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## Forum Original Research Communication

# A Dual Regulatory Role of Apurinic/Apyrimidinic Endonuclease 1/Redox Factor-1 in HMGB1-Induced Inflammatory Responses

Jae–Min Yuk,[1,4] Chul–Su Yang,[1,4] Dong–Min Shin,[1,4] Kwang–Kyu Kim,[1] Sang Ki Lee,[2] Yun Jeong Song,[2] Hye–Mi Lee,[1] Chung–Hyun Cho,[2,3] Byeong Hwa Jeon,[2,3] and Eun–Kyeong Jo[1,3]

### Abstract

Apurinic/apyrimidinic endonuclease 1/Redox factor-1 (APE1) is a multifunctional protein involved in reduction–oxidation regulation. High-mobility group box 1 (HMGB1) is released by necrotic cells and various inflammatory stimuli, acting as an inflammatory marker in sepsis and autoimmune diseases. Here, we report the dual regulatory role of APE1 in inflammatory signaling to extracellular HMGB1 or in the release of endogenous HMGB1 in human monocytes/macrophages. Forced cytoplasmic overexpression of APE1 profoundly attenuated the upregulation of HMGB1-mediated reactive oxygen species generation, cytokine secretion, and cyclooxygenase-2 expression by primary monocytes and macrophage-like THP-1 cell lines. In addition, HMGB1-induced activation of p38 and c-Jun N-terminal kinase (JNK), but not extracellular signal-regulated kinase 1/2, was strongly abrogated by the overexpression of APE1. The activation of apoptosis signal-regulating kinase 1 was required for both the p38 and JNK activation challenge with HMGB1. The extracellular release of HMGB1 by activated macrophages was inhibited by APE1 transfection. Small interfering RNA (siRNA) knockdown of endogenous APE1 impaired HMGB1-mediated cytokine expression and MAPK activation in THP-1 cells. HMGB1 stimulation induced the translocation of APE1 to the nucleus of the cell. In addition, APE1 silencing via siRNA transfection inhibited both the nuclear and cytoplasmic expression of APE1. These data identify APE1 as a novel dual regulator of inflammatory signaling to HMGB1 by human monocytes/macrophages. The modulation of cytosolic APE1 expression might be useful as a potential therapeutic modality for the treatment of inflammatory or autoimmune diseases. *Antioxid. Redox Signal*. 11, 575–588.

### Introduction

EXTENSIVE TISSUE INJURY that occurs as a consequence of mechanical or thermal trauma can result in generalized sterile inflammation or systemic inflammatory response syndrome. During the activation of a repair program, multifunctional alarm signals are generated as a consequence of cell and tissue damage (7). The chromosomal protein high-mobility group box 1 (HMGB1), a ubiquitous and conserved protein (15), is located in all mammalian nuclei at high concentrations, acting as an architectural protein that causes local bending and untwisting of the double helix (6). HMGB1 has special significance, because it is released by necrotic cells and promotes inflammatory properties (42). HMGB1 is released by activated monocytes and macrophages after stimulation with endotoxin, tumor necrosis factor (TNF)-$\alpha$, or interleukin (IL)-1 (53). Indeed, HMGB1 is actively secreted by cells of the innate immune system in response to proinflammatory stimuli (17). Extracellular HMGB1 functions as a proinflammatory cytokine contributing to severe sepsis, and has been identified as a late mediator of severe sepsis (53, 57). Accumulating evidence suggests that extracellular HMGB1 contributes to the pathogenesis of many inflammatory diseases, including autoimmune diseases (40).

Departments of [1]Microbiology and [2]Physiology, and [3]Infection Signaling Network Research Center, College of Medicine, Chungnam National University, Daejeon, South Korea.
[4]These authors contributed equally to this work.

Monocytes/macrophages are principal phagocytes of the innate immune system. Microbial invasion and oxidative stress are often thought to stimulate the innate and inflammatory responses of these cells. The alarm signal HMGB1 is released mainly by activated monocytes and macrophages, resulting in inflammation, and is involved in the pathogenesis of many inflammatory disorders (17, 53). Reactive oxygen species (ROS) mediate the innate immune receptor-mediated production of inflammatory cytokines (35, 55) and serve an important signaling function during inflammation and organ injury (20). It has been reported that Toll-like receptor 4 (TLR4) participates in hypoxia-induced ROS production and HMGB1 release (49). HMGB1/TLR4 signaling plays an important role in the mediation of NADPH oxidase activation as well as increased ROS production (19). Apoptosis signal-regulating kinase 1 (ASK1) is a target of ROS and is required for TLR4-mediated inflammatory mediator release and p38 mitogen-activated protein kinase (MAPK) activation (35).

Growing evidence indicates that the cellular reduction/oxidation (redox) status regulates various aspects of cellular functions, including the activities of transcription factors. Oxidative stress resulting in transcriptional regulation has been suggested to be an important mechanism during the expression of specific genes that are crucial for inflammation, death, and survival (36). Apurinic/apyrimidinic endonuclease 1/Redox factor-1 (APE1), a ubiquitous 37-kDa protein, functions as a redox factor that maintains transcription factors in an active reduced state (18, 47). APE1 stimulates the DNA-binding activities of Fos–Jun heterodimers, Jun–Jun homodimers, and several other transcription factors, including nuclear factor (NF)-$\kappa$B, hypoxia-inducible factor (HIF)-1$\alpha$, cAMP response element-binding protein, p53, and others (18, 47, 52). APE1 contains nuclear transport signals, and possesses apurinic/apyrimidinic endonuclease DNA repair activity, which is critical for the genomic integrity and viability of cells (12). Recent studies have shown that lipopolysaccharide (LPS) stimulates the upregulation and nuclear translocation of APE1 in activated macrophages (43). In addition, APE1 has differential cellular and subcellular expression patterns (14), suggesting potential regulatory and functional roles of this protein in subcellular localization. Although the role of APE1 in DNA repair and transcriptional regulation has been widely investigated, comparative analyses of the extranuclear and nuclear roles of APE1 in the regulation of inflammatory responses by monocytes/macrophages have not been performed. Furthermore, the molecular mechanisms by which APE1 may regulate redox-sensitive inflammation remain largely unknown.

Here, we report a novel dual role of APE1 in regulating the response of monocytes/macrophages to HMGB1-induced inflammatory processes using both overexpression and small interfering RNA (siRNA) knockdown systems. Forced cytoplasmic expression of APE1 inhibited HMGB1-mediated ROS release, inflammatory mediator production, and signaling activation. The negative regulation of HMGB1-induced p38 MAPK and c-Jun N-terminal kinase (JNK) activation by APE1 overexpression was mediated through the modulation of ASK1 activity. In addition, the transfection of APE1 suppressed the extracellular release of HMGB1 in response to LPS or polyinosinic:polycytidylic acid stimulation. In contrast, endogenous APE1 was required for HMGB1-mediated proinflammatory cytokine expression and MAPK activation. Further, we found that HMGB1 stimulation induced the translocation of APE1 to the cell nucleus, and that silencing of APE1 with siRNA transfection inhibited both the nuclear and cytoplasmic expression of APE1. To our knowledge, this is the first demonstration of the differential roles of APE1 using overexpression and siRNA knockdown conditions, coupled with an investigation of protein localization.

## Materials and Methods

### *Primary monocytes and cell lines*

Adherent monocytes were prepared from peripheral blood mononuclear cells donated by healthy volunteers, as previously described (32). This study was reviewed and approved by the Institutional Research Board of Chungnam National University Hospital, and written informed consent was obtained from each participant. Human monocytic cell lines and THP-1 (ATCC TIB-202) were maintained in complete medium [RPMI 1640 (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (Gibco-BRL), sodium pyruvate, nonessential amino acids, penicillin G (100 IU/ml), and streptomycin (100 $\mu$g/ml)]. THP-1 cells were treated with 4 n*M* phorbol-12-myristate-13-acetate (Sigma-Aldrich, St. Louis, MO) for 24 h to induce differentiation into macrophage-like cells and then washed three times with PBS.

### *Reagent, DNA, and antibodies*

LPS (*Escherichia coli* 026:B6) and recombinant HMGB1 used for the *in vitro* assay was purchased from Sigma. NADPH oxidase inhibitor diphenyleneiodium (DPI) and dihydroethidium (DHE) were purchased from Calbiochem (San Diego, CA). Dimethyl sulfoxide (DMSO; Sigma) was added to cultures at 0.1% (vol/vol) as a solvent control.

Wild-type APE1 encoding full-length of APE1 and APE1 C65A/C93A encoding mutant form of APE1 in pCMV-Tag2B mammalian expression vector were generated by standard cloning method. TAT-APE1 and its mutant form of TAT-APE1 were generated by insertion of full-length of APE1 and APE1 C65A/C93A (muAPE1) into pTAT-2.1, as previously described (44). Expression plasmids encoding HA-hASK1-WT and HA-Kinase dead-hASK1 (K709M) in pcDNA3.1 were generous gifts from Dr. H. Ichijo (University of Tokyo, Tokyo, Japan). Cells were transfected using LipofectAMINE 2000, as indicated by the manufacturer (Invitrogen, Carlsbad, CA).

