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Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1

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Abstract

High mobility group box 1 (HMGB1) can be actively secreted by macrophages/monocytes in response to exogenous and endogenous inflammatory stimuli (such as bacterial endotoxin, TNF-α, IL-1, and IFN-γ) or passively released by necrotic cells and mediates innate and adaptive inflammatory responses to infection and injury. Here, we demonstrated that a reactive oxygen species, hydrogen peroxide (H_2O_2), induces active and passive HMGB1 release from macrophage and monocyte cultures in a time- and dose-dependent manner. At nontoxic doses (e.g., 0.0125–0.125 mM), H_2O_2 induced HMGB1 cytoplasmic translocation and active release within 3–24 h. At higher concentrations (e.g., 0.25 mM), however, H_2O_2 exhibited cytotoxicity to macrophage and monocyte cell cultures and consequently, triggered active and passive HMGB1 release. In addition, H_2O_2 stimulated potential interaction of HMGB1 with a nuclear export factor, CRM1, in macrophage/monocyte cultures. Inhibitors specific for the JNK (SP600125) and MEK (PD98059), but not p38 MAPK (SB203580), abrogated H_2O_2-induced, active HMGB1 release. Together, these data establish an important role for oxidative stress in inducing active HMGB1 release, potentially through a MAPK-and CRM1-dependent mechanism.

INTRODUCTION

Oxidative stress is caused by an excessive accumulation of reactive oxygen species (ROS) and has been implicated in the pathogenesis of a growing number of inflammatory diseases, including arthritis, ischemia/reperfusion (I/R) injury, stroke, and sepsis. Excessive production of ROS can lead to damage of macromolecules (such as lipids, proteins, nucleic acids, or carbohydrates), thereby affecting normal cellular functions [1]. MAPKs can be activated by a variety of stimuli, including growth factors, hormones, and ROS, and are involved in a number of physiological (e.g., cell proliferation and differentiation) [2] and pathological (oxidative stress/damage) processes [3,4]. However, the molecular mechanism by which ROS causes oxidative stress/damage is poorly characterized.

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A ubiquitous nuclear protein, high-mobility group box 1 protein (HMGB1), has been established recently as a crucial mediator of lethal systemic inflammatory diseases [5-7]. Residing predominantly in the nucleus of quiescent macophages/monocytes, HMGB1 can be secreted actively by macrophages/monocytes in response to exogenous and endogenous inflammatory stimuli such as endotoxin, CpG DNA, TNF-α, IL-1, and IFN-γ [6,8]. In addition, HMGB1 can be released passively by necrotic cells [9]. Once released, extracellular HMGB1 mediates a wide range of biological responses in diverse cell types and tissues. In vitro, extracellular HMGB1 can activate macrophages, monocytes [10], and promote dendritic cell (DC) maturation [11-13]. In vivo, HMGB1 cause acute lung inflammation, epithelial-cell barrier leaking, and even death [14,15]. Moreover, increased levels of HMGB1 are found in patients with sepsis and other major inflammatory diseases including rheumatoid arthritis [5, 16].

However, the potential role of oxidative stress in the regulation of HMGB1 release has not been investigated previously. These studies were therefore undertaken to determine the effects of hydrogen peroxide (H_2O_2)-induced oxidative stress/damage on the potential active or passive HMGB1 release in macrophage and monocyte cultures. Here, we demonstrated that H_2O_2 stimulates macrophages/monocytes to actively release HMGB1, potentially through a MAPK- and CRM1-dependent mechanism.

MATERIALS AND METHODS

Cell culture and treatment

Murine macrophage-like RAW 264.7 cells were obtained from the Shanghai Type Culture Collection (China) and cultured in RPMI-1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and an antibiotic-antimycotic mix in a humidified incubator with 5% CO_2 and 95% air. Human PBMCs were isolated from healthy donor blood by Ficoll density gradient centrifugation and cultured in RPMI-1640 medium/10% heat-inactivated human serum/2 mM glutamine overnight. Nonadherent cells were subsequently removed, and adherent monocyte-enriched cultures were stimulated with H_2O_2 at various concentrations.

H_2O_2 was diluted in PBS and further diluted in culture medium. The specific MAPK inhibitors PD98059 (Promega, Madison, WI), SB203580 (Promega), and SP600125 (KangChen Biotechnology, China) were dissolved in DMSO. Cells were pretreated for 30 min with or without MAPK inhibitor prior to the addition of H_2O_2.

