HMGB1 affects the development of pulmonary arterial hypertension via RAGE

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Abstract. – OBJECTIVE: To investigate the effect of high-mobility group box 1 (HMGB1) on the proliferation, migration and inflammatory response of human pulmonary artery smooth muscle cells (HPASMCs) and human pulmonary artery endothelial cells (HPAECs) through the receptor for advanced glycation end products (RAGE) and to investigate the mechanism of action underlying the effect of HMGB1 on pulmonary arterial hypertension.

MATERIALS AND METHODS: The effect of HMGB1 on the proliferation of the two cell types was examined using the MTT assay under environmental hypoxia (incubation with 1.5% oxygen) to simulate the condition of pulmonary arterial hypertension in the body. The effect of HMGB1 on HPAEC migration was observed using the scratch assay. The effect of HMGB1 on the inflammatory mediators IL-6 and CXCL8 in the two cell types was assessed using qPCR (Real-time Quantitative PCR) and ELISA, and the RAGE mRNA and protein expression levels were also examined.

RESULTS: Hypoxia promoted the proliferation of both cell types but inhibited the migration of HPAECs. HMGB1 had no obvious effect on the proliferation and migration of the cells. Both hypoxia and HMGB1 promoted the expression of the pro-inflammatory factors IL-6 and CXCL8. HMGB1 significantly promoted RAGE expression compared to the normal control group.

CONCLUSIONS: HMGB1 affects the functions of HPASMCs and HPAECs through RAGE and may affect the course of pulmonary arterial hypertension.

Key Words:
    High-mobility group box 1, Hypoxia, Pulmonary arterial hypertension, RAGE.

Introduction

Pulmonary arterial hypertension (PAH) is a chronic progressive disease that mainly occurs as a result of elevated pulmonary arterial pressure caused by pulmonary vasoconstriction and pulmonary arterial remodeling. PAH is a destructive disease that causes right heart failure and eventually leads to death if not treated in a timely manner[1]. High-mobility group box 1 (HMGB1) is a classic, highly conserved damage-associated molecular pattern (DAMP) with a small size that can be actively secreted by immune cells or passively released by damaged and necrotic cells into the extracellular environment to trigger an inflammatory response[2,3]. Studies[4-6] have shown that HMGB1 is involved in the development of sepsis, cancer, arthritis and other diseases, and this molecule plays a key role in respiratory diseases, including lung injury, chronic obstructive pulmonary disease, pulmonary fibrosis, asthma, lung cancer and PAH. HMGB1 expression causes vascular remodeling in the airway and lung, which eventually leads to PAH. Sadamura-Takenaka et al[7] found that increased HMGB1 expression in a PAH mouse model promoted the inflammatory response and caused thickening of the pulmonary vascular wall. Conversely, an anti-HMGB1 antibody alleviated the inflammatory response in the lung and prevented the thickening of the pulmonary vascular wall. Additionally, the anti-HMGB1 antibody also reduced the endothelin-1 levels in the alveolar lavage fluid and serum, suggesting that HMGB1 might play a pro-inflammatory role in PAH pathogenesis and may thus control inflammation and pulmonary vascular remodeling[8]. Hence, HMGB1 is a potential therapeutic target for PAH. This study attempts to verify the molecular mechanism of HMGB1 in the development of PAH.

Materials and Methods

Experimental Reagents, Cell Lines and Apparatuses

The recombinant human HMGB1 protein was purchased from Elabscience (Elabscience Biotechnology Co., Ltd., Wu Han, Hubei Province, China),

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the MTT Cell Proliferation and Cytotoxicity Assay Kit were purchased from Promega (Promega Corporation, Madison, WI, USA), the anti-RAGE antibody was purchased from Cell Signaling Technology (CST Inc., Boston, MA, USA), and the IL-6 and CXCL8 ELISA kits were purchased from R&D Systems (St. Paul, MN, USA). Human pulmonary artery endothelial cells and human pulmonary artery smooth muscle cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The fluorescence quantitative PCR cycler-7900T was purchased from ABI (Olympia, Washington, WA, USA), the fluorescence inverted phase contrast microscope was purchased from Nikon Corporation (Tokyo, Japan), the Epoch Microplate Spectrophotometer was purchased from BioTek Instruments (Winooski, VT, USA), and the electrophoresis and membrane transfer apparatuses were purchased from Bio-Rad (Los Angeles, CA, USA).