Specific antibodies against phospho-(Thr202/Tyr204)-extracellular signal regulated signal kinase (ERK) 1/2, phospho-(Thr180/Tyr182)-p38, phospho- (Thr183/Tyr185)-JNK, and ASK1 were purchased from Cell Signaling Technology (Cell Signaling, Beverly, MA). Antibodies against cyclooxygenase (COX)-2, HMGB1 (K-12), APE1 (C-20), and anti-$\alpha$-actin (I-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For immunostaining of APE1, anti-APE1 antibodies were purchased from Novus Biologicals, Inc. (Littleton, CO). Antibodies reactive against HA and FLAG were purchased from InvivoGen (San Diego, CA) and Sigma-Aldrich, respectively.

### *Adenoviral transfections*

Empty adenoviral vector control (Ad-null) and recombinant adenovirus encoding APE1 (Ad-APE1) or mutant APE1

(Ad-muAPE1) were generated by homologous recombination in HEK 293 cells, then virus-contained media were purified using the Adeno-X virus purification kit purchased from Clontech (Mountain View, CA), according to the manufacturer's instructions. THP-1 cells were infected with the indicated multiplicity of infection of adenovirus for 18 h.

### *Enzyme-linked immunosorbent assay (ELISA), reverse transcriptase polymerase chain reaction (RT-PCR), Western blot, and* in vitro *kinase assay*

Human primary monocytes or THP-1 cells were treated as indicated and processed for analysis by a sandwich ELISA, semi-quantitative RT-PCR, and Western blot, as previously described (53). For a sandwich ELISA, cell culture supernatants were analyzed for cytokine content using Duoset antibody pairs (PharMingen, San Diego, CA) for detection of tumor necrosis factor (TNF)-$\alpha$ and interleukin (IL)-6, as described previously (53). For semiquantitative RT-PCR analysis, total RNA was extracted from cells using TRIzol (Invitrogen). Primer sequences were as follows: human TNF (forward: 5′-CAGAGGGAAGAGTTCCCCAG-3′, reverse: 5′-CCTTGGTCTGGTAGGAGACG-3′), and human IL-6 (forward: 5′-TGACCCAACCACAAATGC-3′, reverse: 5′-CGAGCTCTGAAACAAAGGAT-3′). Human APE1 primers were purchased from Santa Cruz Biotechnology. For Western blot analysis, specific antibodies for phospho-ERK1/2, phospho-p38, phospho-SAPK/JNK, *COX-2*, $\beta$-actin, and HMGB1 used at a 1:1,000 dilution. *In vitro* kinase assay was performed as described previously (55). In brief, Protein A-sepharose for the ASK1 assay was obtained from Amersham Bioscience (Piscataway, NJ), and [r-$^{32}$P]-ATP was from PerkinElmer (Waltham, Massachusetts). Densitometry was performed on films and fold increase calculated as experimental sample/control sample.

### *Measurement of intracellular ROS and NADPH oxidase activity*

Intracellular ROS levels were measured by fluorescence microscopy and by flow cytometry using the redox-sensitive dye DHE, as previously described (53, 55). For flow cytometric analysis, the cells were stained and immediately acquired for analyses in FACS Calibur (BD Biosciences, San Jose, CA). The data were plotted using CellQuest software (BD Biosciences). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was measured using the lucigenin chemiluminescence method described previously (54, 56). The reaction was initiated by the addition of cell homogenate (150–200 $\mu$g of protein). Luminescence was monitored on a bioluminescence plate.

### *Immunofluorescence staining*

Immunofluorescence staining was carried out for detection of APE1, HMGB1, and *COX-2* in THP-1 cells or human monocytes with a modification of a previously described method (43). The cells were fixed with 4% *p*-formaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and then blocked for 1 h with PBS containing 3% BSA. For APE1 staining, cells were incubated with anti-APE1 antibody (1:100) in PBS containing 3% BSA for 4 h at 4°C. After extensive washing with PBS containing 0.05% Tween20, cells were treated with Cy2-conjugated anti-mouse IgG (1:200) for 1 h. For intracellular HMGB1 staining, cells were stained with anti-HMGB1 antibody (1:1,000) for 1 h at 25°C, washed, and incubated with a 1:1,000 dilution of goat immunoglobulin G-Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 1 h. Nuclei were visualized using 4,6-diamidino-2-phenylindole (DAPI, 1:1,000; Sigma) or propidium iodide (20 $\mu$g/ml). For *COX-2* staining, cells were stained with anti-*COX-2* antibody (1:100) for 3 h, washed, and treated with rhodamine (TRITC)-conjugated anti-rabbit IgG for 1 h. Fluorescent images were observed and analyzed under a laser-scanning confocal microscope (LSM 510, version 2.3; Carl Zeiss, Inc., Thornwood, NY).

### *HMGB1 release assay*

Primary human monocytes or THP-1 cells were stimulated with 100 ng/ml LPS (InvivoGen) or 20 $\mu$g/ml poly I:C (InvivoGen). The culture supernatants were harvested and subjected to Western blot analysis for HMGB1 release, as previously described (54). Supernatants with equal amount of proteins were used for the immunoprecipitation. A total of 50 $\mu$l of 50% slurry of pre-washed protein G sepharose beads was added to each sample, followed by incubation for overnight at 4°C. The immunoprecipitates were washed three times with the lysis buffer, and then the level of released HMGB1 was detected using anti-HMGB1 antibody by Western blot analysis.

### *RNA interference experiments*

THP-1 cells were transfected with hAPE1/ref-1 siRNA (Santa Cruz Biotechnology) or control-siRNA using Lipofectamine 2000 (Invitrogen), as per the manufacturer's instructions. After 24 h, the transfected cells were stimulated with HMGB1 for the times indicated and then were harvested to perform RT-PCR and Western analysis.

### *Nuclear translocation*

After cell stimulation was terminated by the addition of ice-cold PBS, nuclear and cytosolic protein extracts were prepared using the Nuclear Extraction Kit (Active Motif, Carlsbad, CA), as per the manufacturer's instructions. All the steps of subcellular fractionation were carried out at 4°C. Fraction purity was tested by Western blotting using actin as a cytoplasmic marker and p84/N5 as a nuclear marker.

### *Statistical analysis*

For statistical analysis, data obtained from independent experiments are presented as the mean ± SD and they were analyzed using a Student's *t* test with Bonferroni adjustment or ANOVA for multiple comparisons. Differences were considered significant for $p < 0.05$.

## Results

### *HMGB1-induced intracellular ROS generation in human monocytes is attenuated by treatment with APE1*

Recent studies have demonstrated that oxidative stress induces the release of the nuclear protein HMGB1 from hepatocytes (50). However, it has not been reported whether extracellular HMGB1 induces ROS generation by monocytes/macrophages. To determine if HMGB1 stimulation leads to ROS generation, human primary monocytes were cultured

with HMGB1, and superoxide generation was measured by flow cytometry (Fig. 1A) or fluorescence microscopy (Fig. 1B) using the oxidative fluorescent dye DHE. HMGB1 treatment led to an increase in intracellular ROS release at 15 min, an effect that was prevented by the NADPH oxidase inhibitor, DPI (Fig. 1A). To further establish the role of APE1 in HMGB1-dependent ROS generation, primary monocytes were transfected with empty adenoviral vector control (Ad-null) or recombinant adenovirus encoding APE1 (Ad-APE1) or a mutant version of APE1 (Ad-muAPE1). The APE1 C65A/C93A encoding mutant version was constructed, because the cysteine residues 65 and 93 are critically involved in the redox activity of APE1, probably via disulfide bridge formation between two cysteine residues (51). Transfection of primary monocytes with Ad-null or Ad-muAPE1 resulted in the robust activation of ROS production in response to HMGB1 (Fig. 1A and B). In contrast, transfection of primary monocytes with Ad-APE1 almost completely abrogated ROS production to levels comparable to those in cells pre-treated with DPI.

As the HMGB1-induced ROS production was inhibited by DPI, we examined whether APE1 affected HMGB1-mediated NADPH activity. As shown in Fig. 1C, NADPH oxidase activity was significantly activated by HMGB1 treatment in human monocytes transfected with Ad-null. The HMGB1-induced NADPH oxidase activity was profoundly attenuated by transfection of monocytes with Ad-APE1, but not by transfection with Ad-muAPE1 (Fig. 1C). These results indicate that APE1 negatively regulates the HMGB1-induced ROS generation and activation of NADPH oxidase in human monocytes/macrophages.

