 was considered statistically significant.

Results

LPS-mediated HMGB1 translocation

In a previous study, we demonstrated that APC inhibits LPS-mediated secretion of HMGB1 in primary HUVECs and EA.hy926 endothelial cells (8). To understand the mechanism by which APC inhibits HMGB1 release in endothelial cells, we monitored LPS-mediated subcellular localization/translocation of HMGB1 in both EA.hy926 cells and HUVECs in the absence and presence of APC. Western-blot analysis of nuclear and cytoplasmic extracts of LPS-treated EA.hy926 cells revealed that LPS induces translocation of HMGB1 from the nucleus to the cytoplasm by a time-dependent manner and a significant fraction of HMGB1 migrates to the cytoplasm after an hour of LPS treatment (Supplementary Figure 1S). This conclusion was based on a significant decrease in the density of HMGB1 in the blot of the nuclear extract which paralleled with a proportionally enhanced localization of the protein in the cytoplasmic extract of LPS-treated cells. Further analysis indicated that HMGB1 cytoplasmic translocation can be observed at LPS concentration of as low as 10 ng/mL, but it was maximal at 1μg/mL (Supplementary Figure 2S). Thus, we conducted all experiments described below with 1μg/mL LPS. Analysis of nuclear and cytoplasmic extracts of LPS-treated EA.hy926 cells in the presence of APC suggested that APC effectively inhibits LPS-mediated translocation of HMGB1 from the nucleus to the cytoplasm (Figure 1A). LPS-mediated translocation of HMGB1 was also analyzed by immunofluorescent staining of HMGB1 (Figure 1B). In agreement with Western-blots, the immunofluorescence data indicated that LPS signaling induces translocation of HMGB1 from the nucleus to the cytoplasm in EA.hy926 cells and APC effectively inhibits this process (Figure 1B). This response was not specific for EA.hy926 endothelial cells, since essentially identical results were obtained when LPS-mediated translocation of HMGB1 in the absence and presence of APC was analyzed for primary HUVECs (Figure 1C). The cytoplasmic translocation of HMGB1 was detected for approximately 40% of LPS-treated cells, which was effectively inhibited by APC in both primary HUVECs and EA.hy926 cells (Figures 1D and E). In light of essentially identical results for both primary HUVECs and EA.hy926 cells, due to ease of manipulation, the studies described below were conducted in EA.hy926 cells. It is worth noting that LPS also increased expression of HMGB1 after 3h and APC effectively inhibited the expression of HMGB1 in endothelial cells as determined by time course analysis of the whole cell lysate by Western-blot in the absence and presence of APC (Supplementary Figure 3S). In agreement with our previous results (8), APC also inhibited extracellular secretion of HMGB1 in endothelial cells as determined by an ELISA (Supplementary Figure 3S, panel C).

APC inhibits LPS-mediated HMGB1 translocation by EPCR- and PAR1-dependent mechanisms

To investigate the receptor-dependency of the protective effect of APC in response to LPS, Western-blot and immunofluorescence studies were also conducted in the absence and presence of function-blocking and non-blocking antibodies to EPCR and PAR1. Results indicated that the effect of APC in inhibiting nuclear to cytoplasmic translocation of HMGB1 was mediated through EPCR-dependent cleavage of PAR1 since function-blocking anti-EPCR (JRK1535) and anti-PAR1 (ATAP2) antibodies both inhibited protective activity

of APC (Figure 2A-C). The non-blocking anti-EPCR (FL-238) and anti-PAR1 (S-19) antibodies did not influence the function of APC in this assay (Figure 2A-B).

In a series of recent studies, we have demonstrated that the occupancy of EPCR endows a PAR1-dependent cytoprotective effect for thrombin in vascular endothelial cells (6,23). In agreement with our previous results, thrombin also inhibited LPS-mediated translocation of HMGB1 from the nucleus to the cytoplasm if EPCR was occupied by Gla-domain of protein C by pretreating cells with PC-S195A before activating PAR1 with thrombin (Supplementary Figure 4S). In agreement with these results, a prothrombin activation intermediate (meizothrombin), in which its Gla-domain was replaced with protein C Gla-domain (23), exhibited an APC-like HMGB1 inhibitory activity in response to LPS in endothelial cells (Supplementary Figure 5S).

APC inhibits LPS-mediated HMGB1 acetylation in endothelial cells

The cytoplasmic translocation of HMGB1 by activated innate immune cells has been shown to be regulated by acetylation of specific lysine residues within two clusters of nuclear-localization sites on HMGB1 (18,27). It has been found that HMGB1 continuously shuttles between the nucleus and cytoplasm and that the equilibrium is shifted in favor of accumulation in the cytoplasm when HMGB1 is hyperacetylated by the balanced catalytic function of (de)acetylating enzymes (18,19,27). Interestingly, immunoprecipitation of cell lysates of LPS-treated endothelial cells with anti-HMGB1 antibody and subsequent analysis of co-precipitated proteins by Western-blot, using a specific antibody detecting acetylated-lysines, revealed that LPS signaling induces hyperacetylation of HMGB1 and APC inhibits this response (Figure 3A). These results were further confirmed by Western-blotting of cell lysates using a rabbit antibody that directly detects acetylated HMGB1 (Ace-HMGB1) (Figure 3B). Immunofluorescent staining with rabbit anti-Ace-HMGB1 confirmed that LPS signaling induces hyperacetylation of HMGB1, thus favoring its accumulation in the cytoplasm (Figure 3C, second row). APC effectively inhibited LPS-mediated acetylation of HMGB1 in endothelial cells (Figure 3C, third row). In agreement with our hypothesis that the occupancy of EPCR determine the signaling specificity of thrombin, both thrombin and the thrombin receptor activation peptide (TRAP), also promoted acetylation and cytoplasmic translocation of HMGB1 in endothelial cell the absence but not in the presence of PC-S195A (Supplementary Figure 6S).

APC inhibits LPS-mediated HMGB1 translocation by modulating (de)acetylating enzymes

A coordinated catalytic function of histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate the acetylation status of HMGB1, histones and a number of non-histone proteins (19,27). Among several HATs, it has been found that CBP, which has intrinsic histone acetyltransferase activity, can efficiently acetylate HMGB1 as demonstrated by an in vitro acetylation assay (20,28). To investigate whether APC modulates the activity of CBP in LPS-treated endothelial cells, cell lysates were first immunoprecipitated with an anti-HMGB1 antibody and co-precipitated proteins were then analyzed by immunoblotting using an anti-CBP antibody. Results demonstrated that APC downregulates the interaction of CBP with HMGB1 in LPS-treated cells (Figure 3D).

To investigate whether APC also regulates the acetylation status of HMGB1 by regulating expression and/or subcellular localization of HDACs, nuclear and cytoplasmic extracts of LPS-treated cells, before and after pretreatment with APC, were analyzed by immunoblotting using specific antibodies to HDAC1, HDAC2 and HDAC4. Time course analysis indicated that LPS treatment results in translocation of a significant fraction of HDAC2 and HDAC4, but not HDAC1, from the nucleus to the cytoplasm. This conclusion was based on a dramatic increase in cytoplasmic fractions of HDAC2 and HDAC4, which was accompanied by a proportional decrease in the density of their nuclear fractions observed on the Western-blot (Supplementary Figure 7S). These results demonstrated that while these enzymes are primarily localized in the nucleus of non-treated cells, HDAC2 and HDAC4 can be translocated to the cytoplasm by LPS signaling in a time-dependent manner. LPS did not exhibit any effect on translocation of HDAC1 from the nucleus to the cytoplasm. Further analysis of cell lysates demonstrated that APC downregulates LPS-mediated translocation of HDAC2 and HDAC4 from the nucleus to the cytoplasm (Figure 3E-F). Taken together, these results suggest that the inhibitory effect of APC against LPS-mediated translocation of HMGB1 is mediated at least partially through the protective signaling of APC inhibiting interaction/acetylation of HMGB1 by (de)acetylating enzymes, which appears to be the first step in LPS-mediated secretion of HMGB1 to extracellular space in endothelial cells similar to that reported for immune cells. Interestingly, similar to LPS, the HDAC inhibitor, trichostatin A (TSA) promoted the acetylation of HMGB1 and its cytoplasmic translocation of HMGB1 in EA.hy926 cells (Supplementary Figure 8S).