Cell Culture and Hypoxia

The HPAECs were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO_2 until the cells adhered to the culture plate and reached more than 80% confluency. Then, the cells were digested with 0.05% trypsin containing 0.02% EDTA and passaged at a 1:3 ratio; the cells were ready for use after 4-6 h of adhesion. The HPASMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS for 4-6 h after adhesion. After seeding into plates, the cells were incubated under normoxic conditions at 37°C with 5% CO_2 as the normal control or at 37°C with 5% CO_2 and 1.5% O_2 in a hypoxia incubator to simulate hypoxia.

Detection of Cell Proliferation Using the MTT Assay

Cells in logarithmic phase were seeded into 96-well plates at a density of 2×10^4 cells/well and cultured at 37°C and 5% CO_2 under normoxic or hypoxic conditions for 24 h. The culture medium was discarded and replaced with serum-free medium containing 0.1% bovine serum albumin (BSA) at a 100 μL/well concentration overnight to induce serum starvation (the positive control group was cultured in 15% high-serum medium). The medium was discarded and replaced with serum-free medium containing 0.1% BSA at a 100 μL/well concentration, followed by the addition of different HMGB1 concentrations for each group

(final HMGB1 concentrations of 0.1 μg/mL, 1 μg/mL and 10 μg/mL). The HMGB1 was replaced with an equal volume of PBS in the positive control and blank control groups. The cells were cultured for 24 or 48 h, followed by the addition of 20 μL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) to each well. Next, the cells were cultured for 2-4 h to reduce the MTT to formazan. The absorbance (OD) was measured at 490 nm and 630 nm for each well on a microplate reader. Each experiment was set up in triplicate.

Effect of HMGB1 on HPAEC Migration in the Scratch Assay

First, a 6-well plate was marked on the back with evenly distributed horizontal straight lines at 0.5-1-cm intervals. Each well was seeded with 5×10^5 cells, and the cells were serum starved overnight after reaching 100% confluency. Then, three parallel scratches were randomly made using a pipette tip in each well; the scratches were made as perpendicular as possible to the horizontal lines on the back of the plate, and the pipette tip was set as perpendicular as possible to the surface when the scratches were made. Optimally, the same pipette tip was used to score all of the wells. The cells were washed three times with PBS and cultured with starvation medium containing HMGB1. The HMGB1 concentration chosen was 1 μg/mL based on the preliminary experiment. Then, the cells were cultured at 37°C and 5% CO_2 under normoxic or hypoxic conditions and photographed at 0, 2, 4, 8, 12 and 24 h of culture. The number of cells migrating to the blank area was counted for each group.

Evaluation of the mRNA Expression Levels of Inflammatory Factors and the Receptor for RAGE in the Cells Using qPCR

HPAECs and HPASMCs at the 5th passages were seeded into three 100-mm dishes each at a density of 10^6 cells/mL and divided into three groups. The cells were cultured for 1 day at 37°C and 5% CO_2. When the cells reached the logarithmic phase, one group of cells was cultured under the same conditions for 12 h, one group of cells was cultured in normal medium containing HMGB1 (final concentration of 1 μg/mL), and one group was cultured under hypoxic conditions. After 24 h of culture, the media were discarded, and the cells were rinsed with cold PBS (0.01 M, pH 7.2), followed by the addition of 1 mL of the TRIzol reagent into each dish and vigorous

mixing. The mixture was allowed to stand for 10 min and then it was transferred to a 1.5-mL centrifuge tube, followed by the addition of 200 µL of chloroform and mixed by vortexing. The mixture was allowed to stand at room temperature for 10 min and then centrifuged at 12,000 rpm for 15 min at 4°C. The visible flocculent RNA precipitate was collected, and the supernatant was discarded. The precipitate was washed once with 1 mL of pre-cooled 75% ethanol and once with 1 mL of pre-cooled anhydrous ethanol and then air dried. The RNA was dissolved in diethyl dicarbonate (DEPC)-treated water, and the RNA concentration was measured and adjusted to 3 µg/10 µL. The RNA was placed on ice until use. The reverse transcription PCR system was prepared according to the user instruction for the RT-PCR kit from Thermo Fisher (Waltham, MA, USA) (10928042). The cDNA from the reverse transcription was diluted, and a fluorescence quantitative PCR was performed using the fluorescence Real-time Quantitative PCR (qPCR) system prepared according to the qPCR kit from Thermo Fisher (11736059). The primes sequences of relevant genes were listed in Table I.

