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High-mobility group box-1 protein induces mucin 8 expression through the activation of the JNK and PI3K/Akt signal pathways in human airway epithelial cells

Dong Eun Kim [a,1], Kyoung-jin Min [b,1], Jung Soo Kim [c], Taeg Kyu Kwon [b,*]

[a] Department of Otolaryngology, School of Medicine, Keimyung University, 2800 Dalgubeoldaero, Dalseo-Gu, Daegu 704-701, Republic of Korea
[b] Department of Immunology, School of Medicine, Keimyung University, 2800 Dalgubeoldaero, Dalseo-Gu, Daegu 704-701, Republic of Korea
[c] Department of Otolaryngology, Kyungpook National University, School of Medicine, Daegu 700-422, Republic of Korea

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ABSTRACT

High-mobility group box-1 protein (HMGB1), which is produced by immune cells, was recently identified as a proinflammatory mediator in various inflammatory diseases. In this study, we investigated the effect of HMGB1 on the expression of mucin (MUC) genes in human airway epithelial cells. We showed that HMGB1 markedly increased MUC8 expression, and that the expression of other MUC genes was also regulated by HMGB1. HMGB1 activated the JNK and PI3K/Akt signaling pathways, and inhibitors of JNK and PI3K/Akt markedly inhibited HMGB1-induced MUC8 expression. Furthermore, HMGB1 increased the production of intracellular reactive oxygen species (ROS). However, the ROS scavengers Trolox and N-acetylcysteine (NAC) had no effect on MUC8 expression in HMGB1-treated NCI-H292 cells. Taken together, our results suggest that HMGB1 induces MUC8 expression in a JNK and PI3K/Akt signaling pathway-dependent manner but that HMGB1 acts in an ROS-independent manner.

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1. Introduction

In the respiratory tract, mucus maintains hydration and has important protective functions in mucosal defense by entrapping particulates such as bacteria and viruses [1,2]. Mucins are high-molecular-weight glycoproteins that are major components of the mucus produced by the epithelium of the respiratory tract [3]. However, mucus hypersecretion is a common pathologic manifestation of inflammatory airway diseases such as sinusitis, rhinitis, and bronchitis, and this mucus hypersecretion can lead to airway obstruction [4]. Various cytokines and inflammatory mediators activate mucin secretion by up-regulating the expression of mucin (MUC) genes [5]. To date, 20 distinct MUC genes have been identified, and these genes are usually subdivided into three groups: membrane-associated mucins, secreted gel-forming mucins, and secreted non-gel-forming mucins. MUC1, MUC3, MUC4, MUC11, MUC12, MUC13, MUC17, MUC18, and MUC20 are membrane-associated mucins; MUC2, MUC5AC, MUC5B, and MUC6 are secreted gel-forming mucins; and MUC7, MUC8, and MUC9 are secreted non-gel-forming mucins [3,5]. Among the 20 human mucin genes, MUC8 is regarded as one of the important airway mucin genes because its mRNA levels in polyp epithelium are higher than normal epithelium [1]. In addition, *in vitro*, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and a cocktail of inflammatory mediators were found to up-regulate the MUC8 mRNA level in human nasal epithelium [6,7]. These results showed that the MUC8 mRNA level increased both *in vivo* and *in vitro* under inflammatory conditions. However, the mechanisms of MUC8 gene expression during inflammation and the signal molecule(s) involved in human airway epithelial cells have not been fully explained.

High-mobility group box-1 protein (HMGB1) was first identified as a 30 kDa nonhistone chromosomal protein by Goodwin and Johns [8]. HMGB1 has been known for a long time to be a nuclear DNA-binding protein that participates in the regulation of chromatin structure, participating in processes such as DNA replication, transcription, and repair [9]. In contrast, extracellular HMGB1 has been described as the regulator of various biological processes, such as cell differentiation, cell migration, and metastasis [10–12]. Recent studies have demonstrated that HMGB1 acts as an important mediator of inflammation [13]. HMGB1 is released from activated monocyte/macrophages and functions as a proinflammatory mediator, partly through the induction of various proinflammatory mediators [14,15]. However, the effect of HMGB1 on mucin gene expression in human airway epithelial cells has not been determined.

To our knowledge, the results reported here showed, for the first time, the induction of MUC8 expression by HMGB1 in human

* Corresponding author. Fax: +82 53 5803882.
E-mail address: kwontk@dsmc.or.kr (T.K. Kwon).
[1] These authors contributed equally to this work.

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epithelial cells. Our results showed that HMGB1 induces MUC8 mRNA expression in a dose-dependent manner through the activation of the JNK and PI3K/Akt signal pathways in human epithelial NCI-H292 cells.

2. Materials and methods

2.1. Materials and cell culture

NCI-H292 cells were purchased from the American Type Culture Collection (Rockville, MD). The culture medium used throughout these experiments was RPMI-1640 medium containing 10% fetal bovine serum, 20 mM Hepes buffer, and 100 μg/ml gentamicin. Cells were grown in 100 mm culture dishes, and cells were subcultured using 4% trypsin every two or three days. PCR primers were purchased from Bioneer (Daejeon, Korea), and other chemicals were from Sigma (St. Louis, MO). Anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38 MAPK and anti-phospho-Akt were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Western blot analysis

Cells were washed with cold PBS and lysed on ice in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 2 mM EDTA). Lysates were centrifuged at 10,000*g* for 10 min at 4 °C, and the supernatant fractions were collected. Proteins were separated by SDS–PAGE and transferred to Immobilon-P membranes. Specific proteins were detected using an enhanced chemiluminescence (ECL) Western blotting kit according to the manufacturer's instructions.