Cell viability assay

Cells were plated at a density of 10^4 cells/well on 96-well plates in 100 μl RPMI. Cell viability was evaluated using the conventional MTT reduction assays, where cells of each microwell were incubated with 20 μl 0.5% MTT for 2 h at 37°C, and the reaction was stopped by adding 150 μl DMSO. The amount of MTT formazan product was determined by measuring absorbance using a micro-plate reader (Bio-Rad, Hercules, CA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Preparation of cellular extracts

At indicated time-points after the treatment, cells were harvested and washed twice with cold PBS; nuclear and cytoplasmic extracts were prepared according to the method of Schreiber et al. [17]. Briefly, the cell pellet was resuspended in 400 μl cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). The cells were allowed to swell on ice for 15 min, after which 25 μl of a 10% solution of Nonidet P-40 (NP-40) was added, and the tube was vortexed vigorously for 10 s. The homogenate was centrifuged

for 30 s, and the nuclear pellet was resuspended in 50 μl ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After vigorously rocking at 4°C for 15 min on a shaking platform, the nuclear extract was centrifuged for 5 min in a microfuge at 4°C, and the supernatant was frozen in aliquots at −80°C. The protein content of the different fractions was determined by a Bradford method.

Western blotting analysis

After various treatments, cell-conditioned medium was harvested and filtered through Millex-GP (Millipore, Bedford, MA) to remove cell debris and macromolecular complexes. Samples were then concentrated 40-fold with Amicon Ultra-4-10000 NMWL (Millipore) following the manufacturer's instructions. Proteins in the whole-cell lysate, subcellular fractions, or concentrated cell culture supernatants were resolved on 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane. After blocking the membrane at room temperature for 6 h, the membrane was incubated for 2 h with various primary antibodies specific for HMGB1, CRM1, proliferating cell nuclear antigen (PCNA; BD Biosciences, San Jose, CA), β-actin, and GAPDH (KangChen Biotechnology), respectively. After incubation with peroxidase-conjugated secondary antibodies for 1 h at 25°C, the signals were visualized by 3,3′-diaminobenzidine detection (Boster Biotech, China) according to the manufacturer's instruction.

Immunocytochemical analysis

Cells were cultured on glass coverslips and fixed in 4% formaldehyde for 30 min at room temperature prior to detergent extraction with 0.1% Triton X-100, 10 min at 4°C. Coverslips were saturated with PBS containing 2% BSA for 1 h at room temperature and processed for immunofluorescence with anti-HMGB1 antibody (BD Biosciences), followed by Cy3-conjugated Ig (Sigma Chemical Co., St. Louis, MO) and Hoechst 33258 (Sigma Chemical Co.). Between all incubation steps, cells were washed three times for 3 min with PBS containing 0.2% BSA. Coverslips were mounted on slides using Movio (Sigma Chemical Co.). Fluorescence signals were analyzed using a fluorescence microscope (Nikon, Japan).

Coimmunoprecipitation analysis

Nuclear extracts were lysed at 4°C in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, protease inhibitor cocktail). Lysates were cleared by centrifugation at 12,000 g for 10 min and then incubated for 2 h or overnight at 4°C with 5 μg/mL of the appropriate antibody and protein A or G agarose/sepharose beads (Amersham Biosciences, Sweden). Immune complexes were washed extensively with PBS, and proteins were eluted by boiling in 2× SDS sample buffer. Proteins were assayed by Western blotting as above.

Statistical analysis

Significance of differences between groups was determined by two-tailed Student's *t*-test or Fisher's LSD test, as indicated. $P < 0.05$ was considered significant.

RESULTS

H_2O_2 induces HMGB1 release in RAW 264.7 macrophages and human PBMCs

The effects of H_2O_2 on cell viability were first determined by MTT reduction assay (Fig. 1A). At lower (0.0125–0.125 mM) concentrations, H_2O_2 was not toxic to macrophage cultures. At higher (0.25–1.0 mM) concentrations, however, H_2O_2 exhibited a dose-dependent cytotoxicity to macrophage cultures.

To determine if H_2O_2 induces active HMGB1 release in the absence of cell death, macrophage cell cultures and cell-conditioned medium were collected separately and assayed for HMGB1 levels by Western blotting analysis. At nontoxic doses (e.g., 0.0125–0.125 mM), H_2O_2 increased cellular HMGB1 protein levels slightly (Fig. 1B) and induced active HMGB1 release within 3–24 h (Fig. 1, C and D). At higher concentrations (e.g., 0.25 mM), however, H_2O_2 exhibited cytotoxicity to macrophage cell cultures (Fig. 1A) and consequently, triggered a more pronounced, robust HMGB1 release, possibly as a result of active and passive HMGB1 release (Fig. 1C). The inducible nature of HMGB1 release was reproduced in cultures of primary human PBMCs, which actively released HMGB1 within 3 h after stimulation with H_2O_2 at nontoxic concentrations (0.125 mM, data not shown).