Detection of Pro-Inflammatory Cytokine Expression by Enzyme Linked Immunosorbent Assay (ELISA)

The supernatants were collected from the cell culture, and the expression of pro-inflammatory cytokines in the supernatant was measured according to the user instructions for the ELISA kits from R&D Systems.

Changes in RAGE Expression Detected by Immunoblotting

The cells were washed once with cold PBS, followed by the addition of 120 µL of cell lysis buffer. Next, the cells were allowed to lyse on ice for 30 min. The lysate was transferred to an EP tube and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was transferred to a new EP tube, and the protein concentration was measured using the bicinchoninic acid (BCA) method. The lysates of the samples were adjusted to the same protein concentration by adding the corresponding loading buffer and PBS. The lysates were denatured at 95°C for 10 min, followed by cooling on ice, electrophoresis, and membrane transfer. The membrane was blocked with blocking buffer at room temperature for 1 h, followed by incubation with the primary antibody (1:1000 dilution) at 4°C overnight. The membrane was rinsed with Tris-buffered saline with Tween 20 (TBST) three times (5 min per wash) prior to incubation with the secondary antibody (1:5000 dilution) at room temperature for 1 h. The membrane was rinsed with TBST three times (5 min per wash), followed by image development.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 5.0 (La Jolla, CA, USA). The results were expressed as the mean ± standard deviation (mean ± SD). Variance analysis was used to compare multiple groups, and a *t*-test was used to compare the two groups. The difference was considered highly significant when $p<0.001$, significant when $0.001<p<0.05$, and not significant when $p>0.05$.

Results

Effect of HMGB1 on HPASMC and HPAEC Proliferation

The growth and proliferation of pulmonary artery smooth muscle cells and endothelial cells affect the development of PAH. In this study, the

Table I. Primer sequences of relevant genes.

| Gene	Primer sequence (5'-3')
NR-H-HMGB1-F	TGAACATCTGAGTCCTGGATGA
NR-H-HMGB1-R	GGGAAAAACTTTGCCATCCCT
NR-H-CXCL8-F	TAGCCAGGATCCACAAGTCC
NR-H-CXCL8-R	CCACATGTCCTCACAACATCAC
NR-H-RAGE-F	TGAGGCAGGCGAGAGTAGTA
NR-H-RAGE-R	GGAGAGAGTTGGTCTGAGGC
NR-H-IL6-F	CAATAACCACCCCTGACCCAA
NR-H-IL6-R	TGAGGTGCCCATGCTACATTT
TH-GAPDH-F	CCAGGTGGTCTCCTCTGACTTC
TH-GAPDH-R	GTGGTCGTTGAGGGCAATG
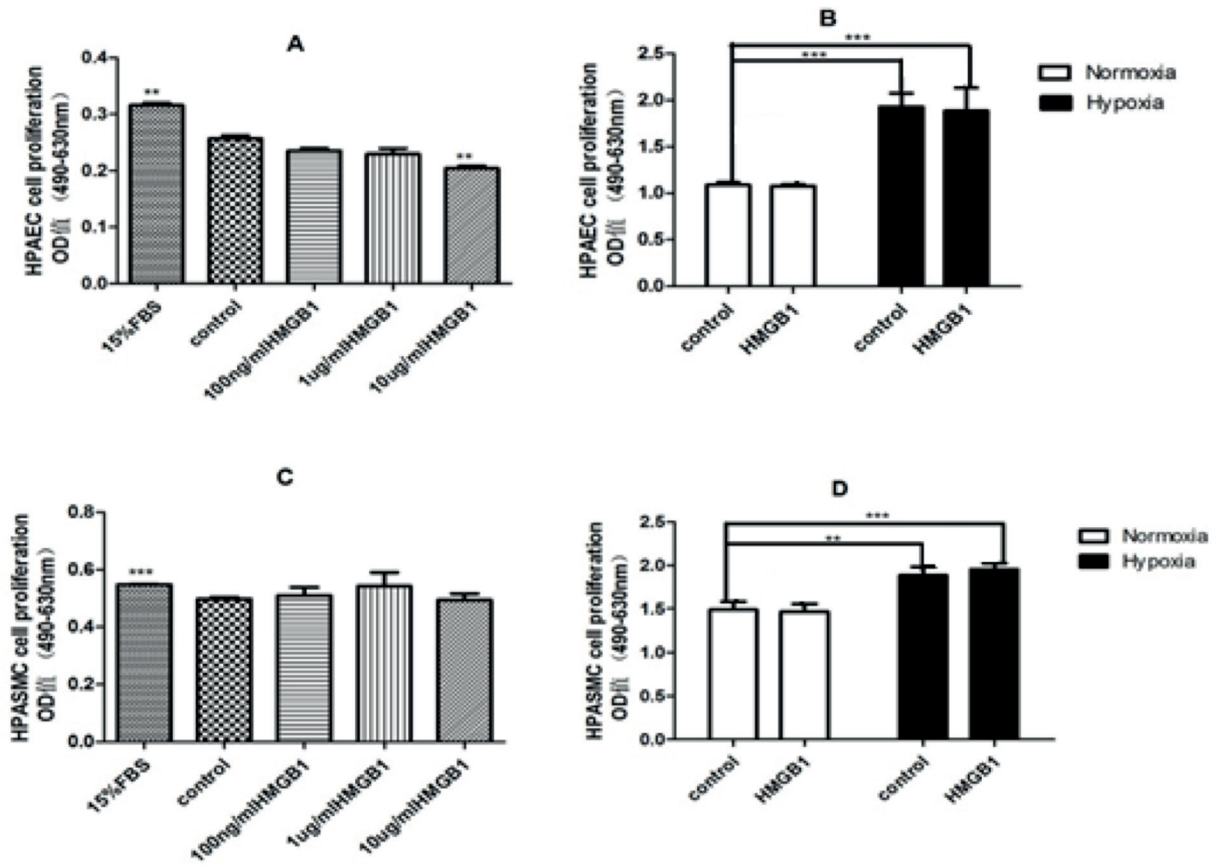
Figure 1. Effect of HMGB1 and hypoxia on HPAEC and HPASMC proliferation detected by the MTT assay. *(A)* Effect of different HMGB1 concentrations on HPAEC and HPASMC proliferation; *(B)* effect of HMGB1 and hypoxia on HPAEC proliferation; *(C)* effect of different HMGB1 concentrations on HPASMC proliferation; *(D)* effect of HMGB1 and hypoxia on HPASMC proliferation. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with the control.

