Biochemical and Biophysical Research Communications 438 (2013) 732–738

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# HMGB1 induces an inflammatory response in endothelial cells via the RAGE-dependent endoplasmic reticulum stress pathway ☆

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ARTICLE INFO

*Article history:*
Received 19 July 2013
Available online 31 July 2013

*Keywords:*
HMGB1
PERK
IRE1
RAGE
ICAM-1
P-selectin

ABSTRACT

The high mobility group 1B protein (HMGB1) mediates chronic inflammatory responses in endothelial cells, which play a critical role in atherosclerosis. However, the underlying mechanism is unknown. The goal of our study was to identify the effects of HMGB1 on the RAGE-induced inflammatory response in endothelial cells and test the possible involvement of the endoplasmic reticulum stress pathway. Our results showed that incubation of endothelial cells with HMGB1 (0.01–1 μg/ml) for 24 h induced a dose-dependent activation of endoplasmic reticulum stress transducers, as assessed by PERK and IRE1 protein expression. Moreover, HMGB1 also promoted nuclear translocation of ATF6. HMGB1-mediated ICAM-1 and P-selectin production was dramatically suppressed by PERK siRNA or IRE1 siRNA. However, non-targeting siRNA had no such effects. HMGB1-induced increases in ICAM-1 and P-selectin expression were also inhibited by a specific eIF2α inhibitor (salubrinal) and a specific JNK inhibitor (SP600125). Importantly, a blocking antibody specifically targeted against RAGE (anti-RAGE antibody) decreased ICAM-1, P-selectin and endoplasmic reticulum stress molecule (PERK, eIF2α, IRE1 and JNK) protein expression levels. Collectively, these novel findings suggest that HMGB1 promotes an inflammatory response by inducing the expression of ICAM-1 and P-selectin via RAGE-mediated stimulation of the endoplasmic reticulum stress pathway.



## 1. Introduction

The development of atherosclerosis is strongly associated with the production and secretion of pro-inflammatory cytokines and chemokines, such as P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), by endothelial cells (ECs), leading to monocyte recruitment and accumulation in the subendothelial spaces of the arteries [1,2].

High mobility group 1B protein (HMGB1), a 30 kDa nuclear protein, can be released by inflammatory cells [3] or necrotic cells [4] to trigger inflammation. Multiple lines of evidence support a role for HMGB1 in atherosclerotic inflammation. These include the following findings: (1) HMGB1 serum levels are higher in patients with acute MI than in controls and are elevated in animal models of ischemia–reperfusion heart injury [5]. (2) HMGB1 is also highly expressed in endothelial cells in atherosclerotic lesions [3]. (3) Administration of neutralizing monoclonal antibodies against HMGB1 attenuates atherosclerosis by 55% in ApoE−/− mice and leads to reductions in the expression of VCAM-1 and MCP-1 [6]. (4) Extracellular HMGB1 is capable of interacting with the receptor for advanced glycation end products (RAGE) and/or toll-like receptors on the surface of inflammatory cells, which activate inflammatory pathways and trigger a cascade of pro-inflammatory cytokines, including ICAM-1 and P-selectin [7,8]. These findings suggest that HMGB1 may be a crucial member of the uncontrolled pro-inflammatory response associated with fatal outcomes. However, the details of the mechanisms regulating HMGB1-induced inflammation are poorly understood. The endoplasmic reticulum (ER) is an organelle that plays an essential role in multiple cellular processes, such as the folding of secretory and membrane proteins, calcium homeostasis, and lipid biosynthesis [9]. A variety of insults can interfere with ER function, thereby triggering the unfolded protein response (UPR) to cope with the resulting ER stress [10]. The



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http://dx.doi.org/10.1016/j.bbrc.2013.07.098

UPR is triggered by 3 upstream proteins: inositol requiring enzyme (IRE) 1, activating transcription factor (ATF) 6, and PERK (an RNA-dependent protein kinase, also known as endoplasmic reticulum kinase). Several recent studies have revealed that ER stress may mediate the inflammatory response. It has been reported that oxidized 1-palmitoyl-2-arachidonyl-sn-3-glycero-phosphorylcholine (oxPAPC) accumulates in atherosclerotic lesions and leads to endoplasmic reticulum stress and UPR activation in human aortic endothelial cells [11]. These studies have further demonstrated that the UPR pathway is a general mediator of vascular inflammation and endothelial cell dysfunction in atherosclerosis and likely other inflammatory disorders. More recently, Gora et al. demonstrated that phospholipolyzed LDL induces an inflammatory response in endothelial cells through endoplasmic reticulum stress signaling [12]. We therefore postulated that ER stress may mediate the inflammatory responses induced by HMGB1, and we further investigated the details of this mechanism.