To gain insight into the mechanisms underlying H_2O_2-mediated HMGB1 release, H_2O_2 was washed out after a brief (2-h) exposure, and HMGB1 levels in the culture medium were determined 24 h after the onset of H_2O_2 stimulation. A brief exposure of macrophage cultures to H_2O_2 at doses nontoxic (0.125 mM) or otherwise low toxic (0.25 mM if exposed persistently) did not reduce cell viability significantly (data not shown) but triggered a marked HMGB1 release (Fig. 1E).

H_2O_2 induces the translocation of HMGB1 in RAW 264.7 macrophages and human PBMCs

To determine if H_2O_2 induces HMGB1 cytoplasmic translocation, macrophage and monocyte cultures were immunostained with HMGB1-specific antibodies. In agreement with previous reports [18-21], HMGB1 was noted predominantly in the nucleus of quiescent macrophages and monocytes (Fig. 2A). At 12 h after H_2O_2 treatment, HMGB1 staining was observed in nuclear and cytoplasmic regions of macrophage and monocyte cultures (Fig. 2A). To confirm HMGB1 cytoplasmic translocation, cytoplasmic and nuclear fractions of macrophage or monocyte cultures were isolated and immunoblotted with antibodies specific for HMGB1, PCNA (a nuclear protein), or β-actin (a cytoplasmic protein), respectively. Consistently, levels of HMGB1 in the cytoplasmic fractions were increased dramatically after treatment with H_2O_2 at a nontoxic concentration (0.125 mM; Fig. 2B).

H_2O_2 increased interaction of HMGB1 with a nuclear export factor, CRM1, in macrophage and monocyte cultures

The nuclear pore complex (NPC) is a 125-MDa nuclear envelope complex, which mediates nucleocytoplasmic shuttling of RNA and protein. NPC is comprised of more than 30 different proteins, including Ran/TC4, importins, and exportins. CRM1 is a widely expressed importin involved in the maintenance of chromatin structure and export of nuclear proteins. To determine the potential involvement of CRM1 in H_2O_2-mediated HMGB1 cytoplasmic translocation, we first determined whether H_2O_2 affects CRM1 levels in macrophage and monocyte cultures. Following H_2O_2 stimulation, the relative CRM1 content (as normalized by a cytoplasmic housekeeping protein, GAPDH) was increased slightly in macrophage and monocyte cultures (Fig. 3A), implicating a possibility that H_2O_2 may induce CRM1 cytoplasmic translocation. To examine potential CRM1-HMGB1 interaction, nuclear proteins were immunoprecipitated with CRM1- or HMGB1-specific antibodies, and the precipitated protein complexes were subsequently assayed for HMGB1 or CRM1 content by Western blotting analysis. In quiescent macrophage or monocyte cultures, a small amount of HMGB1 was coimmunoprecipitated with CRM1 protein, indicating a poor interaction between HMGB1 and CRM1. After H_2O_2 exposure, however, significant, higher amounts of HMGB1 were coimmunoprecipitated with CRM1 (Fig. 3B), indicating an increase in HMGB1-CRM1 interaction in H_2O_2-stimulated macrophage/monocyte cultures. In light of the potential role of CRM1 in LPS-induced HMGB1 release [22], it is plausible that CRM1 may be involved in H_2O_2-mediated HMGB1 cytoplasmic translocation in macrophage and monocyte cultures.

SP600125 and PD98059 inhibit H_2O_2-induced HMGB1 release and translocation in RAW 264.7 macrophages and human PBMCs

In response to various stimuli, the p38, JNK, and ERK MAPKs are phosphorylated quickly. Indeed, H_2O_2 effectively induced phosphorylation of p38, JNK, and ERK MAPK in macrophages and monocyte cultures (data not shown). To determine the potential involvement of various MAPK pathways in H_2O_2-induced HMGB1 release, we determined whether specific inhibitors for p38 (SB203580), JNK (SP600125), or MEK (PD98059) affect H_2O_2-induced HMGB1 release. Pretreatment of macrophage cell cultures with inhibitors for JNK and MEK, but not p38 MAPK, dose-dependently attenuated H_2O_2-induced HMGB1 release (Fig. 4A). Consistently, pretreatment with JNK inhibitor dramatically reduced cytoplasmic HMGB1 levels in H_2O_2-stimulated macrophages (Fig. 4B), suggesting that JNK inhibitor attenuates H_2O_2-induced HMGB1 release through interfering with its cytoplasmic translocation.