effect of HMGB1 on the proliferation of normal HPASMCs and HPAECs was examined. The high-serum culture medium significantly promoted the proliferation of these two cell types. Although HMGB1 did not inhibit cell growth at the low concentration, this molecule inhibited the growth of endothelial cells at the high concentration (10 μg/mL) (Figure 1A and 1C). Under the hypoxic condition (oxygen concentration of 1%), the HPAECs and HPASMCs exhibited increased proliferation compared with the cells grown under the normal condition. Furthermore, the control and HMGB1 (1 μg/mL) experimental groups did not show significant differences in proliferation under the hypoxic condition (Figure 1B and 1D). The above findings indicated that HMGB1 had no obvious promoting effect on HPAEC and HPASMC proliferation, whereas hypoxia promoted HPAEC and HPASMC proliferation. Hence, a hypoxic environment can simulate *in vivo* PAH environment.

Effect of HMGB1 on HPAEC Migration

After damage due to PAH, repair of the vascular endothelium starts with the repair of vascular endothelial cells. The scratch assay showed no effect of HMGB1 on HPAEC migration, whereas HMGB1 exhibited a significant inhibitory effect on HPAEC migration under hypoxic conditions (Figure 2). The migration of HPAECs in the body promotes the repair of the damaged vascular endothelium. However, hypoxia inhibits the migration capability of HPAECs. This finding suggests that hypoxia inhibits the repair of pulmonary artery endothelial injury, which indicates that hypoxia can simulate injuries from PAH in the body.