Another nuclear enzyme that has been reported to be activated by LPS and plays an important role in the acetylation-dependent LPS-mediated secretion of HMGB1 is poly(ADP-ribose) polymerase 1 (PARP1), which can catalyze the transfer of ADP-ribosyl group from nicotine adenine dinucleotide (NAD^+) to HMGB1 and other acceptor proteins in the nucleus (24,29,30). This modification has been demonstrated to play an essential role in acetylation-dependent translocation of HMGB1 from the nucleus to the cytoplasm in activated macrophages (24,29,30). Immunoprecipitation and immunoblotting studies demonstrated that HMGB1 is also associated with PARP1 in LPS-treated cells (Figure 3G). Interestingly, we found that APC also inhibits LPS-mediated association of HMGB1 with PARP1 in endothelial cells (Figure 3G).

LPS-mediated cytoplasmic HMGB1 may be stored in secretory lysosomes

LPS-mediated translocation/secretion of HMGB1 from the nucleus to the cytoplasm and to the extracellular space has been extensively studied for activated monocytes/macrophages (18,21,24). It has been found that the cytoplasmic HMGB1 is stored in secretory lysosomal vesicles of activated cells before secretion to extracellular space by exocytosis (18,21). To determine whether LPS-activated endothelial cells secret HMGB1 by a similar mechanism, confocal microscopy was used to capture immunofluorescence images of HMGB1 and a lysosomal marker, lysosomal-associated membrane protein 1 (LAMP1). Results of double staining, presented in Figure 4A, demonstrate that the translocated HMGB1 is partially colocalized with LAMP1 in the cytoplasm of LPS-treated cells, possibly suggesting that LPS induces secretion of the cytoplasmic HMGB1 in endothelial cells by a similar mechanism through directing HMGB1 to similar secretory lysosomes before secretion to

extracellular space. Immunofluorescent co-staining with Ace-HMGB1 and LAMP1 further suggested that it is the acetylated form of HMGB1 that is partially colocalized with LAMP1 in LPS-treated cells, a localization process which is effectively inhibited by APC (Figure 4B).

APC inhibits LPS-mediated HMGB1 translocation and lysosomal localization in macrophages

To determine whether APC exerts a cytoprotective effect in LPS-activated macrophages, J774A.1 macrophages were pretreated with APC before stimulating them with LPS and analyzing HMGB1 translocation by immunofluorescence. In agreement with previous reports, LPS induced translocation of HMGB1 from the nucleus to the cytoplasm in these cells (Figure 5A). Quantitative analysis indicated that the efficiency of LPS-mediated HMGB1 translocation was the same as that observed for endothelial cells (~40%). Similar to its protective effect in endothelial cells, APC effectively inhibited nuclear to cytoplasmic translocation of LPS-induced HMGB1 in the J774A.1 macrophage cell line (Figure 5A). In agreement with the literature, immunofluorescence imaging for co-staining with LAMP1 further suggested that HMGB1 is stored at least partially in secretory lysosomal vesicles of LPS-stimulated macrophages (Figure 5B). Similar to its inhibitory effect in endothelial cells, APC effectively inhibited localization of HMGB1 to secretory vesicles for subsequent secretion by exocytosis in the macrophage cell line (Figure 5B). Interestingly, analyses of HMGB1 cytoplasmic translocation and lysosomal co-localization in J774A.1 macrophages by utilizing anti-EPCR, anti-PAR1 or anti-MAC-1 function-blocking antibodies revealed that both MAC-1 and PAR1 but not EPCR is required for APC's inhibitory effect against LPS in this cell line (Figure 6). This finding is consistent with the literature (31).

APC inhibits LPS-induced HMGB1 expression and cytoplasmic localization in mouse cremaster muscle

Finally, we evaluated the effect of LPS and APC on the expression of HMGB1 in vivo. The cremaster muscle of mouse was used as a model system to investigate this question since this model has been extensively used for studying inflammation and its effect on microvascular endothelial cells in vivo. Analysis of HMGB1 protein levels in cremaster muscle lysate showed a markedly elevated HMGB1 protein expression level after 1h of LPS treatment. Interestingly, a 30 min APC pretreatment with either wild-type APC or the signaling-selective APC-2Cys nearly completely abolished LPS-induced HMGB1 expression in cremaster muscle tissues (Figure 7A). Confocal immunofluorescence microscopy of frozen sections of cremaster muscle was used to study HMGB1 expression and localization in CD31-positive vascular endothelial cells (Figure 7B). In unstimulated cremaster muscle, HMGB1 was primarily localized to the nucleus but upon LPS treatment a fraction of HMGB1 could be seen widely distributed in the cytoplasm, suggesting that LPS induces translocation of HMGB1 from the nucleus to the cytoplasm. APC completely abolished LPS-induced HMGB1 cytoplasmic localization in CD31-positive cremaster muscle tissues (Figure 7B). The cytoprotective signaling function of APC was responsible for its inhibitory effect on HMGB1 subcellular localization since the signaling-selective variant of APC (APC-2Cys) exhibited a similar HMGB1 inhibitory effect in this assay (Figure 7A and B).

Discussion

As a ubiquitous chromatin associated nuclear protein, HMGB1 is primarily located in the nucleus of all cell types (12,13,27). However, HMGB1 can also be released to extracellular space by injured and necrotic cells or it may be actively secreted by immune cells when they are exposed to pathogen/damage-associated molecular pattern molecules (i.e., LPS, DNA, RNA, etc.) during infection and injury (12,13,27). Inflammatory signaling by LPS through TLR4 induces a nuclear to cytoplasmic translocation of HMGB1 in activated monocytes, macrophages and other innate immune cells before secretion to extracellular space (18,21). It has been demonstrated that the HMGB1 secretion by activated immune cells requires a tightly controlled relocation program involving dislodging of HMGB1 from the nucleosome in the first step followed by its migration to the cytoplasm and subsequent secretion to outside of the cell. It has been shown that this elaborate relocation program is regulated by the acetylation status of HMGB1, mediated by the balanced catalytic action of (de)acetylating enzymes (18,21,24,30). Thus, LPS through regulation of (de)acetylating enzymes, HATs and HDACs, induces hyperacetylation of HMGB1, which culminates in migration of HMGB1 from the nucleus to the cytoplasm in activated innate immune cells. However, because HMGB1 lacks a signal peptide and cannot be secreted through a Golgi apparatus/endoplasmic reticulum–dependent secretory pathway, the translocated HMGB1 has been found to be first stored in specialized organelles referred to as secretory lysosomes before secretion by exocytosis (18,21). The extracellular secretion of HMGB1 has been hypothesized to require a second receptor-mediated proinflammatory signal that results in intracellular calcium rise and fusion of secretory vesicles with the plasma membrane and subsequent exocytosis of HMGB1 and other stored vesicular cargo (21,32). APC is known to alter cytosolic calcium flux in human brain endothelium via EPCR-dependent activation of PAR1 (33). Vascular endothelial cells also express molecular pattern receptors and can respond to LPS and thus secrete HMGB1 to extracellular space by a TLR4-dependent mechanism (8,27). In a previous study, we demonstrated that the cytoprotective signaling activity of APC inhibits LPS-mediated secretion of HMGB1 in HUVECs and transformed EA.hy926 endothelial cells (8). In this study, we investigated the mechanism by which APC inhibits LPS-mediated HMGB1 secretion by endothelial cells and discovered that APC inhibits LPS-mediated acetylation of HMGB1, thereby inhibiting translocation of HMGB1 from the nucleus to the cytoplasm and its subsequent secretion to extracellular space.