As mentioned above, a brief exposure of macrophage cultures to H_2O_2 at a dose (0.25 mM) otherwise slightly toxic if exposed persistently (for 24 h) did not reduce cell viability significantly (data not shown) but triggered a marked HMGB1 release (Figs. 1E and 4C). This HMGB1 release was almost completely abrogated by specific inhibitors for JNK and MEK, but not p38 MAPK (Fig. 4C), when they were used at well-documented, nontoxic concentrations [23-27].

To confirm the role of MAPKs in H_2O_2-induced HMGB1 release, we examined the effects of various MAPK inhibitors on H_2O_2-induced HMGB1 release in human monocyte cultures. Similarly, specific inhibitors for the JNK and MEK, but not p38 MAPK, significantly attenuated HMGB1 release induced by H_2O_2 at nontoxic doses (Fig. 4D). Taken together, these data support a potential role for JNK and ERK, but not p38 MAPK, in H_2O_2-induced HMGB1 release in macrophage and monocyte cultures.

DISCUSSION

HMGB1, a 30-kDa nuclear protein, widely studied as a transcription factor and growth factor, has been identified recently as a cytokine mediator of lethal systemic inflammation (e.g., endotoxemia and sepsis), arthritis, and local inflammation (e.g., hepatic injury after I/R and LPS-induced acute lung injury) [5,14,16,28,29]. In response to exogenous or endogenous inflammatory stimuli (such as bacterial endotoxin, LPS, TNF-α, IL-1, or IFN-γ), monocytes/macrophages and pituicytes actively release HMGB1 [5,18,19,30]. In addition, HMGB1 is released passively from necrotic or damaged cells [9] and triggers an inflammatory response to injury. Extracellular HMGB1 can activate macrophages, monocytes, DC, and T cells and mediates a rigorous innate and adaptive inflammatory response to infection and injury [11-13,31].

Although various HMGB1-inducing, inflammatory stimuli (such as LPS, TNF-α, and IFN-γ) can also stimulate macrophages and monocytes to produce oxidative-free radicals, the potential role of oxidative stress (such as H_2O_2) in the regulation of HMGB1 release was unknown previously. Consistent with the notion that LPS and hypoxia up-regulate HMGB1 expression in vivo [29,32], we found that H_2O_2, at non- to low toxic doses (e.g., 0.0125–0.25 mM), similarly increases cellular HMGB1 expression levels in macrophage cultures. It is currently unknown whether up-regulation of HMGB1 expression influences its subsequent release in response to oxidative stress.

It is more important that we demonstrated that oxidative stress could effectively stimulate macrophage/monocyte cell cultures to actively secrete HMGB1 at nontoxic doses (e.g., 0.0125–0.125 mM). Consistently, H_2O_2 induces dramatic cytoplasmic translocation of HMGB1 in macrophage and monocyte cell cultures. At higher concentrations (e.g., 0.25 mM),

however, H_2O_2 exhibited a dose-dependent cytotoxicity and consequently, triggered a more robust release of HMGB1 as a possible result of active secretion from activated cells and passive leakage from injured cells. It is notable that a brief exposure with H_2O_2 was enough to induce active HMGB1 release, implicating a potential involvement of early signaling molecules (such as MAPKs) in the regulation of H_2O_2-induced HMGB1 release.

Various MAPKs play important roles in physiological and pathological events, including cell-cycle regulation, proliferation, apoptosis, and inflammation [2,33]. We observed that specific inhibitors for JNK (SP600125) [23] and MEK (PD98059) [34], but not for p38 MAPK (SB203580) [35], almost completely abrogated H_2O_2-induced, active HMGB1 release. The suppression of H_2O_2-induced HMGB1 release by JNK and MEK inhibitors was not a result of their cell toxicity, as at concentrations effectively abrogating H_2O_2-induced HMGB1 release, these inhibitors did not affect cell viability of macrophage cultures. Taken together, our experimental results support a potential role for JNK and ERK (but not p38) MAPKs in the regulation of H_2O_2-induced, HMGB1 active release.

The mechanisms underlying regulation of active HMGB1 release are complex and still remain elusive. For instance, early studies indicate that HMGB1 is secreted by macro-phages/monocytes via a nonclassical, vesicle-mediated, secre-tory pathway [22,36], but the identity of the HMGB1-containing vesicles is not yet fully characterized. Although ERK MAPK is not important in the regulation of endotoxin- or IFN-γ-mediated HMGB1 release [18,20], it may be important in H_2O_2-induced HMGB1 translocation and release. It is thus plausible that macrophages/monocytes use distinct signaling pathways to trigger HMGB1 release in response to different inflammatory stimuli.

The CRM1 protein is a leucine-rich nuclear export signal receptor involved in export of nuclear proteins [37] such as HMGB1 [22]. In the present study, we demonstrated that H_2O_2-induced HMGB1 release is accompanied by an increase in CRM1-HMGB1 interaction. In light of the important role of CRM1 in export of nuclear RNA and proteins [38], it will be important to determine whether oxidative stress induces active HMGB1 release through a CRM1-dependent mechanism in future studies.