### *APE1 plays an inhibitory role in HMGB1-induced proinflammatory cytokine secretion by human monocytes/macrophages through ROS generation*

Previous studies have demonstrated that the addition of HMGB1 to monocytes induces the release of proinflammatory cytokines, including TNF-$\alpha$, IL-6, IL-1$\alpha$, and IL-1$\beta$ (1). We next attempted to determine whether HMGB1-induced proinflammatory cytokine production is regulated by treatment with APE1. Stimulation of primary monocytes with HMGB1 induced maximal secretion of TNF-$\alpha$ and IL-6 within 18 h of stimulation (Fig. 2A). To examine the role of APE1 in HMGB1-induced proinflammatory cytokine production, human monocytes were treated with the TAT-APE1 protein or a mutated version of the protein (TAT-muAPE1). Following transduction with TAT-APE1, HMGB1-stimulated TNF-$\alpha$ production was significantly inhibited in human primary monocytes in a dose-dependent manner (Fig. 2B). Compared to the effects on TNF-$\alpha$, HMGB1-induced IL-6 production was partially modulated by transduction with TAT-APE1. In contrast to TAT-APE1, cultures treated with TAT-muAPE1 did not show inhibited secretion of TNF-$\alpha$ or IL-6 (Fig. 2B). The effects of APE1 were further determined in THP-1 cells transfected with an empty vector control (mock) or a recombinant vector encoding APE1 (APE1) or the mutant APE1 (muAPE1). As shown in Fig. 2C, transfection of cells with the mock control or muAPE1 vector actively induced cytokine secretion in response to HMGB1. However, transfection of THP-1 cells with APE1 almost completely abrogated HMGB1-induced TNF-$\alpha$ and IL-6 secretion (Fig. 2C). Collectively, these results indicate that APE1 is a strong inhibitory regulator of HMGB1-induced proinflammatory responses through NADPH oxidase-derived intracellular ROS in monocytes/macrophages.

### *Expression of COX-2 in HMGB1-stimulated monocytes is modulated by transduction with APE1*

Stimulation with recombinant HMGB1 has been found to activate the *COX-2* and prostaglandin E2 synthase genes in vascular smooth muscle cells when incubated with the proinflammatory cytokine IL-1$\beta$ (31). However, the role of APE1 in *COX-2* expression in HMGB1-stimulated monocytes has been not reported. We first examined whether *COX-2* expression is regulated by HMGB1. Primary monocytes were treated with 100 ng/ml of HMGB1 for varying times. HMGB1 stimulation actively induced the expression of *COX-2* in human monocytes in a time-dependent manner (Fig. 3A). Peak *COX-2* expression level was observed within 18 h of HMGB1 stimulation, after which the expression declined. We next examined whether the HMGB1-induced *COX-2* expression is regulated by transduction with APE1. As shown in Fig. 3B and C, HMGB1-induced *COX-2* expression was markedly reduced by the transduction of monocytes with TAT-APE1 protein and Ad-APE1 adenovirus, as measured by fluorescence microscopy and Western blot analysis. However, HMGB1-induced *COX-2* expression was not modulated by the transduction of monocytes with the TAT-muAPE1 or mock control protein and Ad-muAPE1 or Ad-null adenovirus (Fig. 3B and C). Together, these results indicate that APE1 negatively regulates the HMGB1-induced proinflammatory mediator *COX-2* in human primary monocytes.

### *APE1 critically regulates the HMGB1-induced activation of p38 MAPK and JNK, but not ERK1/2, in human monocytes*

MAPK activation plays a critical role in the generation of proinflammatory cytokines such as TNF-$\alpha$ and IL-6 (27). To investigate the HMGB1-induced signaling pathway, we examined whether HMGB1 affects the phosphorylation of MAPKs in monocytes. Primary monocytes were treated with 100 ng/ml of HMGB1 for varying times (Fig. 4A). HMGB1 induced the phosphorylation of JNK, p38 MAPK, and ERK1/2 in a time-dependent manner. Peak phosphorylation of each was observed within 30 min of stimulation, after which the phosphorylation level declined (Fig. 4A).

To examine the role of APE1 in the HMGB1-induced activation of MAPKs, human primary monocytes were transduced with Ad-null, Ad-APE1, or Ad-muAPE1 (Fig. 4B; 50, 100, or 200 MOI). Transduction of monocytes with Ad-APE1 strongly inhibited the activation of HMGB1-induced JNK and p38 MAPK, but not ERK1/2, in a dose-dependent manner. However, HMGB1 did not affect the MAPK activation in monocytes transduced with Ad-null or Ad-muAPE1 (Fig. 4B). Furthermore, HMGB1-induced p38 and JNK activation was dramatically abrogated in THP-1 cells transfected with APE1 plasmid, whereas it was not inhibited in THP-1 cells transfected with the mock control or muAPE1 vector (Fig. 4C). These results indicate that APE1 plays a specific role in the negative regulation of JNK and p38 MAPK induced in response to HMGB1.

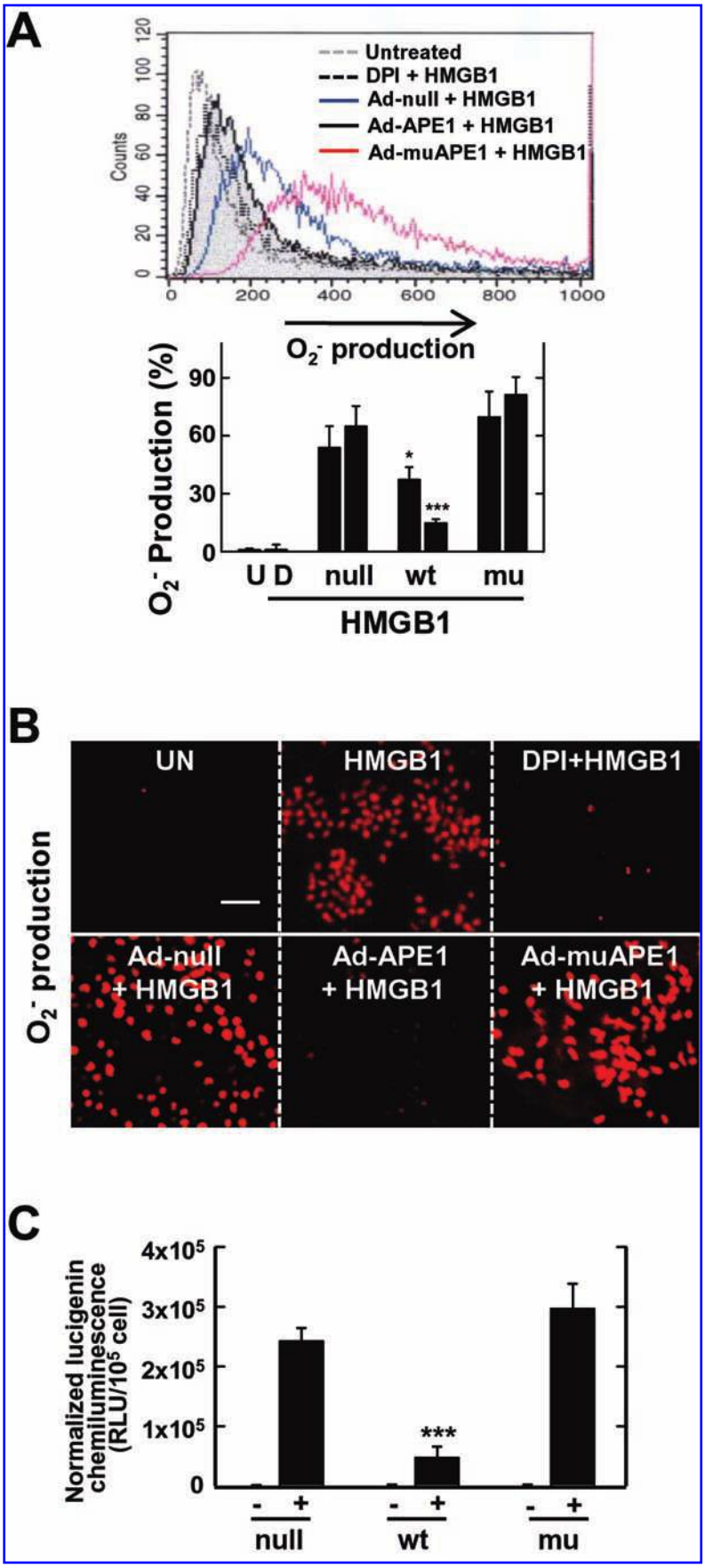


**FIG. 1. HMGB1-induced ROS generation is decreased by APE1 in human monocytes/macrophages.** Monocytes were infected with 200 MOI of empty adenoviral vector control (Ad-null), recombinant adenovirus encoding APE1 (Ad-APE1), or mutant APE1 (Ad-muAPE1). After a 24-h incubation period in normal culture medium, the transfected cells were stimulated with HMGB1 (100 ng/ml) for 15 min (**A**–**C**) in the presence or absence of DPI (20 $\mu M$). (**A**) The formation of ROS was assessed by FACS. The cells were then incubated with DHE (20 $\mu M$) for 30 min, washed rapidly and thoroughly, and analyzed immediately by flow cytometry to determine the ROS levels. The data are expressed as changes in the ROS-sensitive dye fluorescence represented by frequency histograms. Images shown are representative of three independent experiments with similar results. (*Lower panel*) Data shown are the means ± SD of all cases. Significant differences ($*p < 0.05$; $***p < 0.001$) compared with control cultures treated with empty vector are indicated. (**B**) ROS formation was assessed by confocal microscopy. Infected monocytes were treated HMGB1 (100 ng/ml) and incubated with DHE (20 $\mu M$) for 30 min. Live cells were washed with serum-free medium, and imaged using a laser-scanning microscopy. *Bar*, 50 $\mu$m. (**C**) NADPH oxidase activity was measured by lucigenin luminescence. The data are representative of three independent experiments with similar results. Significant differences ($***p < 0.001$) compared with control cultures treated with empty vector are indicated. D, solvent control (0.1% DMSO); U, untreated; null, cells infected with Ad-null; wt, cells infected with Ad-APE1; mu, cells infected with Ad-muAPE1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