Results in the presence of function-blocking anti-EPCR and anti-PAR1 antibodies demonstrated that the cytoprotective activity of APC in preventing LPS-mediated acetylation-dependent cytoplasmic translocation of HMGB1 is mediated via APC interacting with EPCR followed by activating PAR1 in endothelial cells. In addition, we have demonstrated that the occupancy of EPCR by Gla-domain of protein C switches the PAR1-dependent signaling specificity of thrombin from a disruptive to a cytoprotective response (6,23). The observation that pretreatment of endothelial cells with PC-S195A prior to activation by thrombin resulted in inhibition of LPS-mediated relocation of HMGB1 supports our hypothesis that PAR1 signaling by thrombin is protective if EPCR is occupied by Gla-domain of protein C/APC. Results demonstrated that the EPCR occupancy and cleavage of PAR1 by these proteases inhibits LPS-mediated acetylation and cytoplasmic

translocation of HMGB1 in endothelial cells. The acetylation status of HMGB1 is regulated by the coordinated catalytic function of (de)acetylating enzymes collectively referred to as HATs and HDACs (19). Immunoprecipitation followed by immuno-blot analysis of whole cell lysates using appropriate specific antibodies revealed that the CBP histone acetyltransferase is associated with HMGB1 in LPS-stimulated endothelial cells, however, pretreatment of cells with APC prevented this interaction. Further analysis indicated that APC also inhibits LPS-mediated translocation of HDAC2 and HDAC4 from the nucleus to the cytoplasm. These nuclear enzymes maintain the dynamic equilibrium between the acetylated and deacetylated forms of HMGB1 in the nucleus in response to LPS and other inflammatory stimuli under different pathophysiological conditions. Our results suggest that the cytoprotective signaling function of APC, by inhibiting LPS signaling, facilitates the nuclear retention of HDACs, thereby inhibiting hyperacetylation-dependent translocation of HMGB1 to the cytoplasm. In addition, poly(ADP-ribose) polymerase 1 (PARP1), which can catalyze the transfer of ADP-ribosyl group from nicotine adenine dinucleotide (NAD^+) to acceptor proteins in the nucleus, is known to play an important role in regulating acetylation-dependent translocation of HMGB1 to the cytoplasm (29,30). Thus, it has been found that the inhibition of PARP1 prevents LPS-mediated hyperacetylation of HMGB1 by HAT(s), a process that is also associated with the simultaneous reduction in HDAC activity in the nucleus (24,29,30). Our results showing that APC significantly inhibits LPS-induced interaction of HMGB1 with PARP1 indicate that in addition to (de)acetylating nuclear enzymes, APC also inhibits PARP1-dependent acetylation of HMGB1 in LPS-activated endothelial cells.

Previous results have indicated that following translocation to the cytoplasm in response to LPS, HMGB1 is first directed to secretory lysosomal vesicles and then secreted by exocytosis following activated monocytes receiving a second proinflammatory signal (18,21). Results of the current study further indicate that, similar to activated monocytes, endothelial cells have the compartmentalization machinery to allow regulatory secretion of HMGB1 from the cytoplasm to the extracellular space. Thus, it appears that secretory lysosomal vesicles, which has been reported to be almost exclusively present in hematopoietic lineage cells (32), to be also present in vascular endothelial cells. This conclusion is based on the immunofluorescence staining for the lysosomal marker membrane protein, LAMP1, which was found to partially colocalize with the translocated HMGB1 in LPS-stimulated endothelial cells (Figure 4). Results demonstrated that APC also effectively inhibits this process. In addition to endothelial cells, APC also inhibited LPS-mediated HMGB1 translocation and its storage in lysosomal secretory vesicles of activated macrophages before secretion to extracellular space. This elaborate translocation and secretion program may explain the basis for HMGB1 exhibiting its proinflammatory effect with delayed kinetics under different pathological condition as has been documented in various inflammatory and injury models including endotoxemia, sepsis and ischemia/reperfusion injury (12,13,34,35). It has been established that extracellular HMGB1 makes a key contribution to pathogenic mechanisms in most of these models and that APC or its different signaling-selective derivatives exhibit potent cytoprotective and antiinflammatory activities, thereby rescuing experimental animals from severe adverse effects of injuries and proinflammatory cytokines (36–38). As presented in Figure 8 as a hypothetical model, our

novel findings in this manuscript provide some insight into the epigenetic mechanisms through which APC can exert a cytoprotective signaling function in response to LPS and possibly also in response to some other proinflammatory stimuli. Our findings suggest that LPS signaling through TLR4 stimulates the acetylation of HMGB1 by nuclear enzymes HATs (i.e., CBP), PARP1 and HDACs in endothelial cells. Acetylation of HMGB1 results in translocation of the nuclear protein to the cytoplasm and its subsequent association/storage in secretory lysosomal vesicles. The fusion of these vesicles with the plasma membrane, possibly following a second proinflammatory signal, culminates in secretion of HMGB1 to extracellular space in endothelial cells. Results presented here suggest that EPCR- and PAR1-dependent cytoprotective signaling function of APC (also thrombin) inhibits LPS-mediated subcellular localization of HMGB1 and its eventual secretion to extracellular space, apparently by facilitating the regulation of HMGB1 acetylation by nuclear enzymes. We further provided in vivo proof-of-principle support for this hypothesis by showing that APC and its signaling-selective variant effectively inhibit LPS-mediated expression and cytoplasmic translocation of HMGB1 in CD31-positive cremaster muscle tissues, supporting in situ results that the cytoprotective and antiinflammatory function of APC in vascular endothelial cells is mediated at least partially through its ability to epigenetically regulate the acetylation-dependent translocation/secretion of HMGB1 to extracellular space during inflammation and injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Cindy Carter for technical assistance and Audrey Rezaie for editorial work on the manuscript.

Funding Sources

This research was supported by grants awarded by the National Heart, Lung, and Blood Institute of the National Institutes of Health HL 101917 and HL 62565 to A.R.R.

References

1. Esmon CT. Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. FASEB J 1995;9:946–955. [PubMed: 7615164]
2. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc Natl Acad Sci U S A 1996;93:10212–10216. [PubMed: 8816778]
3. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science 2002;296:1880–1882. [PubMed: 12052963]
4. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. Blood 2007;109:3161–3172. [PubMed: 17110453]
5. Mohan Rao LV, Esmon CT, Pendurthi UR. Endothelial cell protein C receptor: a multiliganded and multifunctional receptor. Blood 2014;124:1553–1562. [PubMed: 25049281]
6. Rezaie AR. Protease-activated receptor signaling by coagulation proteases in endothelial cells. Thromb Haemost 2014;112:876–882. [PubMed: 24990498]
7. Isermann B Homeostatic effects of coagulation protease-dependent signaling and protease activated receptors. J Thromb Haemost 2017;15:1273–1284. [PubMed: 28671351]

8. Bae JS, Rezaie AR. Activated protein C inhibits high mobility group box 1 signaling in endothelial cells. Blood 2011;118:3952–3959. [PubMed: 21849480]
9. Mosnier LO, Sinha RK, Burnier L, Bouwens EA, Griffin JH. Biased agonism of protease-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46. Blood 2012;120:5237–5246. [PubMed: 23149848]
10. Bae JS, Rezaie AR. Thrombin inhibits nuclear factor kappaB and RhoA pathways in cytokine-stimulated vascular endothelial cells when EPCR is occupied by protein C. Thromb Haemost 2009;101:513–520. [PubMed: 19277413]
11. Liang HP, Kerschen EJ, Hernandez I, et al. EPCR-dependent PAR2 activation by the blood coagulation initiation complex regulates LPS-triggered interferon responses in mice. Blood 2015;125:2845–2854. [PubMed: 25733582]
12. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248–251. [PubMed: 10398600]
13. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418:191–195. [PubMed: 12110890]
14. DeMarco RA, Fink MP, Lotze MT. Monocytes promote natural killer cell interferon gamma production in response to the endogenous danger signal HMGB1. Mol Immunol 2005;42:433–444. [PubMed: 15607795]
15. Andersson U, Wang H, Palmblad K, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med 2000;192:565–570. [PubMed: 10952726]
16. Sama AE, D'Amore J, Ward MF, Chen G, Wang H. Bench to bedside: HMGB1-a novel proinflammatory cytokine and potential therapeutic target for septic patients in the emergency department. Acad Emerg Med 2004;11:867–873. [PubMed: 15289194]
17. Chen G, Ward MF, Sama AE, Wang H. Extracellular HMGB1 as a proinflammatory cytokine. J Interferon Cytokine Res 2004;24:329–333. [PubMed: 15212706]
18. Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, Agresti A, Bianchi ME. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. EMBO J 2003;22:5551–5560. [PubMed: 14532127]
19. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet 2009;10:32–42. [PubMed: 19065135]
20. Pasheva E, Sarov M, Bidjekov K, Ugrinova I, Sarg B, Lindner H, Pashev IG. In vitro acetylation of HMGB-1 and −2 proteins by CBP: the role of the acidic tail. Biochemistry 2004;43:2935–2940. [PubMed: 15005629]
21. Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, Rubartelli A. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep 2002;3:995–1001. [PubMed: 12231511]
22. Bae JS, Yang L, Manithody C, Rezaie AR. Engineering a disulfide bond to stabilize the calcium-binding loop of activated protein C eliminates its anticoagulant but not its protective signaling properties. J Biol Chem 2007;282:9251–9259. [PubMed: 17255099]
23. Bae JS, Yang L, Manithody C, Rezaie AR. The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. Blood 2007;110:3909–3916. [PubMed: 17823308]
24. Yang Z, Li L, Chen L, Yuan W, Dong L, Zhang Y, Wu H, Wang C. PARP-1 mediates LPS-induced HMGB1 release by macrophages through regulation of HMGB1 acetylation. J Immunol 2014;193:6114–6123. [PubMed: 25392528]
25. Biswas I, Panicker SR, Cai X, Mehta-D'souza P, Rezaie AR. Inorganic polyphosphate amplifies high mobility group box 1-mediated von Willebrand factor release and platelet string formation on endothelial cells. Arterioscler Thromb Vasc Biol 2018;38:1868–1877. [PubMed: 29930000]
26. Panicker SR, Mehta-D'souza P, Zhang N, Klopocki AG, Shao B, McEver RP. Circulating soluble P-selectin must dimerize to promote inflammation and coagulation in mice. Blood 2017;130:181–191. [PubMed: 28515093]

27. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 2005;5:331–342. [PubMed: 15803152]
28. Ong SP, Lee LM, Leong YF, Ng ML, Chu JJ. Dengue virus infection mediates HMGB1 release from monocytes involving PCAF acetylase complex and induces vascular leakage in endothelial cells. PLoS One 2012;7:e41932. [PubMed: 22860034]
29. Walko TD 3rd, Di Caro V, Piganelli J, Billiar TR, Clark RS, Aneja RK. Poly(ADP-ribose) polymerase 1-sirtuin 1 functional interplay regulates LPS-mediated high mobility group box 1 secretion. Mol Med 2014;20:612–624.
30. Ditsworth D, Zong WX, Thompson CB. Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. J Biol Chem 2007;282:17845–17854. [PubMed: 17430886]
31. Cao C, Gao Y, Li Y, Antalis TM, Castellino FJ, Zhang L. The efficacy of activated protein C in murine endotoxemia is dependent on integrin CD11b. J Clin Invest 2010;120:1971–1980. [PubMed: 20458145]
32. Blott EJ, Griffiths GM. Secretory lysosomes. Nat Rev Mol Cell Biol 2002;3:122–131. [PubMed: 11836514]
33. Dömötör E 1, Benzakour O, Griffin JH, Yule D, Fukudome K, Zlokovic BV. Activated protein C alters cytosolic calcium flux in human brain endothelium via binding to endothelial protein C receptor and activation of protease activated receptor-1. Blood 2003;101:4797–4801. [PubMed: 12586611]
34. Evankovich J, Cho SW, Zhang R, Cardinal J, Dhupar R, Zhang L, Klune JR, Zlotnicki J, Billiar T, Tsung A. High mobility group box 1 release from hepatocytes during ischemia and reperfusion injury is mediated by decreased histone deacetylase activity. J Biol Chem 2010;285:39888–39897. [PubMed: 20937823]
35. Hwang JS, Choi HS, Ham SA, Yoo T, Lee WJ, Paek KS, Seo HG. Deacetylation-mediated interaction of SIRT1-HMGB1 improves survival in a mouse model of endotoxemia. Sci Rep 2015;5:15971. [PubMed: 26522327]
36. Kerschen EJ, Fernandez JA, Cooley BC, et al. Endotoxemia and sepsis mortality reduction by non-anticoagulant activated protein C. J Exp Med 2007;204:2439–2448. [PubMed: 17893198]
37. Dinarvand P, Hassanian SM, Qureshi SH, Manithody C, Eissenberg JC, Yang L, Rezaie AR. Polyphosphate amplifies proinflammatory responses of nuclear proteins through interaction with receptor for advanced glycation end products and P2Y1 purinergic receptor. Blood 2014;123:935–945. [PubMed: 24255918]
38. Roy RV, Ardeshirylajimi A, Dinarvand P, Yang L, Rezaie AR. Occupancy of human EPCR by protein C induces β-arrestin-2 biased PAR1 signaling by both APC and thrombin. Blood 2016;128:1884–1893. [PubMed: 27561318]

Essentials

1. APC elicits cytoprotective responses in endothelial cells via EPCR-dependent cleavage of PAR1
2. APC inhibits LPS-mediated translocation and extracellular secretion of HMGB1 in endothelial cells
3. Signaling activity of APC inhibits LPS-mediated acetylation of HMGB1 by epigenetic mechanisms
4. APC inhibits LPS-mediated HMGB1 expression in CD31-positive endothelial cells in cremaster muscle

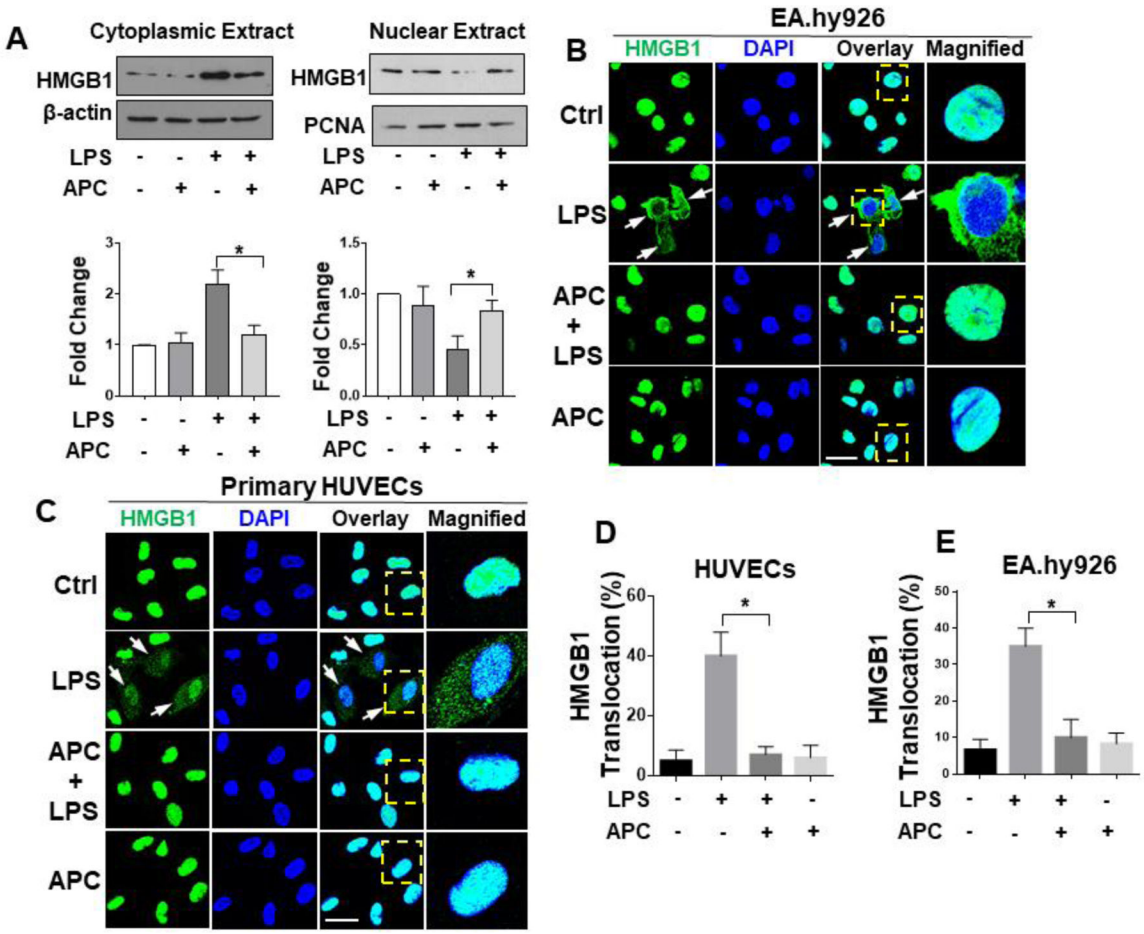

Figure 1. APC inhibits LPS-induced HMGB1 translocation from nucleus to cytoplasm in endothelial cells.