In summary, we demonstrated here that oxidative stress such as H_2O_2 induces active and/or passive HMGB1 release from macrophage and monocyte cultures in a time- and dose-dependent manner. In view of the observations that specific inhibitors for JNK and ERK (but not p38) abrogated H_2O_2-induced HMGB1 release and that H_2O_2 enhanced CRM1-HMGB1 interaction significantly, we propose that oxidative stress induces active HMGB1 release in a MAPK and CRM1-dependent mechanism. Thus, it may be important to explore the therapeutic potential of many antioxidant agents in the treatment of various inflammatory diseases.

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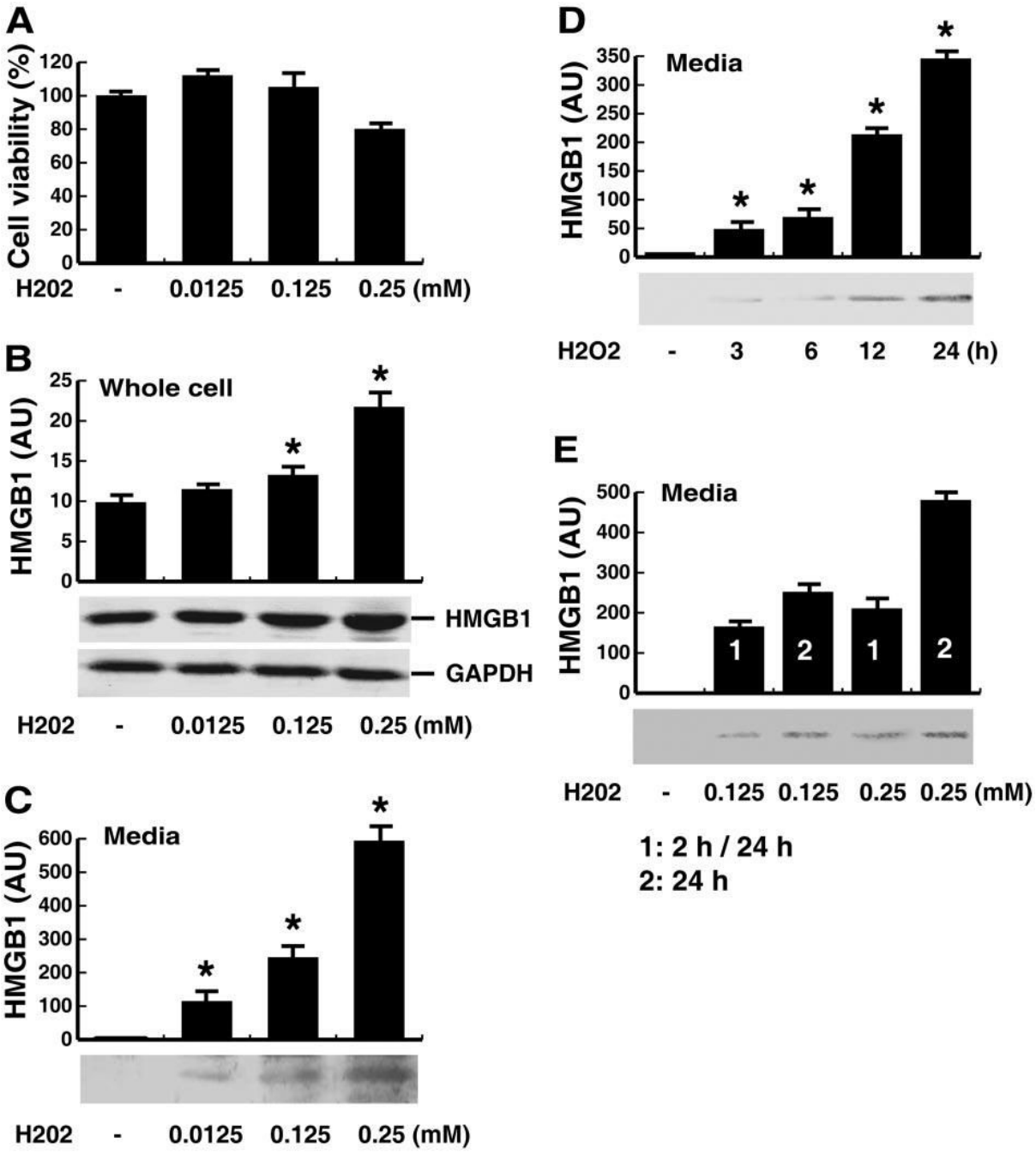

Fig. 1.