HMGB1 Promotes the Release of Pro-Inflammatory Cytokines

Among the HMGB1-mediated pro-inflammatory cytokines, IL-6 and CXCL8 showed more significant expression in the two cell types. The mRNA
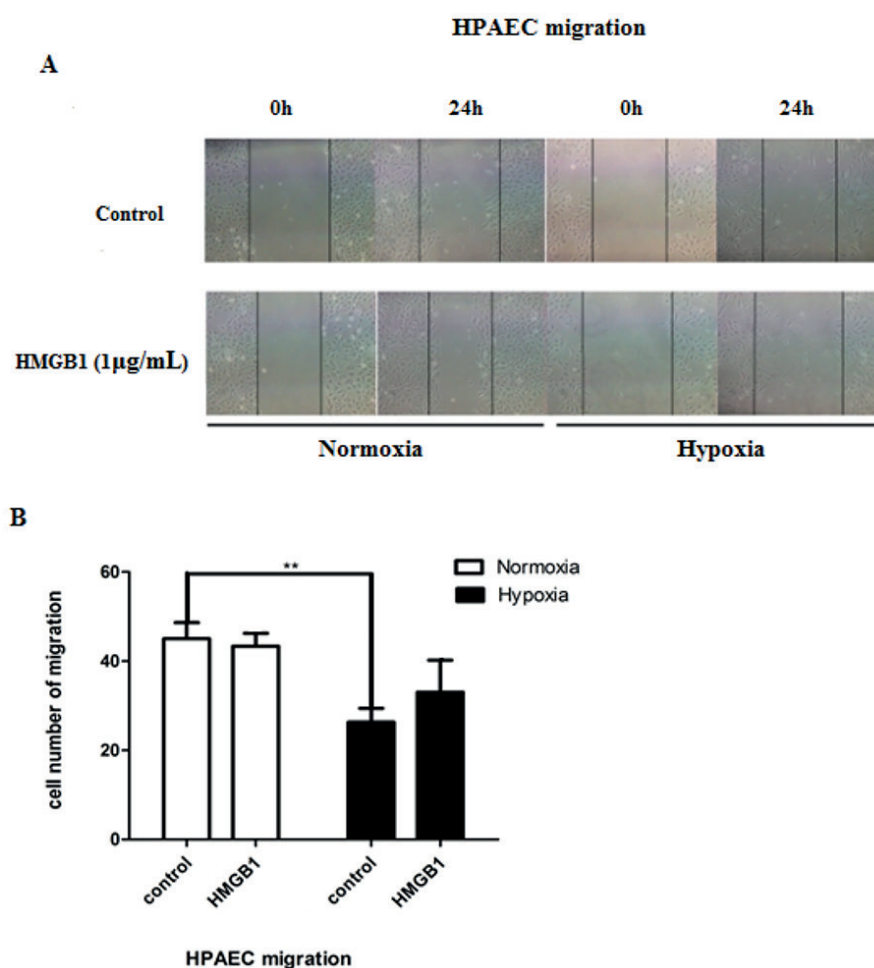
Figure 2. Effect of hypoxia and HMGB1 on HPAEC migration. *(A)* Migration of HPAECs under a microscope; *(B)* Statistical representation of the numbers of migrating cells. **$p<0.01$.

and protein expression levels of these two pro-inflammatory cytokines were significantly increased in the HPASMCs under hypoxic conditions or following treatment with HMGB1 (1 μg/mL) (Figure 3A-B). However, although the IL-6 and CXCL8 mRNA and protein expression levels were slightly increased in the HPAECs under hypoxic conditions, no significant difference was found compared with the blank control group (Figure 3C-D). This experiment demonstrated that hypoxia and HMGB1 induced elevated inflammatory responses in the cells.

HMGB1 Regulates the Development of PAH Through RAGE

First, HMGB1 mRNA expression was examined in the two cell types under the normal culture and hypoxic conditions. The HMGB1 mRNA was found in both cell types under both conditions, with increased expression under the hypoxic con-

dition (Figure 4A-B). Examination of the RAGE mRNA and protein expression levels under the normal culture condition, hypoxic condition, and normal culture condition plus HMGB1 treatment, showed increased RAGE mRNA and protein expression levels when the cells were under the hypoxic conditions or treated with HMGB1 under the normal condition (Figure 4C-4F), indicating that HMGB1 stimulated RAGE secretion.