(A) EA.hy926 endothelial cells were pretreated with APC (20 nM for 3h) followed by stimulation with LPS (1 μg/mL for 1h). Cytoplasmic and nuclear fractions were isolated and HMGB1 levels were analyzed by immunoblotting. β-actin and PCNA were used as loading controls for cytoplasmic and nuclear fractions, respectively. **(B)** The same as panel A except that following treatment of cells with APC and LPS, cells were fixed and permeabilized followed by staining HMGB1 with rabbit anti-HMGB1 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. Arrows indicate cytoplasmic translocation of HMGB1. The magnified insets correspond to the cells marked with yellow dashed boxes. **(C)** The same as panel B except that primary HUVECs, instead of EA.hy926 cells, were used for the HMGB1 staining. **(D and E)** The quantification of LPS-mediated translocated cells from the nucleus to the cytoplasm for HUVECs (panel C) and EA.hy926 cells (panel B), respectively. Scale bar: 50 μm. Results are shown as means ± SE. **p < 0.05.*

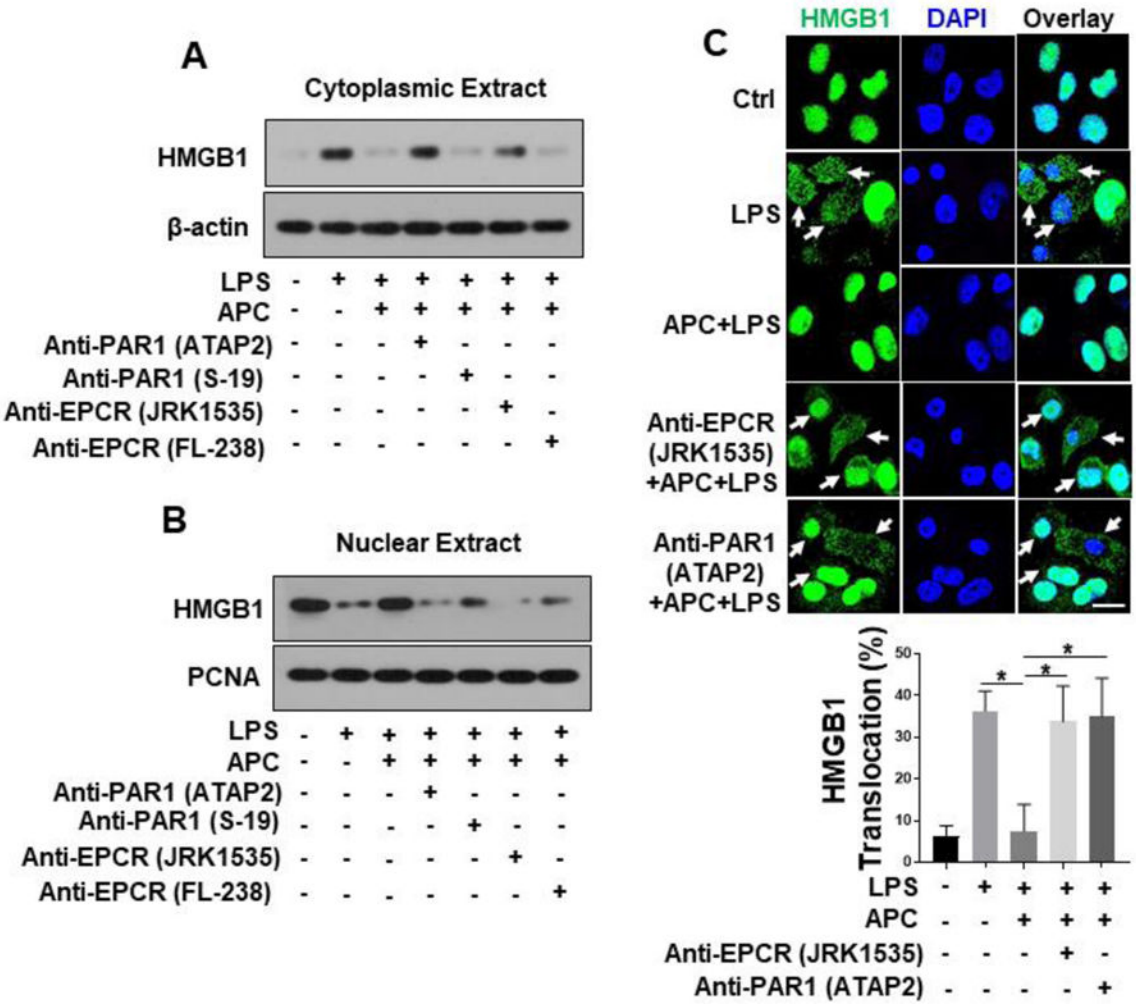

Figure 2. EPCR and PAR1 is required for APC inhibition of LPS-induced HMGB1 cytoplasmic translocation in endothelial cells.

(A and B) EA.hy926 cells were pretreated with PAR1 or EPCR function-blocking or non-blocking antibodies (15–20 μg/mL for 1h) followed by treatment with APC (20 nM for 3h). Cells were then treated with LPS (1 μg/mL for 1h) and cytoplasmic (panel A) and nuclear fractions (panel B) were prepared. HMGB1 level in cytoplasmic and nuclear fractions were analyzed by immunoblotting. β-actin and PCNA were used as loading controls for cytoplasmic and nuclear fractions, respectively. **(C)** The same as above except that following treatment of cells with APC and LPS, cells were fixed and permeabilized followed by staining HMGB1 with rabbit anti-HMGB1 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. Arrows indicate cytoplasmic translocation of HMGB1. Quantification of translocated cells in the absence and presence of function-blocking EPCR (JRK1535) and PAR1 (ATAP2) antibodies is shown below panel C. Scale bar: 20 μm. Results are shown as means ± SE. *$p < 0.05$.