Effects of H_2O_2 on the release of HMGB1 in macrophage cultures. (A) RAW 264.7 macrophages were stimulated with H_2O_2 at indicated doses for 24 h, and cell viability was determined by MTT assay and expressed as mean ± SEM of four experiments in duplicate. In parallel experiments, HMGB1 levels in the whole-cell lysate (B) or culture medium (C–E) were determined by the relative optical intensity [in arbitrary units (AU)] of the immunoreactive bands on Western blots and expressed as mean ±SEM of three experiments in duplicate. *, Statistically significant versus control group; $P < 0.05$. (D) Levels of HMGB1 in the culture medium at various time-points following stimulation with H_2O_2 at a nontoxic dose (0.125 mM). (E) Levels of HMGB1 in the culture medium at 24 h after the onset of a brief (for

2 h, after which H_2O_2 was washed out; marked as “1” or “2 h/24 h”) or a persistent (for 24 h; marked as “2” or “24 h”) exposure to H_2O_2 at indicated concentrations.

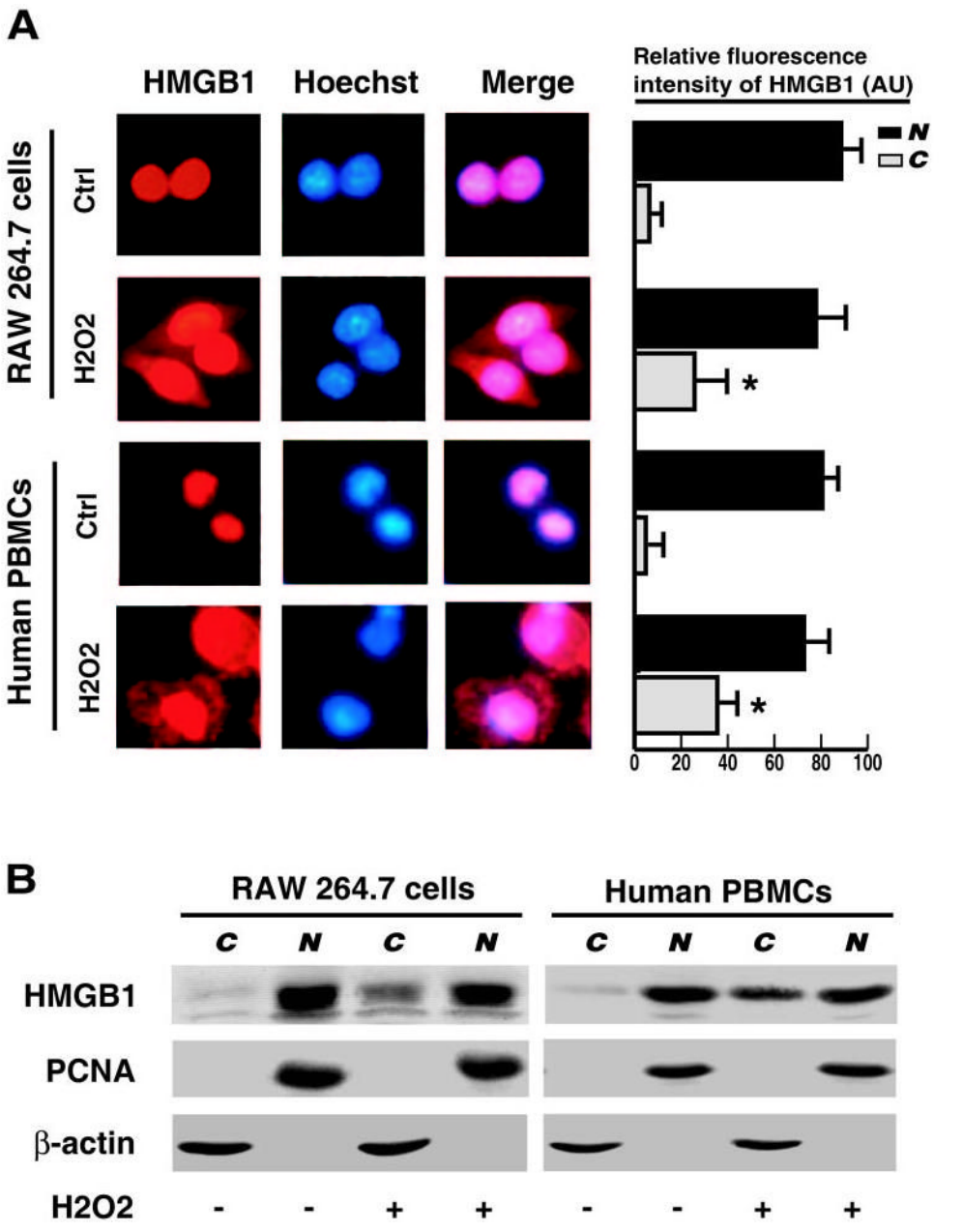

Fig. 2.