## 2. Materials and methods

### 2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Hyclone. Recombinant HMGB-1 was purchased from R&D Systems. PERK and ATF-6 antibodies were purchased from Abgent. IRE1 antibodies were purchased from Abcam. JNK, ICAM-1 and P-selectin primary antibodies and IgG were purchased from Santa Cruz Biotechnology. The anti-RAGE antibody was purchased from Biovision. The antibodies for eIF2α, p-eIF2α, and β-actin were purchased from Cell Signaling Technology. ELISA kits for measurement of sICAM-1 and P-selectin levels were obtained from Senxiong Biological Limited Corporation (Shanghai, China). All other biochemicals used were of the highest purity available.

### 2.2. Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) were originally purchased from ATCC. HUVECs were cultured according to standard procedure, as previously described [13]. Cells were seeded at a density of $3 \times 10^5$ per 100 mm dish in DMEM supplemented with 20 mM HEPES and 10% FBS. The cultures were maintained at 37 °C with a gas mixture of 5% $CO_2$/95% air. The medium was supplemented with 5 U/ml heparin, 100 IU/ml penicillin and 100 μg/ml streptomycin. Endothelial cells of the forth to sixth passages in the actively growing condition were used for the experiments.

The PERK and IRE1 siRNAs were synthesized by Ambion. Their targeting sequences were as follows: PERK (NM_004836, EIF2AK3, targeting sequence: sense GCAUGCAGUCUCAGACCCAtt and antisense UGGGUCUGAGACUGCAUGCtt); IRE1 (NM_001433, ERN1, targeting sequence: sense GAUGUCCCACUUUGUGUCCtt and antisense GGACACAAAGUGGGACAUCtt). A non-targeting siRNA (also purchased from Ambion) was used as a negative control in our experiments. As previously described [14], HUVECs grown to 60–70% confluence were transfected with the GeneSilencer transfect ion reagent plus IRE1 siRNA, PERK siRNA or control non-targeting siRNA in FBS-free DMEM medium, according to the manufacturer's instructions. Four hours after transfection, fresh DMEM medium supplemented with 5% FBS and without endothelial growth supplement was added, and the cells were cultured in the presence or absence of HMGB1 for an additional 24 h. The infection efficiency was evaluated by IRE1 or PERK protein expression using Western blot analysis.

### 2.3. Experimental protocol

First, to investigate the effect of HMGB1 on ER stress in endothelial cells, the endothelial cells were exposed to HMGB1 (0, 0.01, 0.1, 1 and 10 μg/ml) for 24 h, and the expression of PERK and IRE1 or nuclear transfer of ATF6 was subsequently measured.

Second, in the following parallel studies, to further determine the critical effect of the ER stress pathway in HMGB1-induced ICAM-1 and P-selectin expression, PERK or IRE1 siRNA was used. Otherwise, to further examine the role of the RAGE/ER stress pathway in HMGB1-induced inflammation, endothelial cells were pretreated for 30 min with an anti-RAGE Ab (20 μg/ml), IgG Ab (100 μg/ml), Salubrinal (100 μM), or SP600125 (10 μM) before being exposed to Ang II. All incubation concentrations and durations listed above were chosen on the basis of published data and modified based on pilot experiments.

### 2.4. Western blot analysis

The cells were lysed for 30 min at 4 °C in a lysis buffer. Total cell protein concentration was determined using the bicinchoninic acid reagent. Total protein (50–100 μg) was resolved by SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis. Primary antibodies for PERK (1:1000), eIF2α (1:1000), p-eIF2α (1:1000), IRE1 (1:1000), JNK (1:500), P-selectin (1:3000), ICAM-1 (1:3000) or β-actin (1:5000) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) were used. The bands were visualized using enhanced chemiluminescence reagents and analyzed using a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst version 1.1). The results are representative of six independent experiments.

### 2.5. Enzyme-linked immunosorbent assay (ELISA) for P-selectin and sICAM-1

The levels of P-selectin and sICAM-1 in the endothelial cell-conditioned medium were measured by ELISA. The measurements of P-selectin and sICAM-1 were performed step-by-step based on the protocol booklets provided with the ELISA kits (Senxiong Biotech Co., Ltd., China).