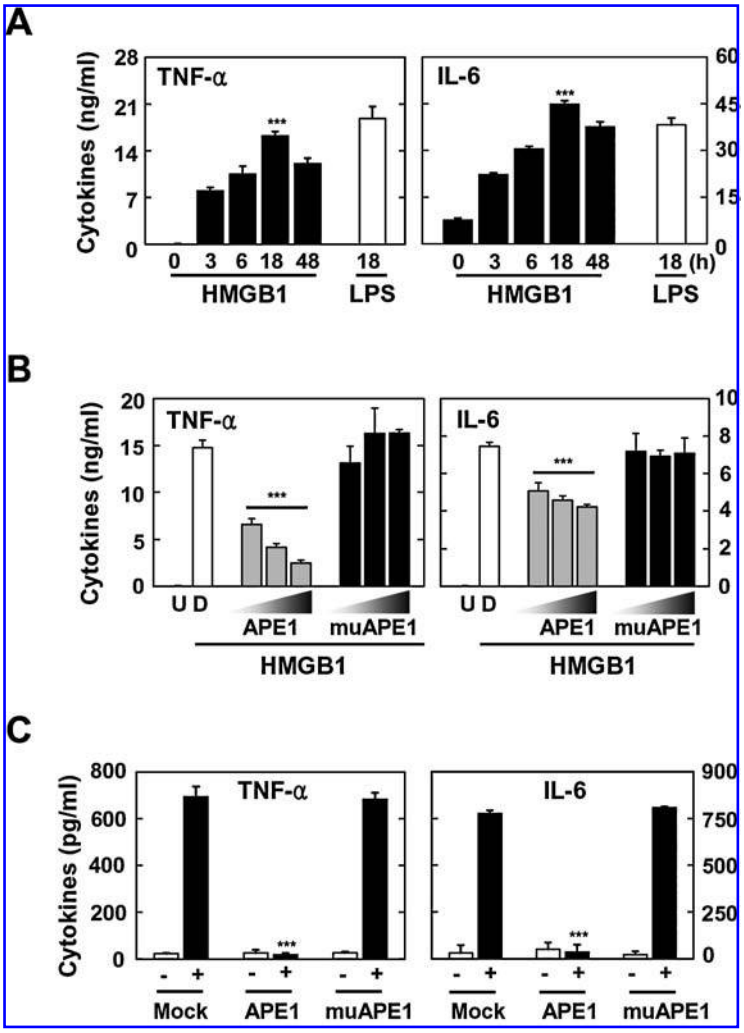


**FIG. 2. HMGB1-induced proinflammatory cytokine production is abrogated by APE1 in human monocytes/macrophages.** (**A**) Human primary monocytes were stimulated with HMGB1 (100 ng/ml) or LPS (100 ng/ml). Supernatants were collected at the times indicated, and the levels of TNF-$\alpha$ and IL-6 were measured by ELISA. Data are the mean ± SD of three experiments. (**B**) Human primary monocytes were pretreated with TAT-APE1 (APE1; 0.1, 1, 10 n*M*) or mutant TAT-APE1 (muAPE1; 0.1, 1, 10 n*M*) for 1 h, and then stimulated with HMGB1 (100 ng/ml) for 18 h. Culture supernatants were harvested, and the levels of TNF-$\alpha$ and IL-6 were measured, as described in (**A**). Significant differences (***$p < 0.001$) compared with control cultures treated with empty protein solvent are indicated. (**C**) THP-1 cells were transfected with FLAG-tagged empty vector control (mock) or recombinant plasmid encoding APE1 (APE1) or mutant APE1 (muAPE1). After 24 h, the transfected cells were stimulated with HMGB1 (100 ng/ml) for 18 h, and the supernatants from the monocytes were collected for assessment of TNF-$\alpha$ and IL-6 using ELISA. The data are the means ± SD of five experiments. Significant differences (***$p < 0.001$) compared with control cultures treated with empty vector are indicated. D, solvent control (0.1% DMSO); U, untreated.

### *HMGB1-induced ASK1 activation is upstream of MAPK and is negatively regulated by APE1*

ASK1 mediates the activation of both the MKK4/MKK7-JNK and MKK3/MKK6-p38 pathways (30, 46). We first examined whether HMGB1 stimulation induces the activation of ASK1 in monocytes using an ASK1 kinase assay. When human monocytes were stimulated with HMGB1 for varying times, the ASK1 activity levels peaked at 15 min and declined thereafter (Fig. 5A). Next, in order to investigate whether HMGB1-induced ASK1 activity is inhibited by APE1 transduction, THP-1 cells were transfected with FLAG-tagged APE1, muAPE1, or mock control vector alone. As shown in Fig. 5B, HMGB1-induced ASK1 activation was almost completely abolished in THP-1 cells transfected with APE1 plasmid (Fig. 5B). However, it was not inhibited in THP-1 cells transfected with the mock control or muAPE1 vector (Fig. 5B).

We next investigated the role of ASK1 in HMGB1-induced MAPK activation in THP-1 cells transfected with HA-tagged ASK1-WT (WT-ASK1), dominant-negative ASK1 (DN-ASK1), or empty control (mock) vector alone. As shown in Fig. 5C, the phosphorylation of p38 and JNK1/2 was significantly increased in ASK1-transfected cells, but substantially reduced in DN-ASK1-transfected cells. The ERK1/2 activation was not modulated by transfection with WT-ASK1, DN-ASK1, or mock control. Taken together, these results indicate that ASK1 is required for the HMGB1-induced activation of p38 MAPK and JNK, but not ERK1/2, in human monocytic cells.

### *The extracellular release of HMGB1 induced by proinflammatory cytokines is inhibited by APE1*

Monocytes and macrophages secrete HMGB1 in response to interaction with TLR ligand, which acts to enhance both the innate and adaptive immune responses (50). We investigated whether the extracellular release of HMGB1 in response to TLR4 (LPS, 100 ng/ml) or TLR3 (poly I:C, 20 $\mu$g/ml) ligand is modulated by APE1. Human primary monocytes were treated with LPS or poly I:C for varying times, and then the levels of extracellularly released HMGB1 were determined by Western blot analysis after immunoprecipitation of the culture supernatants (Fig. 6A). Following stimulation of human monocytes with LPS or poly I:C, the extracellular release of HMGB1 peaked at 18 h (Fig. 6A).

Next, we examined whether APE1 modulates the TLR4/LPS-induced extracellular release of HMGB1. THP-1 cells were transfected with FLAG-tagged APE1, muAPE1, or mock control vector alone. The cells were stimulated with