Effects of H_2O_2 on HMGB1 cytoplasmic translocation in macrophage and monocyte cultures. RAW 264.7 macrophages and human PBMCs were stimulated with H_2O_2 at a nontoxic concentration (0.125 mM) and monitored for HMGB1 cytoplasmic translocation by immunocytochemistry (A) or cell fractionation/Western blot (B) at 12 h poststimulation (A). The relative fluorescence intensity in the nuclear ("N") or cytoplasmic ("C") regions of multiple representative cells was determined using the ImageProPlus software and expressed as mean ± SEM (in arbitrary units) of three independent experiments. Red, HMGB1; blue, nuclei; pink, Merge (original magnification, ×400). (B) Following cell fractionation, HMGB1 content in the cytoplasmic ("C") or nuclear ("N") fraction was determined by Western blotting analysis. Equal loading of samples was confirmed by Western blotting analysis of each fraction with

antibodies specific for a nuclear (PCNA, BD Biosciences) or cytoplasmic (β-actin, KangChen Biotechnology) protein. Blots are representative of three independent experiments with similar results.

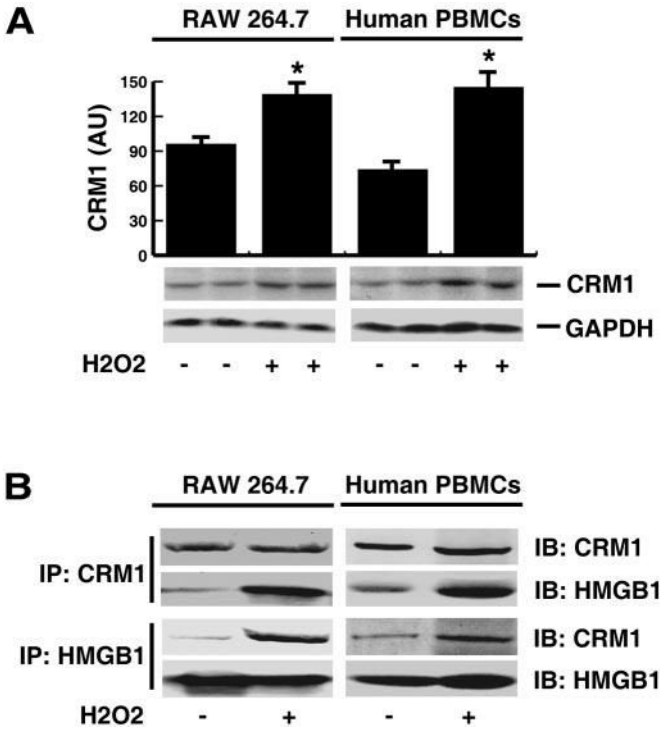

Fig. 3.

Effects of H_2O_2 on CRM1 expression and interaction with HMGB1 in macrophage and monocytes cultures. (A) RAW 264.7 macrophages and human PBMCs were stimulated with H_2O_2 at a nontoxic concentration (0.125 mM) for 24 h, and whole-cell lysate assayed CRM1 content by Western blotting analysis. Shown in the bar graph was the relative optical intensity (in arbitrary units) of the CRM1-immunoreactive band. A cytoplasmic housekeeping protein, GAPDH, was used as a loading control. In parallel experiments, nuclear fractions were isolated and immunoprecipitated ("IP") with CRM1- or HMGB1-specific antibodies. The precipitated proteins were subsequently immunoblotted ("IB") with CRM1- or HMGB1-specific antibodies. Blot shown is representative of three experiments with similar results.