### 2.6. Measurement of intracellular ATF6

The cells were fixed in 4% paraformaldehyde for 15 min and then permeabilized with 0.5% Triton X-100 for 15 min at room temperature. After incubation with the ATF6 antibody and then with a Dylight fluor 594 secondary antibody (EarthOx), the cells were observed under a fluorescence microscope (Olympus, Japan).

### 2.7. Statistical analysis

The results are expressed as the means ± SEM. Data were analyzed by ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. The significance level was set at $P < 0.05$.

## 3. Results

### 3.1. HMGB1 induces the activation of ER stress in endothelial cells

ER stress is characterized by the activation of ER stress sensors, leading to UPR induction and the expression of ER resident proteins bearing the unique C-terminal sequence KDEL. Incubation of endothelial cells with HMGB1 (0, 0.01, 0.1, or 1 μg/ml) for 24 h induced dose-dependent activation of the ER stress transducers,

as assessed by PERK and IRE1 expression (Fig. 1A and B). Moreover, the presence of ATF6 in the nucleus of cells treated with HMGB1 is indicative of the cleavage of ATF6 by site 1 and site 2 proteases, allowing the release of its cytosolic domain and prompting nuclear translocation (Fig. 1C). Moreover, a higher concentration of HMGB1 (10 μg/ml) was observed to cause less PERK and IRE1 protein expression. Based on these findings and those of previous studies, we selected the optimal concentration of HMGB1 (1 μg/ml) for use in subsequent experiments.

### 3.2. ER stress is involved in HMGB1-induced inflammation in endothelial cells

HMBG1 is known to mediate inflammation in endothelial cells. However, the mechanism is poorly understood. In the present study, we expected HMGB1-induced inflammation, including the increases ICAM-1 and P-selectin expression, to be activated by the ER stress pathway. To elucidate the contribution of ER stress to HMGB1-induced ICAM-1 and P-selectin up-regulation, we first examined whether HMGB1 induced ER stress in endothelial cells. As shown in Fig. 1, exposure of endothelial cells to HMGB1 (0, 0.01, 0.1, or 1 μg/ml) for 24 h induced dose-dependent activation of ER stress, as assessed by PERK and IRE1 expression. To confirm the critical role of ER stress, we conducted experiments using knockdown methodology. As shown in Fig. 2A and C, PERK gene silencing by PERK siRNA markedly reduced HMGB1-induced ICAM-1 and P-selectin expression, as determined by Western blotting and ELISA, respectively, while the non-targeting siRNA had no effect. PERK siRNA or non-targeting siRNA itself had no effect on the expression of adhesion molecules. HMGB1-induced increases in ICAM-1 and P-selectin expression (assessed by Western blotting and ELISA) were also inhibited in cells treated with siRNA specific for IRE1 (Fig. 2B and D). IRE1 siRNA or non-targeting siRNA alone had no effect on the expression of adhesion molecules.

ER stress signaling is often referred to as the unfolded protein response (UPR). PERK, phosphorylates eIF2α, and IRE1 promotes the expression of JNK; these changes slow protein translation, allowing perturbations in protein translation to be corrected in an optimal manner. To further confirm whether HMGB1 up-regulates ICAM-1 and P-selectin via the unfolded protein response, the specific eIF2α inhibitor salubrinal and the specific JNK inhibitor SP600125 were used. As shown in Fig. 3, treatment of the cells with either salubrinal (100 μM) or SP600125 (10 μM) significantly abrogated the HMGB1-induced increases in ICAM-1 and P-selectin (assessed by Western blotting and ELISA). Salubrinal (100 μM) or SP600125 (10 μM) alone had no effect on the expression of these adhesion molecules.

### 3.3. The role of RAGE in HMGB-induced inflammation in endothelial cells

To further determine the relationship between RAGE and the ER stress pathway in mediating HMGB1-induced ICAM-1 and P-selectin expression in endothelial cells, we used a specific blocking antibody targeted against RAGE (anti-RAGE antibody). As shown in Fig. 4, treatment with the anti-RAGE antibody (20 μg/ml) markedly suppressed HMGB1-induced expression of the ER sensors PERK and IRE1 and subsequent signaling (as read out by eukaryotic initiation factor 2α phosphorylation and increased expression of JNK). Also, the increases in the expression of ICAM-1 and P-selectin induced by HMGB1 were also inhibited by the anti-RAGE antibody (20 μg/ml). Treatment with a non-specific IgG (60 μg/ml) had no effect on these HMGB1-induced increases in protein expression.