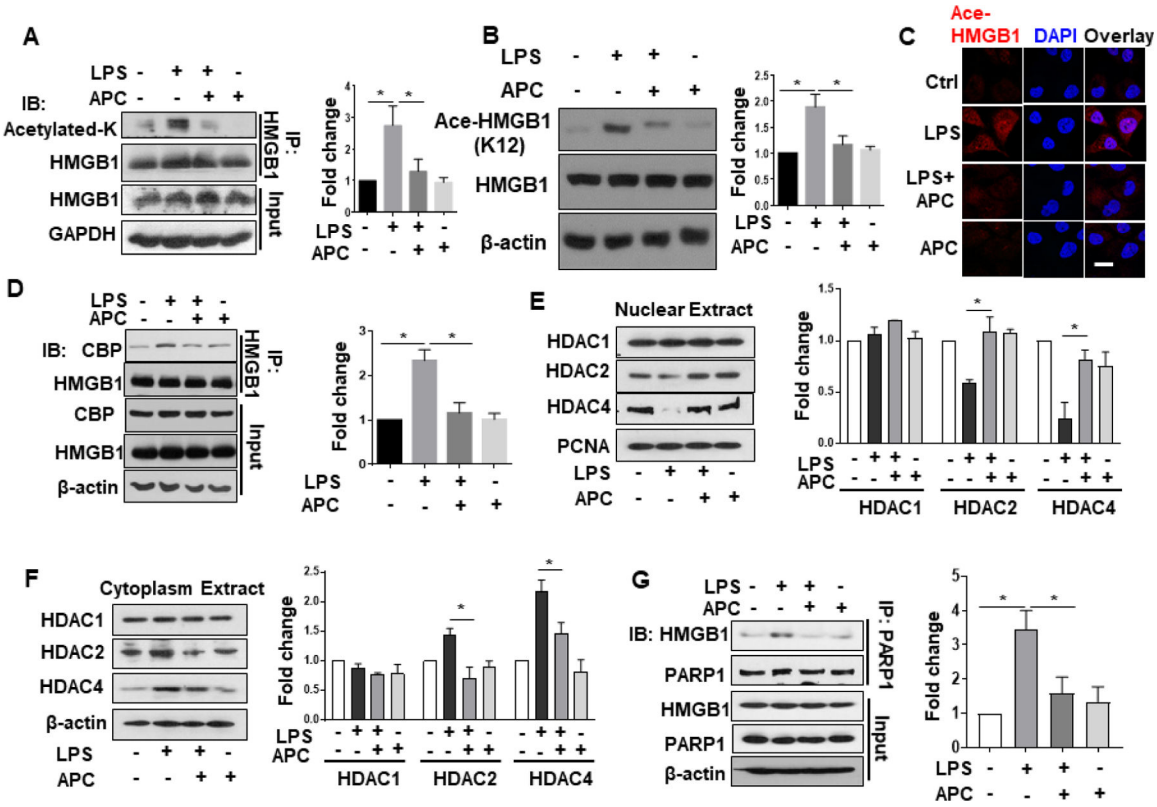

Figure 3. APC inhibits LPS-mediated HMGB1 acetylation by modulating (de)acetylating enzymes in endothelial cells.

(A) EA.hy926 cells were pretreated with APC (20 nM for 3h) followed by stimulation with LPS (1 μg/mL for 1h). Cell lysates were immunoprecipitated with an anti-HMGB1 antibody and co-precipitated protein was analyzed by Western blot employing anti-acetylated-lysine (Acetylated-K) antibody. **(B)** The same as panel A except that cell lysates were immunoblotted and probed with an antibody specific for acetylated HMGB1 (Ace-HMGB1). **(C)** Cells were fixed, permeabilized and stained with the rabbit anti-Ace-HMGB1 antibody followed by Cyanine 3 (Cy3)-conjugated goat anti-rabbit IgG. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. Scale bar: 20 μm. **(D)** The same as panel A except that cell lysates were immunoprecipitated with mouse anti-HMGB1 antibody and co-precipitated protein analyzed by Western blot using an antibody specific for histone acetyltransferase CBP. **(E and F)** Cells were pretreated with APC (20 nM for 3h) followed by stimulation with LPS (1 μg/mL for 3h). Nuclear and cytoplasmic fractions were prepared and HDAC1, HDAC2 and HDAC4 in nuclear (panel E) or cytoplasmic (panel F) fractions were analyzed by immunoblotting. PCNA and β-actin were used as loading controls for nuclear and cytoplasmic fractions, respectively. **(G)** The same as panel A except that cell lysates were immunoprecipitated with an antibody for poly [ADP-ribose] polymerase 1 (PARP1) and co-precipitated protein was analyzed by Western blot for HMGB1 using a mouse anti-HMGB1. Quantification of Western blots is shown on the right side of each panel. Results are shown as means ± SE. *$p < 0.05$.

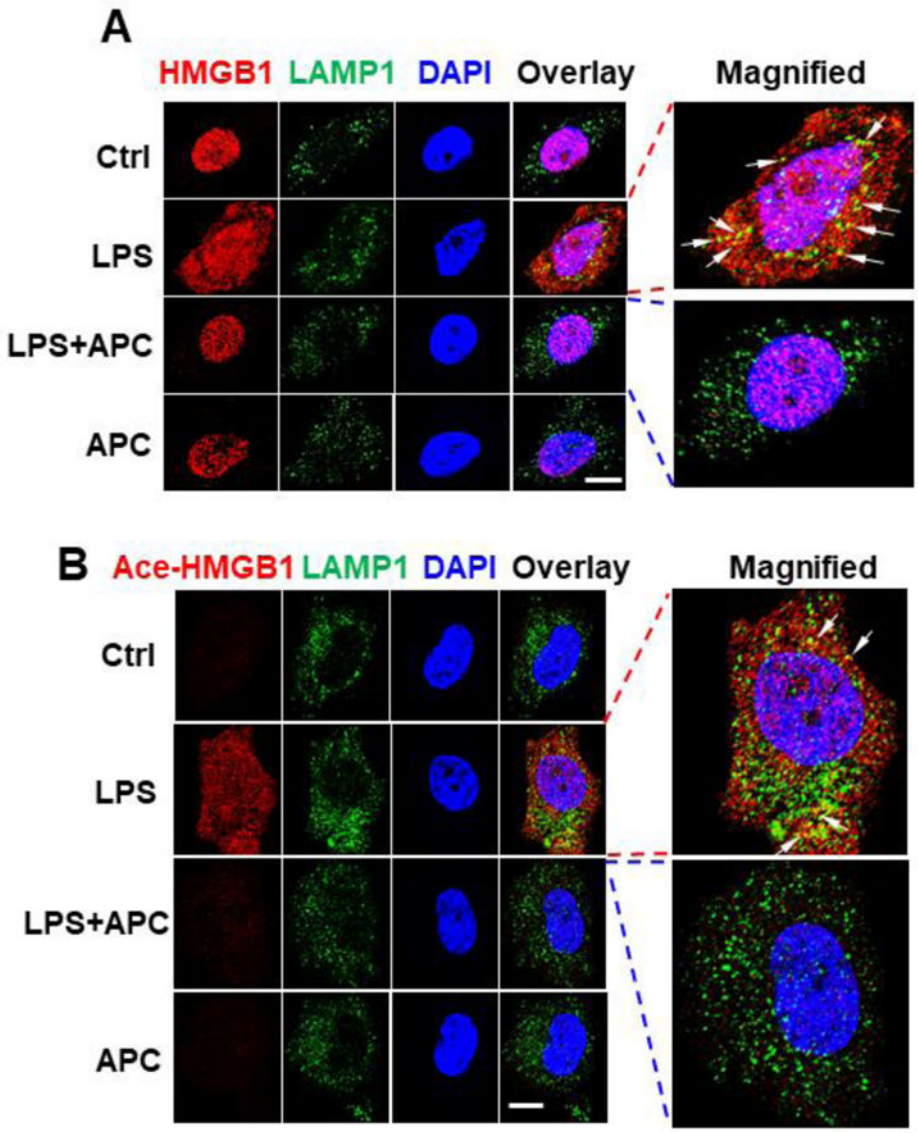

Figure 4. APC inhibits LPS-mediated HMGB1 lysosomal localization in endothelial cells.

(A) EA.hy926 ells were pretreated with APC (20 nM for 3h) followed by stimulation with LPS (1 μg/mL for 1h). Cells were then fixed and permeabilized and HMGB1 was stained with rabbit anti-HMGB1 and Cy3-conjugated goat anti-rabbit IgG. LAMP1, a lysosome marker, was stained with mouse anti-LAMP1 antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. **(B)** The same as panel A except that HMGB1 was stained employing rabbit anti-acetylated HMGB1 (Ace-HMGB1) and Cy3-conjugated goat anti-rabbit IgG. Arrows indicate colocalization of HMGB1 or acetylated-HMGB1 with LAMP1. The magnified insets correspond to the cells from LPS and APC+LPS groups. Scale bar: 10 μm.