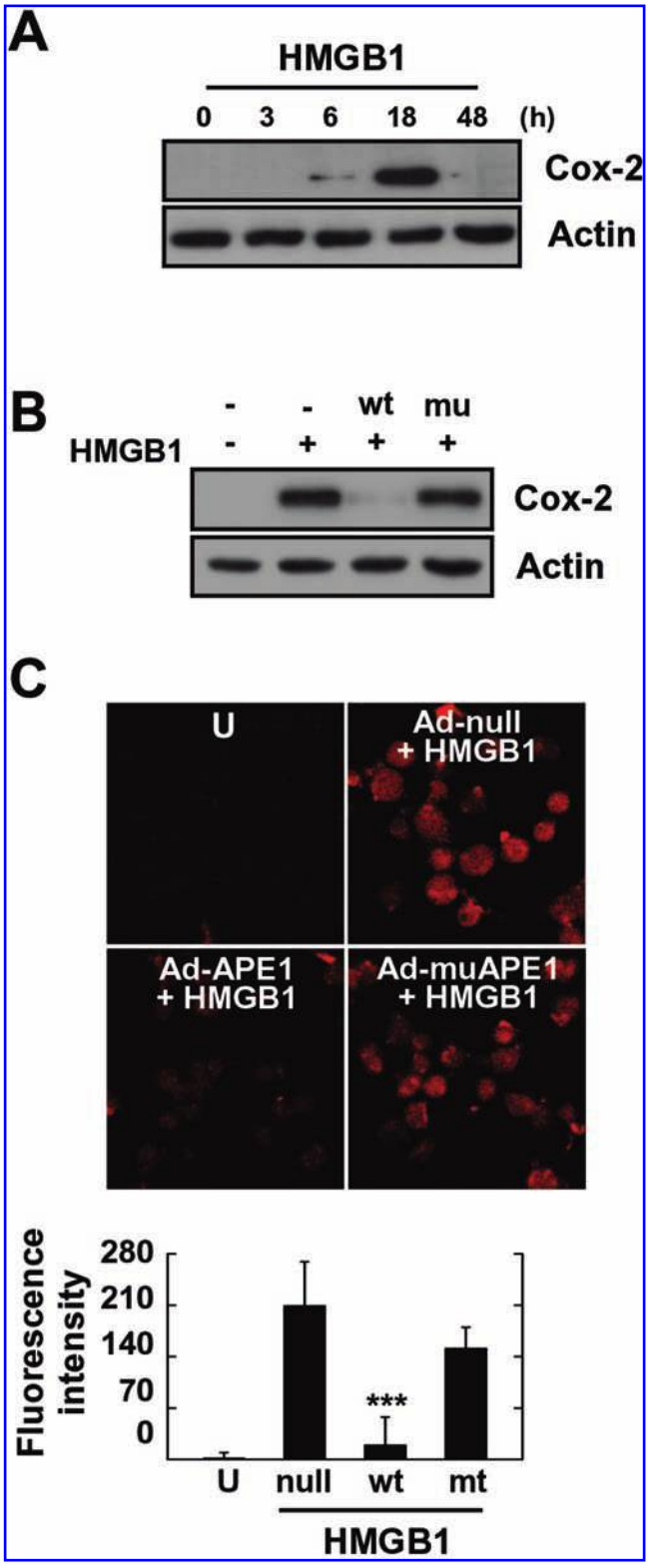


**FIG. 3. HMGB1-induced COX2 expression is attenuated by APE1 in human primary monocytes.** (**A**) Serum-starved human monocytes were treated with HMGB1 (100 ng/ml) for the times indicated. The cells were harvested and subjected to Western blot analysis using anti-COX-2 antibody. The same membranes were stripped and reprobed with $\beta$-actin as a loading control. (**B**) Human monocytes starved for 24 h were pretreated with 1 n*M* TAT-APE1 (wt) or the mutant TAT-APE1 (mu) peptide for 1 h, and then stimulated with HMGB1 (100 ng/ml) for 18 h. The stimulated cells were harvested, and the levels of activated *COX-2* were determined by immunoblotting with anti-COX-2 antibody. The same membrane was stripped and reprobed with anti-$\beta$-actin antibody. (**C**) Human primary monocytes starved for 24 h were pretreated with 200 MOI of empty adenoviral vector control (Ad-null), recombinant adenovirus encoding APE1 (Ad-APE1), or mutant APE1 (Ad-muAPE1). After a 24-h incubation in normal culture medium, the transfected cells were stimulated with HMGB1 (100 ng/ml) for 18 h. Images of *COX-2* immunofluorescence are shown. The data are representative of three independent experiments with similar results. Significant differences (***$p < 0.001$) compared with control cultures treated with empty vector are indicated. U, untreated; null, cells infected with Ad-null; wt, cells infected with Ad-APE1; mu, cells infected with. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

LPS and then subjected to Western blot analysis. The extracellular release of HMGB1 in response to LPS was significantly inhibited by the transfection of cells with APE1 plasmid (Fig. 6B). However, LPS-induced HMGB1 release was not inhibited in cells transfected with muAPE1 or mock control vector (Fig. 6B).

We next used immunofluorescent microscopy to assess whether the HMGB1 in the supernatant that originated from macrophages was modulated by APE1. THP-1 cells were transiently transfected with FLAG-tagged APE1, muAPE1, or mock control vector alone and then stimulated with LPS for 24 h. As shown in Fig. 6C, in THP-1 cells transfected with mock control or muAPE1 vector, HMGB1 was translocated into the cytosol from the nucleus. In contrast, in THP-1 cells transfected with APE1 plasmid, the translocation of HMGB1 protein into the cytosol was significantly suppressed, suggesting that APE1 causes the nuclear retention of HMGB1 by human monocytes/macrophages.

### *SiRNA gene knockdown of endogenous APE1 inhibited the HMGB1-mediated cytokine mRNA expression and MAPK activation in activated macrophage-like cells*

Song *et al*. (43) recently reported that APE1 critically mediates both the translocation of NF-$\kappa$B to the nucleus and the expression of inducible nitric oxide synthase by murine macrophage RAW264.7 cells after stimulation with LPS. We examined whether APE1 affects HMGB1-induced proinflammatory cytokine expression and MAPK activation in macrophages. Specifically, we used siRNA directed against APE1. The siRNA achieved ~95% knockdown of APE1 mRNA expression at a concentration of 20 n*M* (Fig. 7A), and was gene specific [*i.e*., the expression of other proteins was unaffected (data not shown)]. Surprisingly, TNF-$\alpha$ and IL-6 mRNA expression by HMGB1 was markedly attenuated in APE1-depleted THP-1 cells relative to that in cells transfected with control siRNA (Fig. 7B), thus confirming a role for APE1 in HMGB1-stimulated macrophages.

We confirmed the role of APE1 in HMGB1-induced MAPK

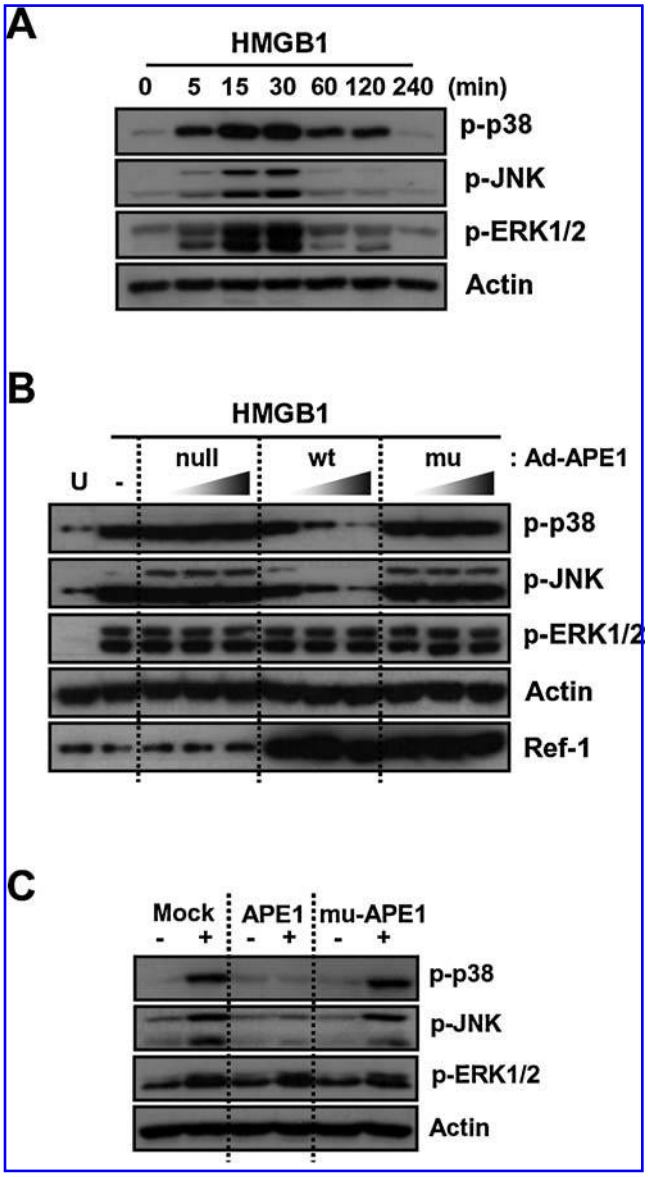


**FIG. 4. APE1 affects the HMGB1-induced activation of JNK and p38 MAPK in human monocytes.** (**A**) Serum-starved human primary monocytes were stimulated with HMGB1 (100 ng/ml) for the times indicated. The cells were harvested and subjected to Western blot analysis for phosphorylated JNK, ERK1/2, and p38. The same membranes were stripped and reprobed for total JNK, ERK1/2, and p38 as loading controls. The data are representative of three independent experiments with similar results. (**B**) Human monocytes starved for 24 h were pretreated with 50, 100, or 200 MOI of empty adenoviral vector control (Ad-null), recombinant adenovirus encoding APE1 (Ad-APE1), or mutant APE1 (Ad-muAPE1). After a 24-h incubation period in normal culture medium, the transfected cells were stimulated with HMGB1 (100 ng/ml) for 30 min. The cells were harvested and subjected to Western analysis as described in (**A**). (**C**) THP-1 cells were transfected with FLAG-tagged APE1, muAPE1, or mock control vector alone. After 24 h, the transfected cells were stimulated with HMGB1 (100 ng/ml) for 30 min. The cells were harvested and subjected to Western blot analysis for phosphorylated JNK, ERK1/2, and p38. The data are representative of three independent experiments with similar results. U, untreated.

activation by transfecting THP-1 cells with APE1-specific siRNA. Interestingly, APE1 knockdown markedly attenuated the HMGB1-induced phosphorylation of MAPKs (p38, JNK1/2, and ERK1/2; Fig. 7C). Thus, endogenous APE1 plays a separate role from APE1 that is forcibly expressed and may be an important endogenous regulator of HMGB1-mediated inflammatory cytokine expression and MAPK activation in human macrophage-like cells.