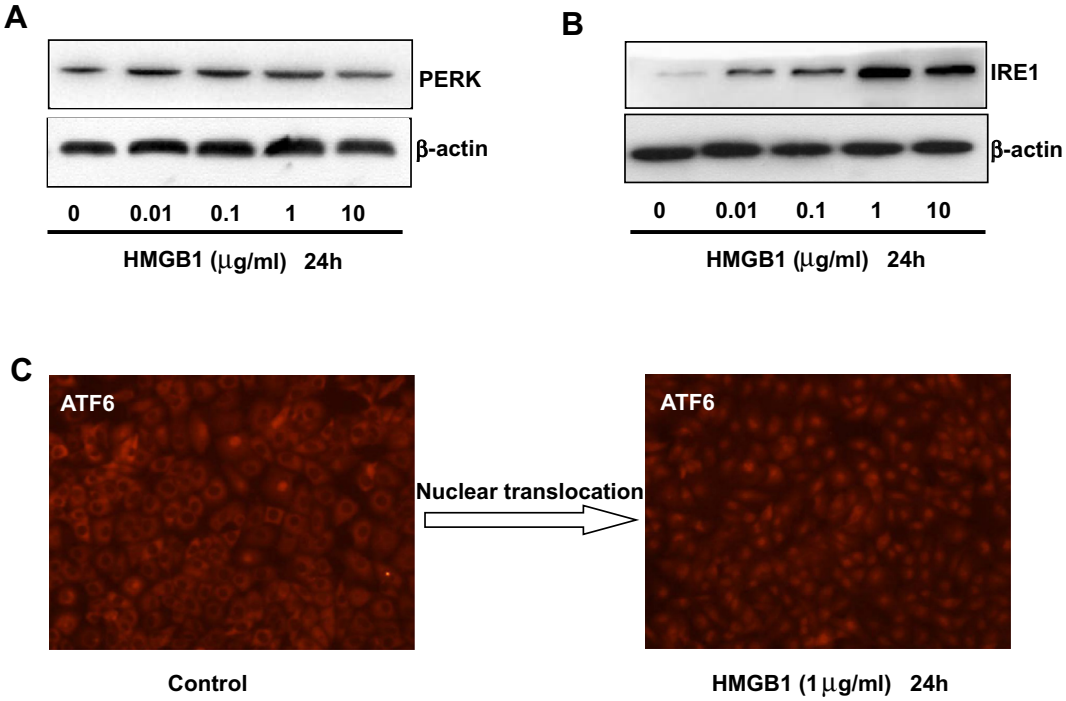



**Fig. 1.** HMGB1 triggers ER stress activation in endothelial cells. Panels A and B, the dose response of the ER stress sensors PERK and IRE1 in endothelial cells treated with HMGB1 was measured. The endothelial cells were exposed to HMGB1 at various concentrations (0, 0.01, 0.1, 1 or 10 μg/ml) for 24 h. HMGB1 at concentrations of 0.01–1 μg/ml can induce ER stress, including PERK and IRE1 protein expression, in a dose-dependent manner. However, higher concentrations of HMGB1 (10 μg/ml) were noted to cause less protein expression. Panel C, immunocytochemistry experiments showing the nuclear translocation of ATF6 after 24 h of treatment with HMGB1 (1 μg/ml). These data are representative of 6 separate experiments.