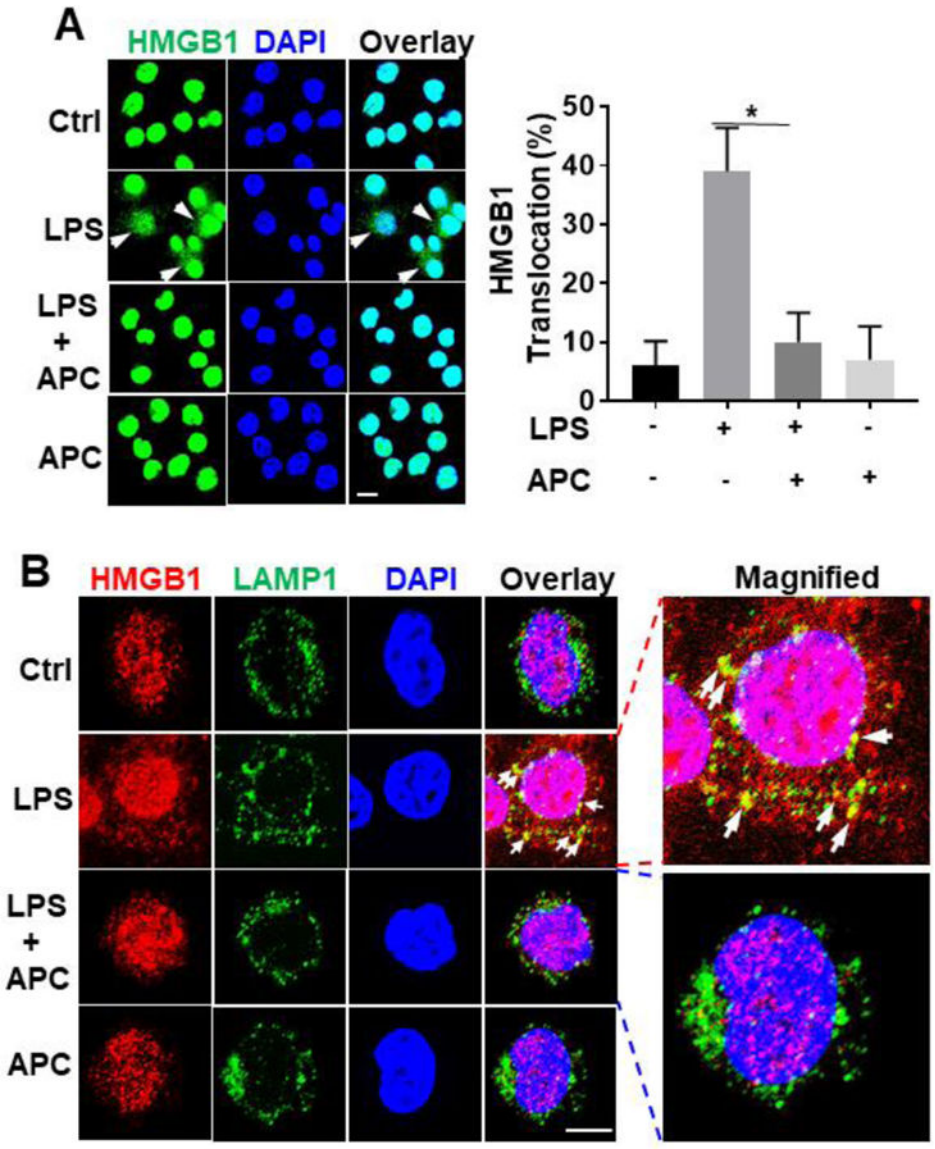

Figure 5. APC inhibits LPS-mediated HMGB1 translocation and lysosomal localization in macrophages.

J774A.1 macrophages were pretreated with APC (20 nM for 3h) followed by stimulation with LPS (1 μg/mL for 1h). Cells were then fixed, permeabilized and HMGB1 was stained with rabbit anti-HMGB1 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG. Quantification of translocated cells is shown on the right. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. Arrows indicate cytoplasmic translocation of HMGB1. **(B)** The same as panel A except that HMGB1 was stained with rabbit anti-HMGB1 followed by Cy3-conjugated goat anti-rabbit IgG. LAMP1 was stained with mouse anti-LAMP1 antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. Arrows indicate colocalization of HMGB1 with LAMP1. The magnified insets correspond to the cells from LPS and APC+LPS groups. Scale bar: 10 μm. Results are shown as means ± SE. *$p < 0.05$.

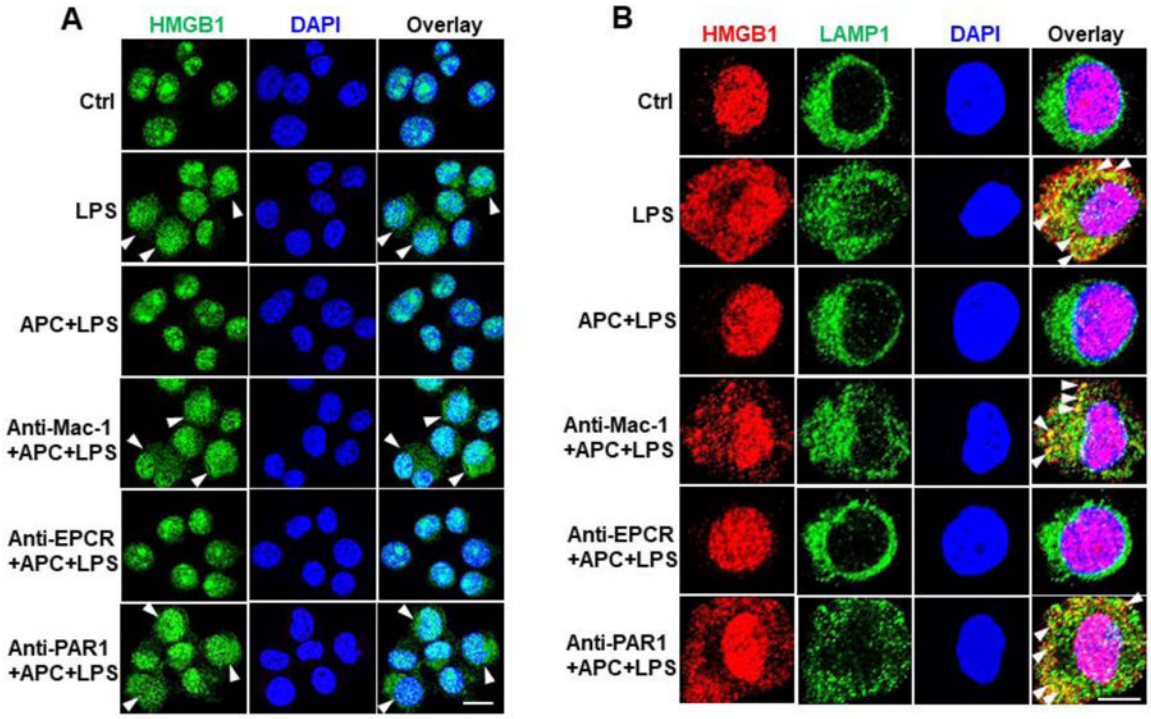

Figure 6. Mac-1 and PAR1 but not EPCR is required for APC inhibition of HMGB1 translocation and lysosomal localization in macrophages.

(A) J774A.1 macrophages were pretreated with Mac-1, EPCR or PAR1 function-blocking antibody (15–20 μg/mL for 1h) followed by treatment with APC (20 nM for 3h) before stimulation with LPS (1 μg/mL for 1h). Cells were then fixed, permeabilized and HMGB1 was stained with rabbit anti-HMGB1 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. Arrows indicate cytoplasmic translocation of HMGB1. **(B)** The same as panel A except that HMGB1 was stained with rabbit anti-HMGB1 followed by Cy3-conjugated goat anti-rabbit IgG. LAMP1 was stained with mouse anti-LAMP1 antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. Arrows indicate colocalization of HMGB1 with LAMP1. Scale bar: 10 μm (A and B).