Discussion

The results from this study showed that HMGB1 had no effect on the proliferation of HPASMCs and HPAECs, which indicated that HMGB1 could not affect the development of PAH by enhancing the proliferation of pulmonary artery smooth muscle cells and endothelial cells. This finding was consi-
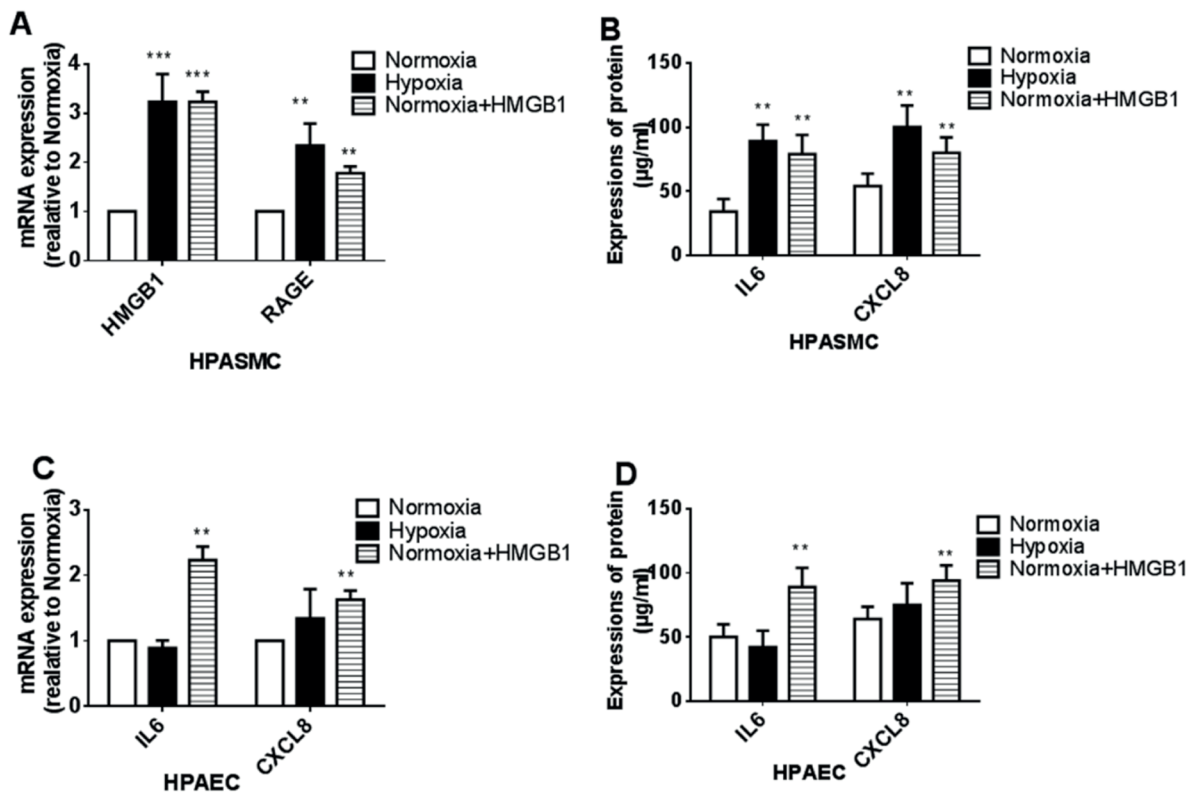
Figure 3. IL-6 and CXCL8 expression in HPAECs and HPASMCs. The normoxia group refers to the normal control, the hypoxia group refers to the group under hypoxic conditions, and the normoxia+HMGB1 group refers to the group treated with HMGB1 (1 µg/mL) under the normoxic condition. The IL-6 and CXCL8 mRNA and protein expression levels under the hypoxic state and HMGB1 (1 µg/mL) treatment are shown for the HPASMCs *(A,B)* and HPAECs *(C,D)*. $^*p<0.05$, $^{**}p<0.01$ compared to the normoxia group.

stent with the results of Bauer et al[9,10], who showed that HMGB1 had no effect on HPASMC proliferation, indicating an indirect role for HMGB1 in vascular remodeling. In this study, HMGB1 had no obvious effect on HPAEC migration, but it had an inhibitory effect on HPAEC migration under the hypoxic condition. Contradictory results exist in the literature concerning the effects of HMGB1 on the proliferation and migration of HPASMCs and HPAECs. Some studies reported that HMGB1 promoted HPASMC and HPAEC proliferation[11,12] and HPASMC migration[10]. The differences in the results were likely caused by the use of different HMGB1 concentrations. Some reports[13,14] showed these two different effects of HMGB1 on proliferation. Therefore, determining whether HMGB1 acts through smooth muscle cells to affect the development of PAH requires further study. HMGB1 has been shown to function as an inhibitor of endothelial cell migration, suggesting that this molecule may be an inhibitor of the endothelial cell repair mechanism. In this study, hypoxia promo-

ted HPASMC and HPAEC proliferation, and inhibited HPAEC migration. Increased endothelial cell and smooth muscle cell proliferation lead to thickening of the blood vessel wall and luminal narrowing, whereas hypoxia inhibits endothelial cell migration and reduces the repair capability following vascular endothelial damage. Endothelial injury induces inflammation and thrombosis, which leads to endothelial dysfunction. Therefore, hypoxia leads to pulmonary artery endothelial cell injury, increased permeability, proliferation and hypertrophy of endothelial cells and proliferation of medial smooth muscle cells caused by endothelial dysfunction and eventually results in pulmonary vascular remodeling. Endothelial dysfunction and inflammation promote vasoconstriction and pulmonary vascular remodeling, which contribute to the development of PAH. Some markers of endothelial dysfunction and inflammation exhibit increasing levels in human and experimental animals with PAH, including intercellular adhesion molecule 1 (ICAM-1) and endothelin-1 (ET-1)[15,16].