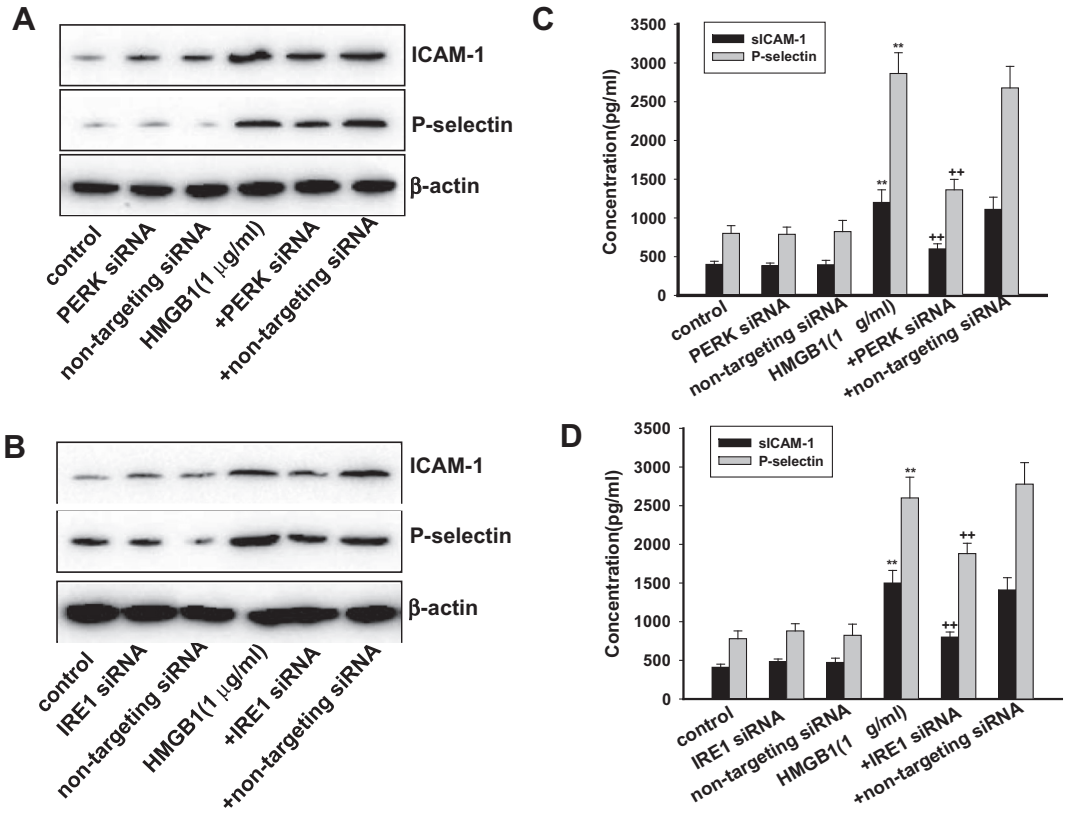



**Fig. 2.** The role of the ER stress pathway in HMGB1-induced inflammation in endothelial cells. To determine the critical roles of the ER stress sensors PERK and IRE1 in HMGB1-induced inflammation in endothelial cells, gene silencing technology was used. Panel A and C shows the inhibitory effect of PERK siRNA on the HMGB1-induced increases in ICAM-1 and P-selectin generation (as shown by Western blotting and ELISA). Non-targeting siRNA and PERK siRNA alone had no effect. In keeping with the above results, IRE1 siRNA also markedly reduces the ICAM-1 and P-selectin expression activated by HMGB1 (as shown by Western blotting and ELISA). IRE1 siRNA alone also had no effect. The data are expressed as the means ± SEM, $n = 6$ each, and the tests were performed in triplicate. Compared with the control, $^{**}P < 0.01$. Compared with HMGB1 (1 μg/ml), $^{++}P < 0.01$.