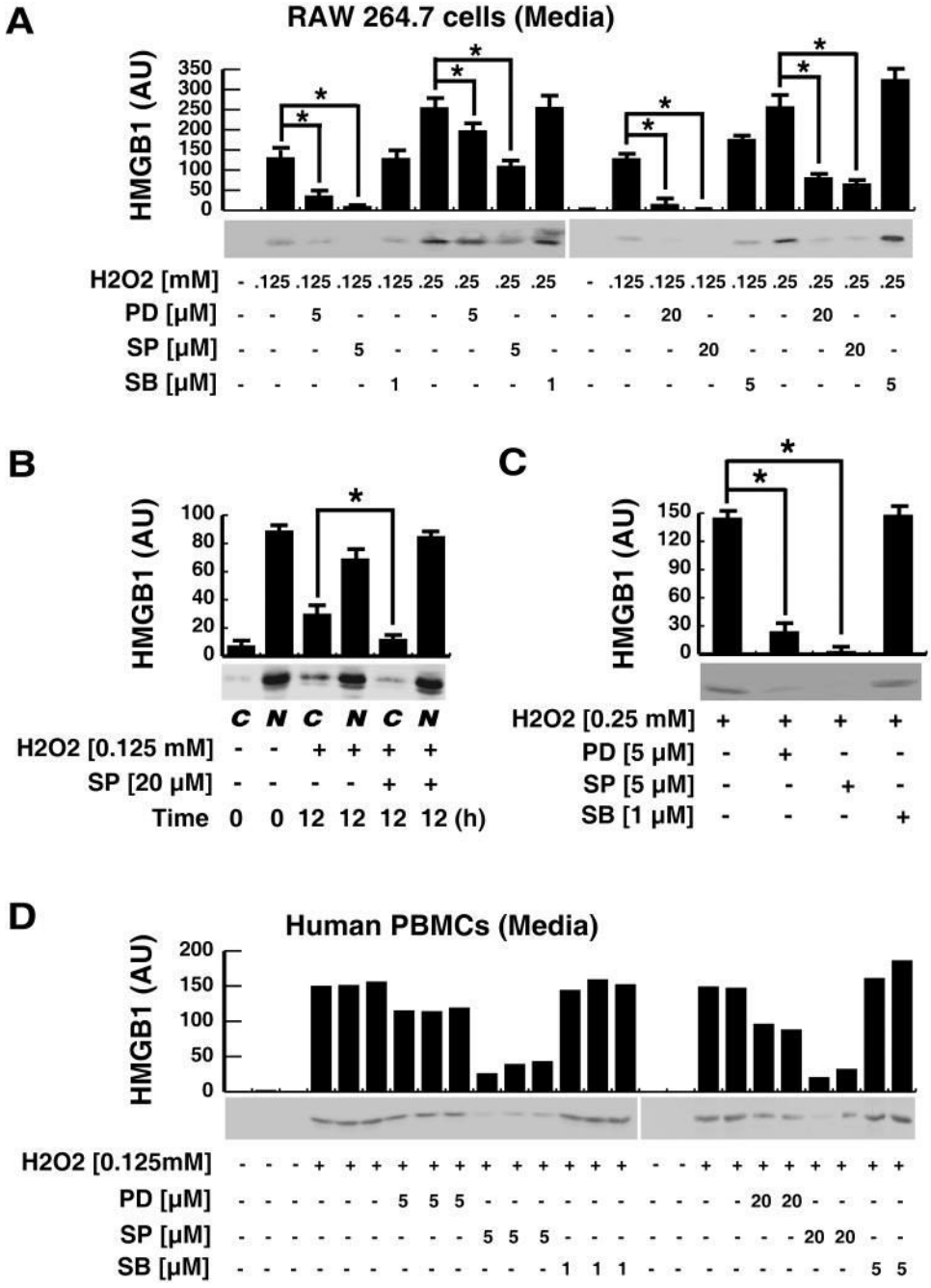

Fig. 4.

Effects of MAPK inhibitors on H_2O_2-induced HMGB1 translocation and release in macrophage and monocyte cultures. (A, B) RAW 264.7 macrophages were pretreated with various MAPK inhibitors [SB203580 (SB), SP600125 (SP), PD98059 (PD)] at indicated concentrations for 30 min before stimulation with H_2O_2 at indicated doses. At 12 h post-H_2O_2 stimulation, levels of HMGB1 in the culture medium (A) or cellular cytoplasmic or nuclear fractions (B) were determined by Western blotting analysis, and the relative optical intensity of HMGB1-immunoreactive band (in arbitrary units) was expressed as mean ± SEM of three experiments in duplicate. *, $P < 0.05$. (C) RAW 264.7 macrophages were pretreated with various MAPK inhibitors for 30 min before exposure to 0.25 mM H_2O_2. At 2 h post-H_2O_2

stimulation, H_2O_2 was washed out, and HMGB1 levels in the culture medium were determined at 24 h after the onset of H_2O_2 exposure. The relative optical intensity of HMGB1-immunoreactive band (in arbitrary units) was expressed as mean ± SEM of three experiments in duplicate. *, $P < 0.05$. (D) Human PBMCs ($n = 5$ per group) were similarly pretreated with various MAPK inhibitors (SB203580, SP600125, PD98059), at indicated concentrations for 30 min before addition of H_2O_2 (0.125 mM). At 6 h post-H_2O_2 stimulation, the levels of HMGB1 in the culture medium were assayed by Western blotting analysis and expressed as the relative optical intensity of a HMGB1-immunoreactive band (in arbitrary units). Blots are representative of three independent experiments with similar results.