Drugs targeting the ET-1 receptor have been used to treat PAH. The ICAM-1 and ET-1 levels were not increased when mice maintained under chronic hypoxia conditions, and they were treated with an α-HMGB1 antibody, indicating that HMGB1 facilitates endothelial dysfunction and inflamma-

tion in PAH. Hypoxia has been shown to enhance platelet-derived growth factor (PDGF)-A and B chain mRNA expression in pulmonary vascular endothelial cells, smooth muscle cells, and to increase PDGF activity in PASMC culture medium, suggesting that hypoxia increases PDGF autocrine
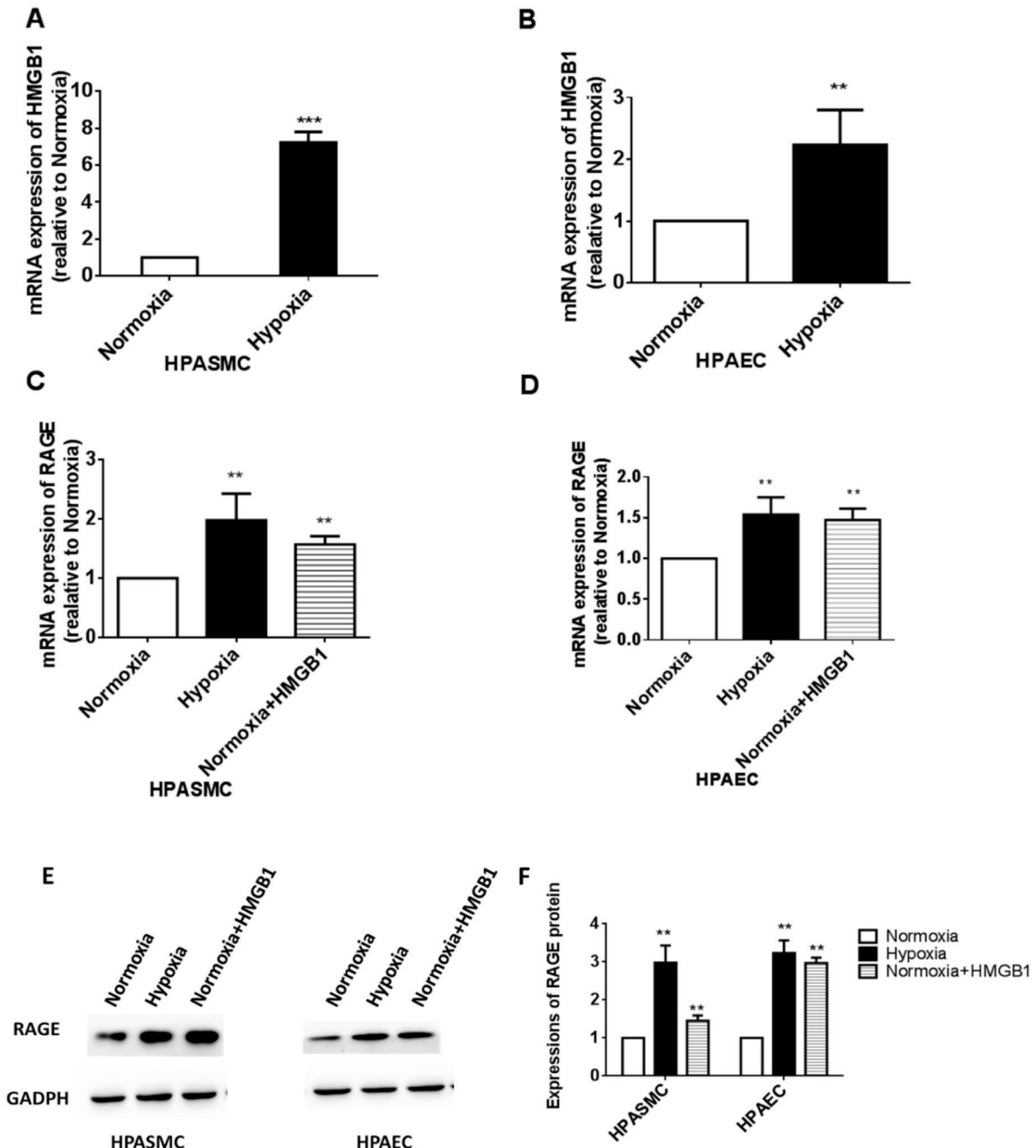