### *HMGB1-mediated nuclear translocation of APE1 is inhibited by siRNA transfection in activated macrophage-like cells*

Because endogenous APE1 leads to the activation of HMGB1-mediated inflammatory cytokine expression and signaling responses, it raises the possibility that HMGB1 play dual roles in mediating inflammatory responses. To assess the potential molecular mechanisms controlling the differential regulatory functions between endogenous and overexpressed APE1 proteins, we further examined the subcellular distribution of APE1 in several experimental conditions within THP-1 cells following HMGB1 stimulation. The subcellular localization of endogenous APE1 after HMGB1 stimulation was determined by confocal microscopy using immunofluorescence staining. APE1 exhibited a weak and diffuse cytosolic distribution under basal conditions (green fluorescence) and not in the nucleus (blue fluorescence; Fig. 8A). When THP-1 cells were incubated with HMGB1 for 12 h, cytoplasmic APE1 proteins were substantially translocated into nucleus, as indicated by the considerable increase in APE1 proteins in the nucleus. In addition, an exclusive nuclear fluorescence pattern of APE1 was observed at 24 h after HMGB1 treatment (Fig. 8A).

Nuclear translocation of APE1 was also confirmed by Western blotting after subcellular fractionation. Consistent with the confocal microscopy findings, Western blot analysis showed that the APE1 proteins were significantly translocated from cytoplasmic to nuclear fractions in THP-1 cells at 24 h after HMGB1 stimulation (Fig. 8B). Furthermore, silencing of APE1 using siRNA transfection abrogated protein expression in both the cytoplasmic and nuclear fractions (Fig. 8B). In contrast, the overexpression of APE1 in human primary monocytes with adenoviral transduction led to abundant protein expression in the cytoplasmic and nuclear fractions (Fig. 8C). Upon HMGB1 stimulation, cytoplasmic APE1 proteins were significantly translocated into the nucleus of monocytes; however, detectable levels of APE1 remained in the cytoplasm. Thus, HMGB1 stimulation induces the nuclear translocation of APE1 protein, and siRNA transfection and adenoviral transduction results in differential expression and localization patterns in activated macrophages.

## Discussion

Our previous studies have shown that the transduction of TAT-APE1 protein inhibits TNF-$\alpha$-induced monocyte adhesion and vascular cell adhesion molecule-1 expression in cultured endothelial cells (44). The overexpression of APE1 inhibits superoxide production and p38 MAPK activation by TNF-activated human umbilical vein endothelial cells (34). These studies strongly suggest that APE1 may be useful in suppressing vascular inflammatory processes. An additional role for APE1 has also been reported previously; specifically, APE1 is an important upstream regulator of transcriptional

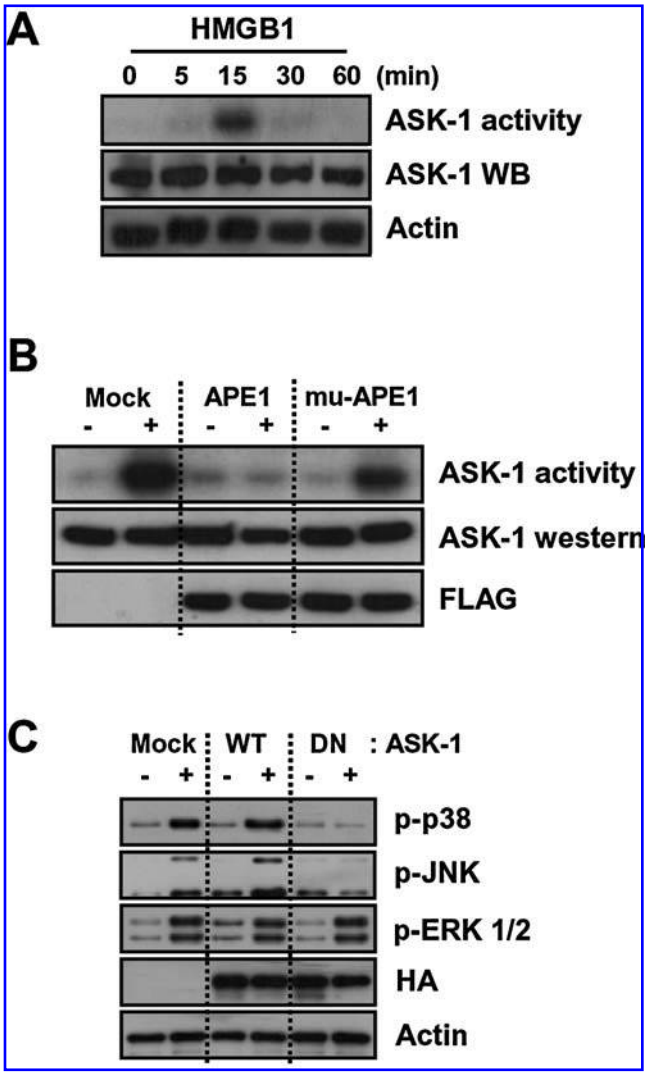


**FIG. 5. APE1 plays an inhibitory role for the HMGB1-mediated activation of p38 and JNK *via* a modulation of ASK1 activation.** (**A**) Human primary monocytes were stimulated with HMGB1 (100 ng/ml) for the times indicated. The cells were harvested and subjected to an ASK1 kinase assay and Western blot analysis for ASK1. The same blots were washed and blotted for $\beta$-actin as the loading control. (**B**) THP-1 cells were transfected with FLAG-tagged APE1 (APE1), mutant APE1 (muAPE1), or empty control (mock) vector alone. After 24 h, the transfected cells were stimulated with HMGB1 (100 ng/ml) for 15 min. The cells were harvested and subjected to an ASK1 kinase assay and Western blot analysis for phosphorylated ASK1. (**C**) THP-1 cells were transfected with HA-tagged ASK1-WT (WT-ASK1), dominant-negative ASK1 (DN-ASK1), or empty control (mock) vector alone. After 24 h, the transfected cells were stimulated with HMGB1 (100 ng/ml) for 30 min. The cells were harvested and subjected to Western blot analysis for phosphorylated JNK, ERK1/2, p38, HA, and actin. The data are representative of three independent experiments with similar results.

activities mediated by NF-$\kappa$B, a crucial transcriptional factor involved in immune and inflammatory signaling, as well as cell survival (26, 33). We examined whether APE1 modulates inflammatory responses and ROS release in response to HMGB1, one of the key alarm molecules released during various stimuli, such as cellular necrosis. Our data show that overexpressed APE1 plays an inhibitory role in HMGB1-induced ROS release, NADPH oxidase activation, inflammatory mediator release, and ASK1-p38/JNK MAPK signaling activation. In contrast, endogenous APE1 activates the

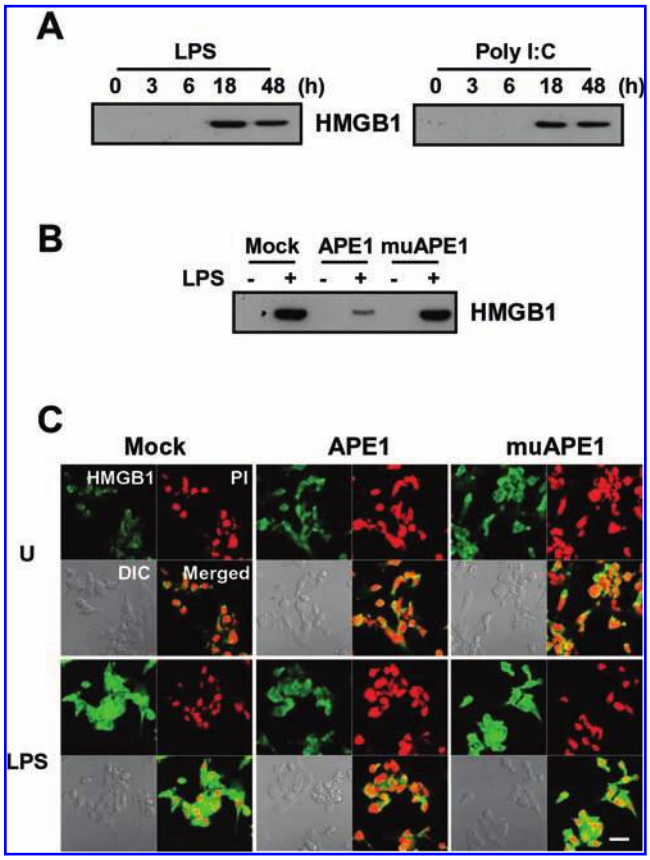


**FIG. 6. The extracellular release of HMGB1 induced by inflammatory stimuli is decreased by APE1.** (**A**) Serum-starved human primary monocytes were stimulated with 100 ng/ml of LPS (*left*) or 20 $\mu$g/ml poly I:C (*right*) for the times indicated. The culture supernatants were collected, and supernatants containing equal amounts of proteins were subjected to immunoprecipitation with 50 $\mu$l of 50% slurry of pre-washed protein-G Sepharose beads. The samples were washed three times in lysis buffer, and then subjected to Western blot analysis with anti-HMGB1 antibody. The data are representative of three independent experiments with similar results. (**B**) THP-1 cells were transfected with FLAG-tagged APE1 (APE1), mutant APE1 (muAPE1), or empty control (mock) vector alone for 24 h. The transfected cells were starved in serum-free media and stimulated with LPS (100 ng/ml) for 24 h. The culture supernatants were collected and subjected to immunoprecipitation and Western blot analysis as described in (**A**). The data are representative of three independent experiments with similar results. (**C**) THP-1 cells were transfected with FLAG-tagged APE1, muAPE1, or mock control vector alone for 24 h. The transfected cells were starved in serum-free media and stimulated with LPS (100 ng/ml) for 24 h. The cells were detected by incubation with anti-HMGB1 antibody for immunofluorescent staining. Nuclei were visualized using confocal microscopy after a 15-min incubation with 20 $\mu$g/ml propidium iodide. *Bar*, 20 $\mu$m. U, untreated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