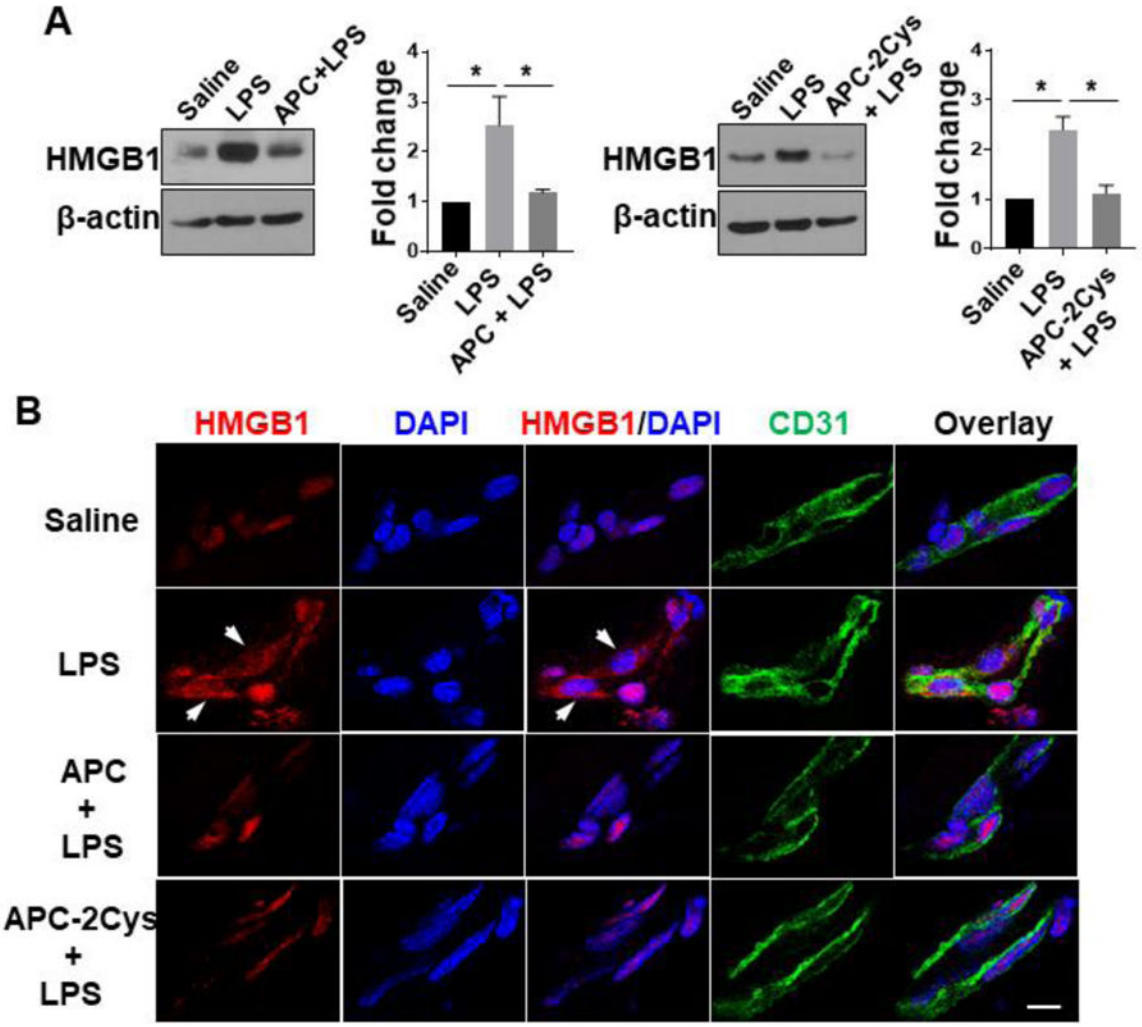

Figure 7. APC inhibits LPS-mediated HMGB1 expression and cytoplasmic translocation in mouse cremaster muscle.

(A) Mice were intrascrotally injected with APC or APC-2Cys (0.2 mg/kg) followed by LPS injection (0.5 mg/kg). After 1 hour, tissue was harvested for lysis or histological process. Tissue lysates were immunoblotted for HMGB1 and β-actin. **(B)** Cryosection of cremaster muscle tissue were fixed and permeabilized. HMGB1 was stained with biotin-conjugated rabbit anti-HMGB1 antibody and Cy3-conjugated streptavidin; CD31 was stained with rat anti-CD31 antibody and Alexa Fluor 488-conjugated donkey anti-rat IgG. Nucleus was stained with DAPI. Immunofluorescence images were taken by confocal microscopy. Arrows indicate cytoplasmic translocation of HMGB1. Scale bar: 10 μm. Results are shown as means ± SE. *$p < 0.05$.

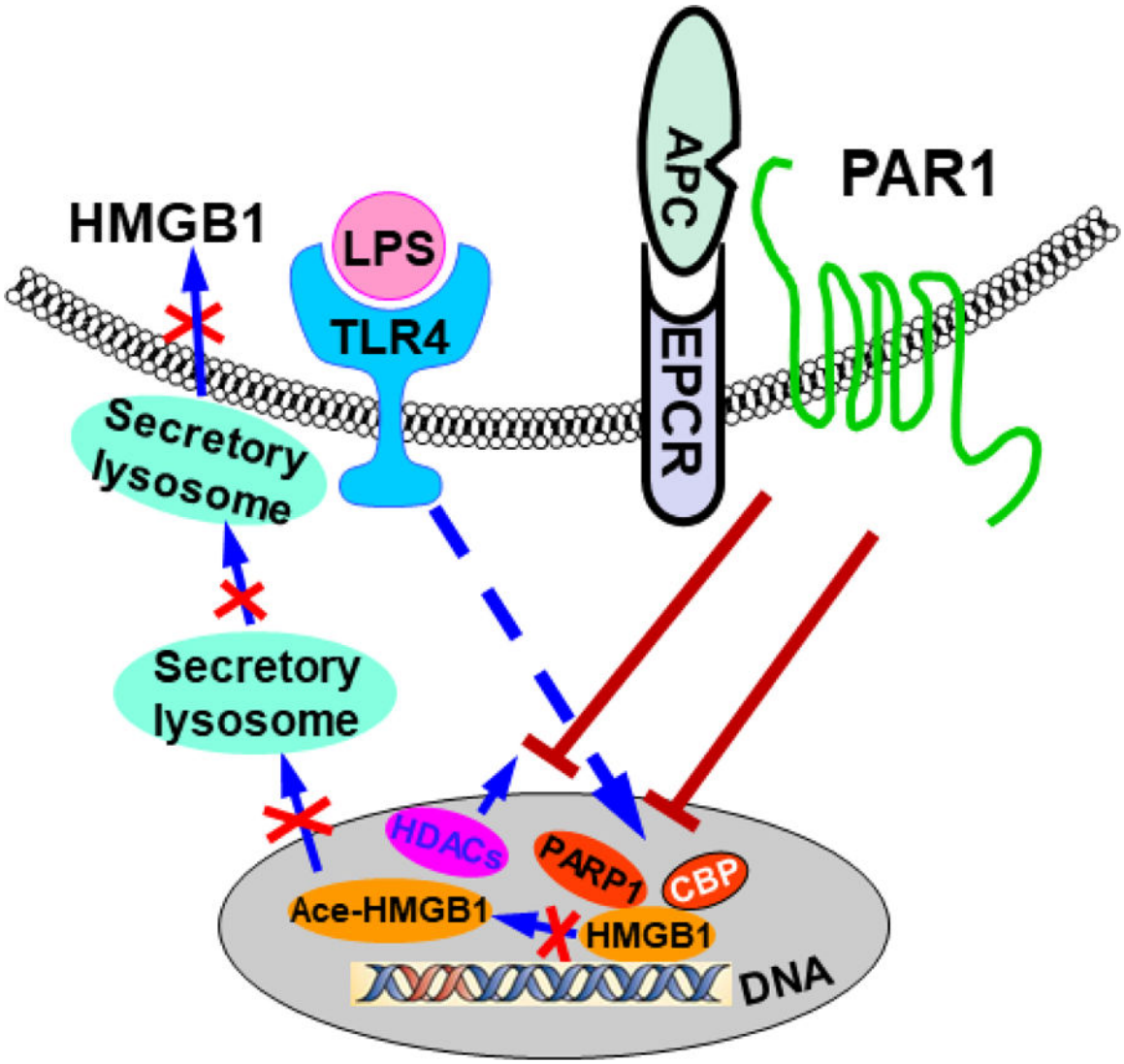

Figure 8.

Hypothetical model of APC inhibition of LPS-mediated HMGB1 translocation/expression in endothelial cells. LPS upon interaction with TLR4 stimulates the acetylation of HMGB1 by histone acetyltransferase(s) (i.e., CBP), the nuclear enzyme poly [ADP-ribose] polymerase 1 (PARP1) and cytoplasmic translocation of histone deacetylases (HDACs). The acetylated HMGB1 (Ace-HMGB1) exits the nucleus and associates with cytoplasmic secretory lysosomes. The fusion of secretory lysosomal vesicles with the plasma membranes following an inflammatory signal culminates in the secretion of HMGB1 to extracellular space (the LPS-mediated pathways are shown by blue arrows). The EPCR- and PAR1-dependent cytoprotective signaling function of APC inhibits all LPS-mediated pathways mentioned above (shown by red solid lines). See the text for more details.