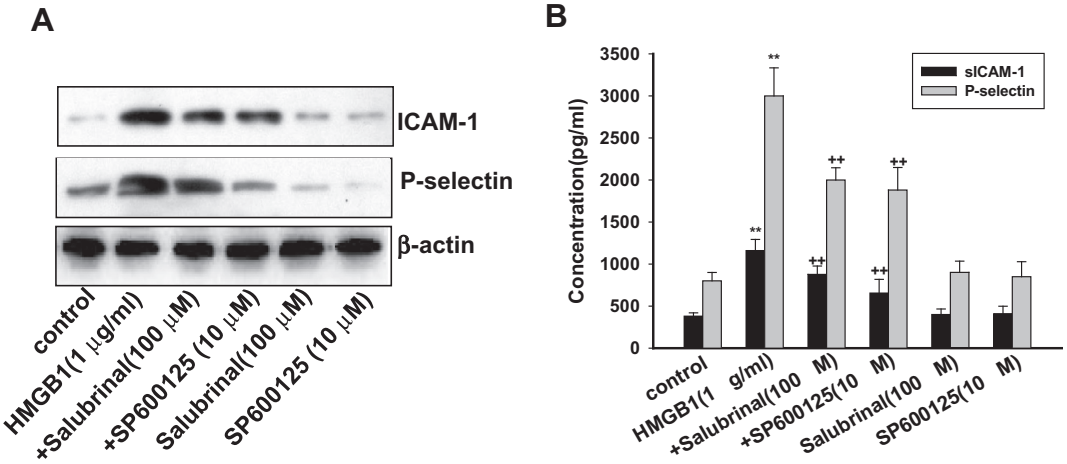



**Fig. 3.** The role of the unfolded protein response in HMGB1-induced inflammation in endothelial cells. ER stress signaling is often referred to as the unfolded protein response (UPR). To further elucidate whether HMGB1 up-regulates ICAM-1 and P-selectin via the unfolded protein response, the specific eIF2α inhibitor salubrinal and the specific JNK inhibitor SP600125 were used. Representative inhibitory effects of salubrinal (the specific eIF2α inhibitor) and SP600125 (JNK specific inhibitor) on HMGB1-induced ICAM-1 and P-selectin protein expression were measured by Western blot (A) and ELISA (B). The data are expressed as the means ± SEM, $n = 6$ each, and the tests were performed in triplicate. Compared with the control, $^{**}P < 0.01$. Compared with HMGB1 (1 μg/ml), $^{++}P < 0.01$.

## 4. Discussion

The present study had the following major findings: (1) this study, for the first time (to our knowledge), determined that exposing endothelial cells to HMGB1 results in the induction of endoplasmic reticulum stress and that 2 key ER stress sensors, PERK and IRE1, directly participated in modulating the inflammatory responses important for atherosclerosis; (2) the endoplasmic reticulum stress induced by HMGB1 was RAGE dependent.

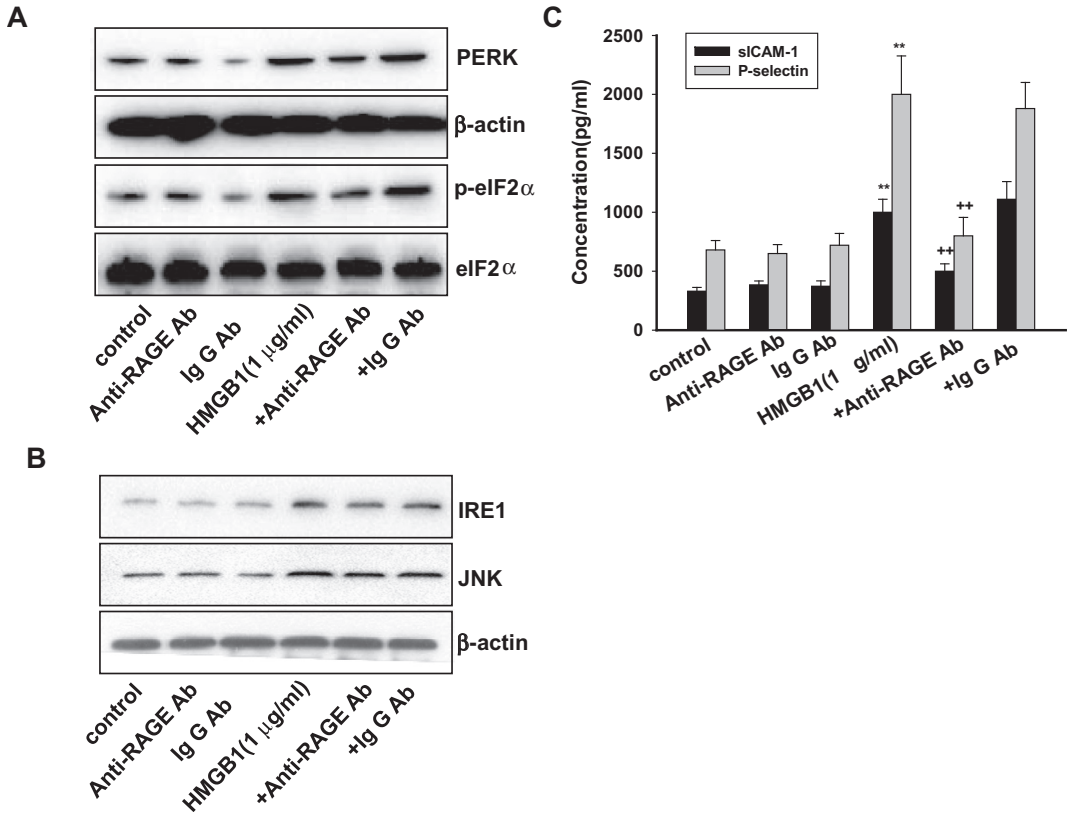
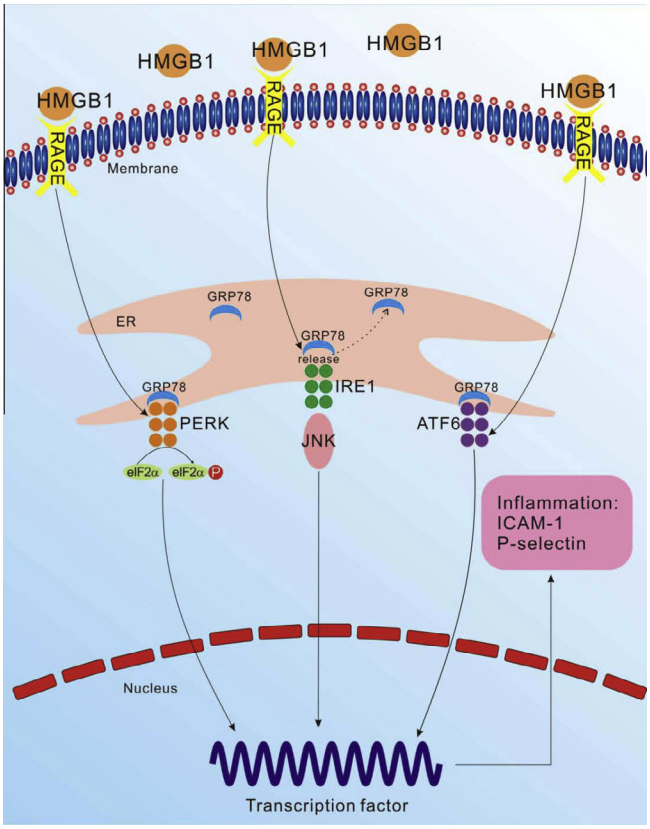