Figure 4. HMGB1 promotes RAGE secretion. The normoxia group refers to the normal control, the hypoxia group refers to the group under hypoxic conditions, and the normoxia+HMGB1 group refers to the group treated with HMGB1 (1 μg/ mL) under the normoxic condition. *(A-C)* HMGB1 mRNA expression in HPAECs and HPASMCs. *(B-D)* Effect of different treatments on RAGE mRNA expression in the HPAECs and HPASMCs. (E, F) Effects of the different treatments on RAGE protein expression in the HPAECs and HPASMCs and statistical analysis. $^*p<0.05$, $^{**}p<0.01$ compared to the normoxia group.

activity by HPASMCs. Also, upregulation of the PDGF receptor is an important route to enhance proliferation[17]. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that is widely found in mammalian cells and plays a key role in the regulation of gene expression induced by hypoxia. Increased HIF-1 expression promoted the hypoxic proliferation of pulmonary artery smooth muscle cells[18]. The role of inflammation in human PAH is based on the presence of inflammatory cells at the site of vascular injury in PAH patients, with increased peripheral inflammatory factor levels. A case study of patients with PAH indicated improved symptoms after treatment with anti-inflammatory drugs. Increased HMGB1 expression in HPASMCs and HPAECs was found in this study compared to the normal control group; this effect was especially evident in the HPASMCs, indicating that endothelial cells and smooth muscle cells might be the sources of the increased peripheral HMGB1 in PAH patients. HMGB1 has been described as an early pro-inflammatory factor that plays a critical role in many pathophysiological processes, including sepsis, rheumatoid arthritis, and liver injury. In this work, RAGE expression was elevated with the increase in HMGB1 expression in HPASMCs and HPAECs under hypoxic conditions, following the expression of the inflammatory factors IL-6 and CXCL8. This was indicating that RAGE was most likely the main HMGB1 receptor in the HPASMCs and HPAECs. RAGE is very abundant in lung tissues[19] and is a very important HMGB1 receptor[20]. Although RAGE had no effect on increasing pulmonary vascular resistance in hypoxic PAH, it played a role in the right ventricular hypertrophy caused by increased pulmonary vascular resistance. This result was consistent with previous studies showing a role for RAGE in cardiac hypertrophy in mice fed with a Western diet[21]. The role of the HMGB1-Toll-like receptor 4 (TLR4) interaction in driving immune pathology was initially described in 2005. A previous paper found that TLR4 gene silencing blocked the increase in ET-1 and sICAM-1 induced by hypoxia, whereas RAGE did not have this effect. These data indicate that TLR4, instead of RAGE, plays a critical role in PAH development. Additionally, TLR4 was found on platelets, and activation of TLR4 on platelets led to platelet aggregation and promoted *in situ* thrombosis, which are characteristics of idiopathic PAH[22]. IL-6 is a cytokine that can be produced by a variety of cells, including monocytes, epithelial cells, vascular endothelial cells, and vascular smooth muscle cells[23]. The IL-6 level was increased under hypoxia compared to its expression level under normoxic conditions. IL-6 overexpression promoted PAH development, whereas IL-6 gene silencing suppressed PAH development[24]. HMGB1 and endotoxin promote synergistic IL-6 production by smooth muscle cells and monocytes in co-culture. A recent work[25] showed that HMGB1 increased the permeability of endothelial cells. In endothelial cells, E-cadherin plays a key role in endothelial cell integrity and the control of vascular permeability[26]. Previous studies have shown the involvement of inflammatory factors in the role of vascular E-cadherin in the permeability and endothelial cell gap formation mediated by cytokines.

Conclusions

This investigation showed that hypoxia promoted HPASMC and HPAEC proliferation and inhibited HPAEC migration. Under hypoxic conditions, the expression levels of HMGB1 and its receptor RAGE were elevated with the increased release of the relevant inflammatory factors IL-6 and CXCL8. These results indicate that hypoxia can be used as an *in vitro* simulation model of PAH. Additionally, HMGB1 significantly enhanced the expression of RAGE and inflammatory factors, but showed no effect on the proliferation and migration of the two cell types, indicating that HMGB1 did not affect the development of PAH by directly acting on the proliferation and migration of smooth muscle and endothelial cells. Instead, HMGB1 promoted the release of inflammatory factors through RAGE to facilitate the development of PAH.

Conflict of interest

The authors declare no conflicts of interest.

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