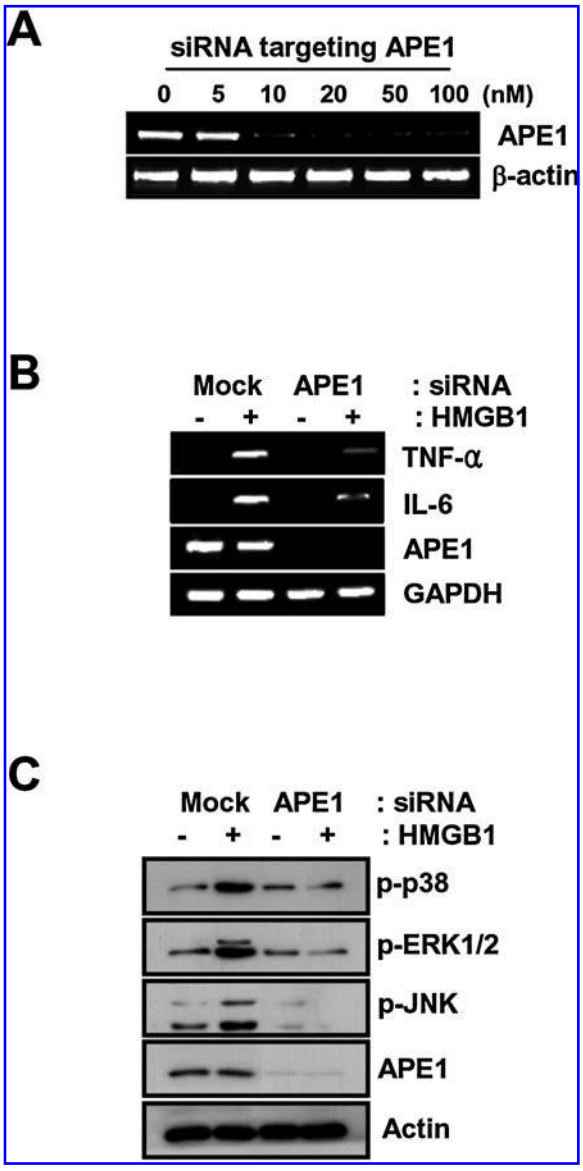


**FIG. 7. Effect of APE1 knock-down on the HMGB1-induced cytokine mRNA expression and MAPK activation in THP-1 cells.** (**A**) THP-1 cells were transfected with APE1 siRNA at indicated concentration, and the levels of APE1 mRNA were then determined by RT-PCR analysis 24 h after transient transfection. (**B**, **C**) THP-1 cells were transfected with APE1 or control siRNA (20 n*M*, each) and cells were incubated for 24 h. Then the cells were stimulated with or without HMGB1 (100 n*M*). The levels of mRNA for TNF-$\alpha$ and IL-6 were determined by RT-PCR 6 h after stimulation (for **B**). The cells were stimulated with or without HMGB1 for 30 min, harvested and subjected to Western blot analysis for phosphorylated MAPKs (ERK1/2, p38, and JNK) and APE1 levels. The same blots were washed and blotted for $\beta$-actin as loading controls (for **C**).

HMGB1-mediated inflammatory responses in macrophages. We also examined the localization patterns of endogenous or overexpressed APE1 before and after HMGB1 stimulation, which might affect the distinct roles of APE1 in HMGB1-mediated inflammatory responses.

HMGB1 acts as a late mediator of endotoxic shock and exerts a variety of proinflammatory activities. HMGB1 simultaneously acts as a chemoattractant and activator of dendritic cells, and is thus becoming increasingly recognized as an immune alarmin (56). Previous studies have suggested that hypoxia- or oxidant-induced HMGB1 release from hepatocytes is an active process that is regulated by ROS, because antioxidants abrogate $H_2O_2$-mediated HMGB1 release (49). In the current study, APE1 adenoviral transduction profoundly inhibited HMGB1-induced ROS formation and NADPH oxidase activation, and these effects were abrogated by the transduction of cells with an Ad-muAPE1 construct containing a substitution in the redox-potential signal (see Fig. 1). The excessive generation of ROS may lead to alterations in normal cell function and cause adverse responses, including sepsis and inflammation (8, 25). Therefore, these observations strongly suggest that the redox-competent APE1 plays a key inhibitory role in HMGB1-induced ROS generation and NADPH oxidase activation through its redox-modulating activities.

Our data demonstrate that APE1 attenuates cytokine secretion and *COX-2* expression in primary monocytes in response to HMGB1. Once activated by inflammatory stimuli, monocytes synthesize and release eicosanoids that mediate inflammatory and immune responses (16). *COX-2* synthesis, an essential step in this event (13), is responsible for the high-level production of prostanoids in response to proinflammatory agents (16). Activation of NF-$\kappa$B is an important component of *COX-2* expression (11, 29), and is assumed to be greatly sensitive to redox imbalance, making this a ROS-responsive target system (3, 4). In fact, APE1 functions as a redox factor that maintains numerous transcription factors in an active reduced state (18, 21, 47), thus affecting eicosanoid biosynthesis through the modulation of the transcription factor NF-$\kappa$B. Previous studies have also demonstrated that apocynin, which affects the assembly of NADPH oxidase (45), markedly inhibited *COX-2* synthesis and activity induced in monocytes, suggesting that this compound exerts a protective effect in inflammation (5). In addition, other studies have demonstrated that APE1 modulates *IL-8* expression in *Helicobacter pylori*-infected human gastric epithelial cells (39). Our data are the first to show the action of overexpressed APE1 in modulating *COX-2* expression and proinflammatory cytokine production in human primary monocytes/macrophages in response to HMGB1. As HMGB1 secretion is stimulated by numerous infectious and inflammatory stimuli, our data suggest a potential therapeutic use for APE1 in numerous cell and animal models of inflammation.

The MAPK signaling cascade plays an essential role in the initiation of cellular processes involved in the transduction of externally derived signals that regulate cell growth, differentiation, and apoptosis (51). The MAPK pathways comprise at least three distinct and parallel MAPK cascades, including ERK1/2, p38 MAPK, and JNK. The activation of MAPK signaling pathways is believed to be crucial for the generation of proinflammatory cytokines, such as TNF-$\alpha$, IL-6, IL-1, and IL-12, as well as chemokines (27). Previous studies have shown that TNF-induced p38 MAPK activation is partially blocked by the overexpression of APE1 in endothelial cells (34). However, the regulatory role exerted by APE1 in MAPK activation has not been widely studied. The current data present evidence that overexpressed APE1 in-