**Fig. 4.** HMGB1-triggered ER stress and inflammation in endothelial cells is RAGE dependent. A: Protein expression of PERK, eIF2α, p-eIF2α and β-actin by Western blot. B: Protein expression of IRE1, JNK and β-actin by Western blot. C: sICAM-1 and P-selectin levels in supernatant by ELISA. Control: wild-type cells. Anti-RAGE Ab: wild-type cells treated with 20 μg/ml anti-RAGE antibody alone for 24 h. IgG Ab: wild-type cells treated with 60 μg/ml IgG antibody alone for 24 h. HMGB1: wild-type cells treated with 1 μg/ml HMGB1 for 24 h. +Anti-RAGE Ab: cells were pretreated with 20 μg/ml anti-RAGE antibody (RAGE blocker) for 1 h and then exposed to HMGB1 (1 μg/ml) for 24 h. +IgG Ab: cells were pretreated with 60 μg/ml IgG antibody for 1 h and then exposed to HMGB1 (1 μg/ml) for 24 h. The data are expressed as the means ± SEM, $n = 6$ each, and the tests were performed in triplicate. Compared with the control, $^{**}P < 0.01$. Compared with HMGB1 (1 μg/ml), $^{++}P < 0.01$.

It was well known that atherosclerosis is a chronic inflammatory disorder [15]. Enhanced adhesion of monocytes to the endothelium in the arterial wall is one of the earliest signs of atherogenesis. P-selectin is thought to mediate monocyte attachment to and rolling along the activated vascular endothelium, and ICAM-1 is believed to be associated with the adhesion and transendothelial migration of monocytes [16]. It has been shown that the levels of soluble forms of P-selectin and sICAM-1 are elevated in hypercholesterolemic humans and in cultured endothelial cells [17]. Increased expression of P-selectin and ICAM-1 has been observed in atherosclerotic plaques from animals and humans. The severity of atherosclerosis in hypercholesterolemic mice is markedly decreased by blocking ICAM-1 action using specific antibodies [18]. ICAM-1 knockout results in the attenuation of the atherosclerotic process in ApoE knockout mice [19]. Administering an anti-P-selectin antibody decreases leukocyte rolling and attachment to vascular endothelium [20], and P-selectin-deficient mice completely lack leukocyte rolling and show reduced atherosclerotic lesion formation [21]. These observations collectively suggest that adhesion molecules, such as P-selectin and ICAM-1, play important roles in atherosclerosis. The available evidence suggests that HMGB1 plays a pivotal role in inflammation and atherosclerosis and that it is a key target for the suppression of vascular inflammation and atherosclerosis. In addition, extracellular HMGB1 activates inflammatory pathways and triggers a cascade of pro-inflammatory cytokines, including ICAM-1 and P-selectin, in endothelial cells [22]. However, the detailed molecular mechanisms are poorly understood.

In the present study, we provide evidence that HMGB1 can induce ICAM-1 and P-selection expression in endothelial cells by activating the endoplasmic reticulum stress pathway. Recent studies have shown that ER stress, with the concomitant activation of the UPR, is associated with a number of pathological conditions; it has been linked to major cardiovascular events, including myocardial ischemia, heart failure [23], and accelerated atherosclerosis [24]. ER stress markers are markedly increased in advanced atherosclerotic lesions in ApoE−/− mice [24] and in humans, whereas their expression is weak in stable plaques and absent in control mammary arteries. ER stress signaling, often referred to as the unfolded protein response, is triggered by 3 upstream proteins: IRE1, ATF6, and PERK [25]. IRE1 promotes the expression of JNK, which is closely associated with the inflammatory response [26]. PERK, by phosphorylating eIF2α (eukaryotic initiation factor 2α), temporally slows protein translation, allowing perturbations in protein translation to be corrected in an optimal manner [27], and ATF6 plays key role in chaperone production. Under normal circumstances, these three transmembrane sensors constitutively bind to chaperone GRP78 in an inactive form. Under ER stress, when unfolded proteins accumulate in the lumen of the ER, GRP78 is released from the UPR sensors, leading to their activation [28]. The results from the present study showed that exogenous HMGB1 (0.01–1 μg/ml) can activate PERK and IRE1 protein expression in a dose-dependent manner in endothelial cells. Moreover, the detection of ATF6 in the nuclei of cells treated with HMGB1 is indicative of the cleavage of ATF6 by site 1 and site 2 proteases, allowing the release of its cytosolic domain and its nuclear translocation. These results demonstrate that exogenous HMGB1 can trigger ER stress. Additionally, we demonstrated that the activation of PERK and IRE1 is necessary for ICAM-1 and P-selectin in endothelial cells induced by HMGB1,




**Scheme 1.** Potential mechanisms of HMGB1-induced ER stress and inflammation in endothelial cells.

because PERK siRNA or IRE siRNA significantly attenuated HMGB1-induced ICAM-1 and P-selectin (as assessed by Western blotting and ELISA). To confirm that HMGB1 up-regulates ICAM-1 and P-selectin production, we investigated the influence of inhibiting their downstream targets (eIF2α and JNK) using the specific eIF2α inhibitor salubrinal and the specific JNK inhibitor SP600125. As expected, salubrinal or SP600125 markedly reduced HMGB1-induced ICAM-1 and P-selectin production in endothelial cells. These data demonstrate that ER stress plays a critical role in HMGB1-induced inflammatory responses in endothelial cells. However, the as yet unknown mechanism by which ER stress regulates the HMGB1-triggered inflammation response deserves further investigation in the future.

RAGE is a transmembrane protein that belongs to the immunoglobulin superfamily of cell surface receptors and has been isolated as a receptor for HMGB1. RAGE signaling accounts for both the physiological and pathophysiological consequences of HMGB1/cell surface interactions [29]. In keeping with our hypothesis, extracellular HMGB1 regulates cells through RAGE, which is by far the most widely studied HMGB1 receptor at the cell surface. This study confirms previous observations that HMGB1 induces the inflammatory response in endothelial cells in a manner that is mediated by RAGE activation. This conclusion is based on our observation that pretreatment of HUVECs with a RAGE blocking anti-RAGE antibody markedly suppressed HMGB1-induced ICAM-1 and P-selectin production, while pretreatment with a negative control IgG antibody had no effect on the HMGB1-induced inflammatory response. Moreover, we postulated that HMGB1 might trigger ER stress via the RAGE pathway. Indeed, our study provides evidence of this process. In the present study, we demonstrated that the anti-RAGE antibody markedly attenuates HMGB1-induced PERK or IRE1 protein expression and their subsequent signaling (through eIF2α or JNK).

In conclusion, the present study indicates that HMGB1 promotes inflammatory responses by inducing the expression of ICAM-1 and P-selectin via RAGE-mediated stimulation of the ER stress pathway. A schematic illustration of the proposed pathway is shown in Scheme 1. These findings may explain the important mechanism through which HMGB1 causes inflammatory responses and provide a novel approach for preventing atherosclerosis.

## Funding sources

This study was supported by a grant from the Natural Science Foundation of China (No. 81172558).

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