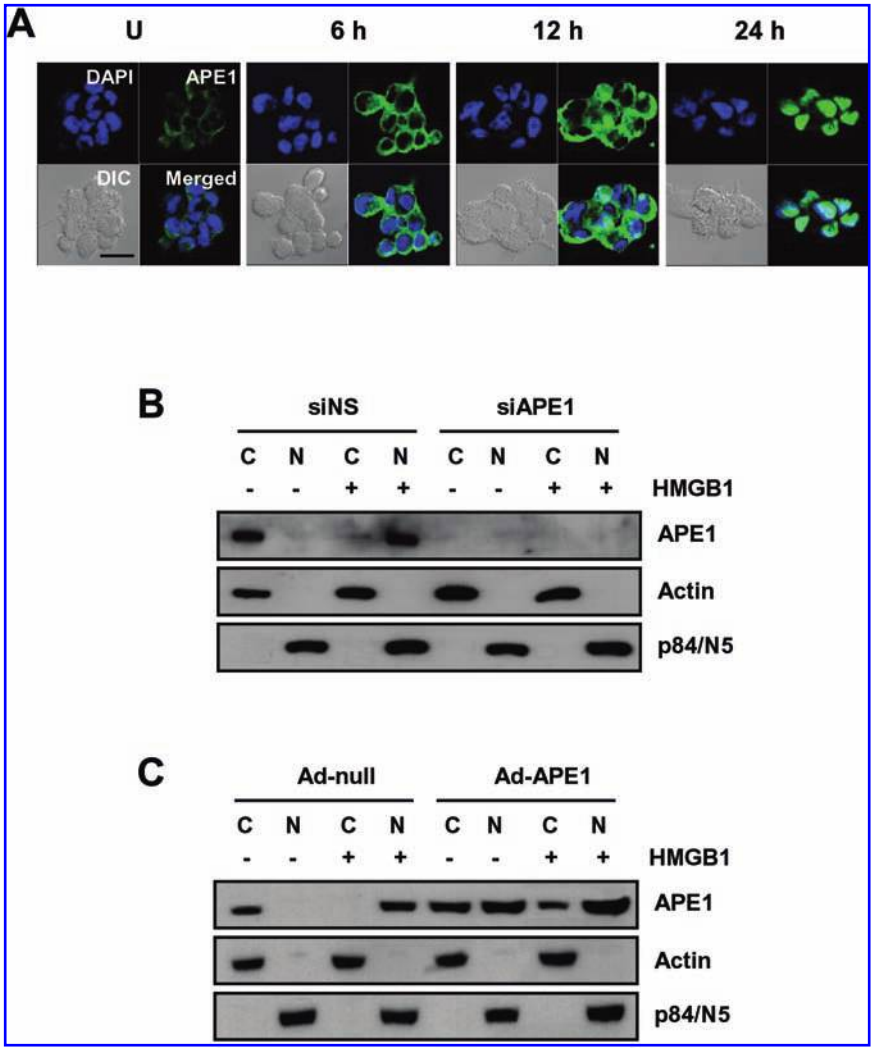


**FIG. 8. APE1 nuclear translocation by HMGB1 stimulation in macrophages.** (**A**) THP-1 cells were stimulated with HMGB1 (100 ng/ml) at the indicated time points. The cells were fixed, and then stained with anti-APE1 (*green*) and DAPI (for nucleus; *blue*) for analysis by confocal microscopy. The *bottom panels* show superposition of DAPI and anti-APE1 immunoreactivity (Merged). *Bar*, 20 $\mu$m. (**B**, **C**) THP-1 cells were transfected with APE1 or control siRNA (20 n*M* each; for **B**). Human primary monocytes were transduced with 200 MOI of empty adenoviral vector control (Ad-null) or recombinant adenovirus encoding APE1 (Ad-APE1; for **C**). Then the cells were incubated with or without HMGB1 for 24 h. Nuclear (N) and cytoplasmic (C) proteins were isolated and used for Western blot analysis for APE1 translocation. The same blots were washed and blotted for $\beta$-actin as loading controls. U, untreated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

hibits the activation of p38 and JNK, but not ERK1/2, in response to HMGB1.

Of special interest is also the fact that the activation of ASK1 is regulated in coordination with the activation of p38 and JNK by APE1 during the response to HMGB1. ASK1 mediates the activation of both the MKK4/MKK7-JNK and MKK3/MKK6-p38 pathways (30, 46). ASK1 is activated by various types of stress, including oxidative stress, endoplasmic reticulum stress, and receptor-mediated infectious and inflammatory signals (35, 37, 41). Endogenous ASK1 constitutively forms a high-molecular-mass complex that includes the redox regulatory protein thioredoxin (Trx), which is designated as an ASK1 signalosome (38). Trx is a negative regulator of the ASK1-JNK/p38 pathway and was found to be a binding protein of ASK1 through yeast two-hybrid screening (22, 41). Upon $H_2O_2$ treatment, the oxidized form of Trx (Trx-$S_2$), which contains a disulfide bridge between Cys32 and Cys35 in the active center, is dissociated from ASK1, resulting in the activation of ASK1 (41). In response to TLR4/LPS stimulation, the formation of a complex of the adaptor molecule TRAF6 with ASK1 was dependent on intracellular ROS generation and was required for the subsequent activation of the ASK1-p38 pathway (35). Previous study demonstrated that APE1 can interact directly with Trx (28). Therefore, APE1 may be a key modulator that regulates ASK1 activity through a redox-sensitive interaction with Trx (Fig. 9). Further studies are urgently needed to clarify the molecular basis mechanisms of APE1 modulation of ASK1 activation in response to HMGB1.

The present study also demonstrates that the extracellular release of HMGB1 from activated macrophages cultured with lipopolysaccharide or polyinosinic:polycytidylic acid is significantly inhibited by overexpressed APE1, causing the nuclear retention of HMGB1. The nonhistone chromatin-binding protein HMGB1 (8, 24) is an important architectural facilitator of the assembly of nucleoprotein complexes, resulting in the regulation of gene recombination and transcription (8, 48). HMGB1 is released by necrotic cells (42), and its release is also triggered by a number of cellular stress events, such as LPS treatment of activated monocytes/macrophages (10, 23, 50). Previous studies have shown that HMGB1 release from cultured hepatocytes is dependent on TLR4-mediated ROS production and downstream calcium/calmodulin-dependent kinases (49). The ability of overexpressed APE1 to regulate the secretion of HMGB1 might shed new light on the contribution of APE1 to the modulation of inflammation in noninfectious settings, such damaged tissue.

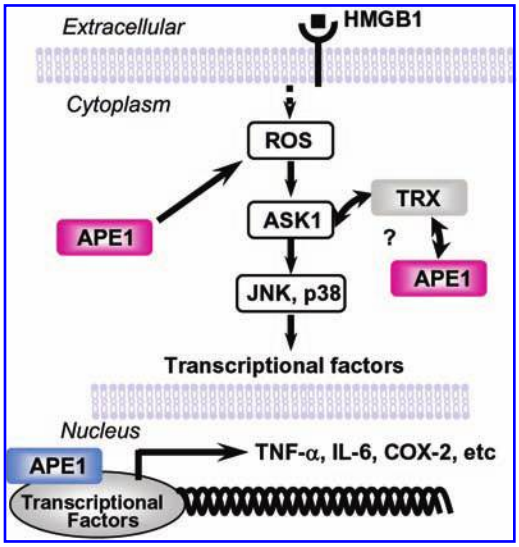


**FIG. 9. A schematic model outlining the distinctive roles of 'endogenous APE1' and 'overexpressed APE1' proteins.** Engagement of receptors by HMGB1 initiates a series of intracellular responses, which activate MAPK signaling pathways involved in the expression of inflammatory mediators. Mammalian MAPK cascade is composed of a sequential activation pathway comprising MAP3K, MAP2K, and MAPK; ASK1 is known as a MAP3K upstream to the JNK and p38 pathways. HMGB1-dependent activation of ASK1 requires the generation of ROS as second messengers.Forced cytoplasmic expression of APE1 (*red-colored*) inhibits ROS-dependent ASK1-p38/JNK pathway activation, presumably through a Trx-dependent redox-sensitive regulatory mechanism. The activation of pathways involving p38 and JNK target the transcriptional factors, such as AP-1. In addition, inflammatory stimuli such as HMGB1 induce a nuclear translocation of endogenous APE1 (*blue-colored*). Once endogenous APE1 reaches the nucleus, it contributes to the maintenance of Jun and Fos in the reduced state and promotes binding to AP-1, thus activating inflammatory responses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

The silencing of APE1 via siRNA transfection resulted in the marked inhibition of HMGB1-induced inflammatory responses. This finding partly correlates with those of recent studies showing that the TLR4/LPS-dependent nuclear translocation of APE1 is critically involved in inducible nitric oxide synthase expression within murine macrophage RAW264.7 cells (43). Indeed, APE1 plays dual and opposing roles in the regulation of NF-κB activation and cellular apoptosis (2, 26). Different conclusions by previous researchers might be related to differences in methodological approaches, including overexpression and monitoring the subcellular compartmentalization of APE1 (2). We found that HMGB1 stimulation causes the nuclear translocation of APE1 by macrophages (see Fig. 8). Further, siRNA transfection resulted in the depletion of endogenous APE1 within both the nuclear and cytosolic fractions, whereas adenoviral transduction markedly upregulated cytoplasmic APE1 in activated macrophages.

Taken together with the results of previous studies, our results suggest that APE1 may play differential roles depending on whether the protein is endogenously or forcibly expressed. To clarify this concept, we propose a summarized model outlining the distinctive roles of 'endogenous APE1' and 'overexpressed APE1' proteins (see Fig. 9). Cytoplasmic forced expression of APE1 may play a role in the inhibition of ROS-dependent ASK1-p38/JNK pathway activation, presumably through a Trx-dependent redox-sensitive regulatory mechanism. Once APE1 reaches the nucleus, it contributes to the maintenance of Jun and Fos in the reduced state and promotes binding to AP-1 (58), thus activating inflammatory responses. Therefore, APE1 acts as a fine tuning instrument within highly orchestrated cellular responses that may determine the net effect of cellular response activation and cell fate during responses to various inflammatory stimuli. Further studies will clarify the molecular mechanisms governing the spatiotemporal behavior of APE1, which influences the differential functionality of APE1 attributed to different cellular compartments.

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## Abbreviations

Ad-APE1, recombinant adenovirus encoding APE1; Ad-muAPE1, mutant version of APE1; Ad-null, adenoviral vector control; APE1, Apurinic/apyrimidinic endonuclease 1/Redox factor-1; ASK1, apoptosis signal-regulating kinase 1; COX-2, cyclooxygenase-2; DAPI, 4,6-diamidino-2-phenylindole; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; DN-ASK1, dominant-negative ASK1; DPI, diphenyleneiodium; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase 1/2; HIF-1α, hypoxia-inducible factor-1α, HMGB1, high-mobility group box 1; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; mock, empty vector control; MOI, multiplicity of infection; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-κB; redox, reduction/oxidation; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase chain reaction; siRNA, small interfering RNA; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; Trx, thioredoxin; WT-ASK1, HA-tagged ASK1-WT.

## Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
*Eun-Kyeong Jo*
*Department of Microbiology*
*College of Medicine*
*Chungnam National University*
*6 Munhwa-dong, Jungku*
*Daejeon 301-747, S. Korea.*

*E-mail:* hayoungj@cnu.ac.kr

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