2.3. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR)

Total RNA was isolated using TriZol reagent (Life Technologies, Gaithersburg, MD), and cDNA was prepared using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. The primers and reaction conditions for qPCR and RT-PCR are shown in Table 1. For qPCR, cDNA and forward/reverse primers (200 nM) were added to 2× KAPA SYBR Fast master mix, and reactions were performed using an RG-6000 real-time amplification instrument (Corbett Research). The threshold cycle number (Ct) of each gene was calculated, and actin was used as the reference gene. The delta–delta Ct values of the genes are presented as the relative fold induction. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized under UV light.

2.4. DNA transfection and luciferase assay

Transient transfection was performed in 6-well plates. One day before transfection, NCI-H292 cells were plated to maintain approximately 60–80% confluence. Plasmids encoding NF-κB and CRE were transfected into cells using the Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA). To assess promoter-driven expression of the luciferase gene, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris–phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol), and aliquots of the supernatants were analyzed by measuring the luciferase activity according to the manufacturer's instructions (Promega, Madison, WI).

Table 1
Primers sequence for RT-PCR and/or qPCR.

Gene name	Primer sequence
Human MUC2 (Forward)	5′-TGCCTGGCCCTGTCTTTG-3′
Human MUC2 (Reverse)	5′-CAGCTCCAGCATGAGTGC-3′
Human MUC4 (Forward)	5′-TTCTAAGAACCACCAGACTCAGAGC-3′
Human MUC4 (Reverse)	5′-GAGACACACCTGGAGAGAATGAGC-3′
Human MUC5AC (Forward)	5′-TGATCATCCAGCAGGGCT-3′
Human MUC5AC (Reverse)	5′-CCGAGCTCAGAGGACATATGGG-3′
Human MUC5B (Forward)	5′-CTGCGAGACCGAGGTCAACATC-3′
Human MUC5B (Reverse)	5′-TGGGCAGCAGGAGCACGGAG-3′
Human MUC6 (Forward)	5′-TCACCTATCACCACACAAC-3′
Human MUC6 (Reverse)	5′-GGAGAAGAAGGAAAAAGAG-3′
Human MUC7 (Forward)	5′-CCACACCTAATTCTTCCC-3′
Human MUC7 (Reverse)	5′-CTATTGCTCCACCATGTC-3′
Human MUC8 (Forward)	5′-ACAGGGTTTCTCCTCATG-3′
Human MUC8 (Reverse)	5′-CGTTTATTCCAGCACTGTTC-3′
Human MUC19 (Forward)	5′-TTTAGAGGCACTGGGACCAC-3′
Human MUC19 (Reverse)	5′-ACCATTGCCCAAAGAAGTTG-3′
Human RAGE (Forward)	5′-TACTAGCTAGCGCCCGGATTGGCGAGCCAC-3′
Human RAGE (Reverse)	5′-ATAGTTTAGCGGCCGCCTGGTAGACACGGACTC-3′
Human MUC8 (Forward)-qPCR	5′-GGCTGGTCTCGAACTCCTGA-3′
Human MUC8 (Reverse)-qPCR	5′-TCGTGCTGTAATCCCAACACTT-3′
Human ACTIN (Forward)	5′-GGCATCGTCACCAACTGGGAC-3′
Human ACTIN (Reverse)	5′-CGATTTCCCGCTCGGCCGTGG-3′

2.5. Measurement of reactive oxygen species (ROS)

The intracellular accumulation of ROS was assessed using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). H2DCFDA is commonly used to measure ROS generation [16]. Cells were seeding in 6-well plates and incubated for 24 h prior to treatment with 50 ng/ml HMGB1, and then the cells were treated with 5 μM H2DCFDA for 30 min. Then, the cells were trypsinized and resuspended in PBS. The fluorescence was measured at the desired time intervals using a flow cytometer (Becton–Dickinson, Franklin Lakes, NJ).

2.6. Statistical analysis

Data were analyzed with one-way ANOVA followed by post-hoc comparisons (Student–Newman–Keuls) using the Statistical Package for Social Sciences 8.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. HMGB1 induces MUC8 expression

HMGB1 is an important signaling molecule that is associated with the expression of inflammatory mediators [13,17], and inflammation is associated with mucin (MUC) gene expression or secretion [18,19]. Therefore, to assess the role of HMGB1 in the induction of MUC8 gene expression in human epithelial NCI-H292 cells, cells were treated with 10 or 50 ng/ml HMGB1 for 12 h. We detected MUC8 mRNA expression using RT-PCR and quantitative PCR. As shown in Fig. 1A and B, HMGB1 increased MUC8 mRNA expression in a dose-dependent manner. In addition, MUC8 mRNA expression was first detected at 3 h, and gradually increased over the next 12 h (Fig. 1C). Next, we wondered whether HMGB1 could induce the expression of other MUC genes. HMGB1 induced slight increases in the expression levels of MUC2, MUC5AC, MUC6, and MUC7 mRNA. However, HMGB1 had no effect on the expression levels of MUC4, MUC 5B, and MUC19 mRNA (Fig. 1D). Therefore, these data suggest that HMGB1 could regulate MUC gene expression in human epithelial NCI-H292 cells.

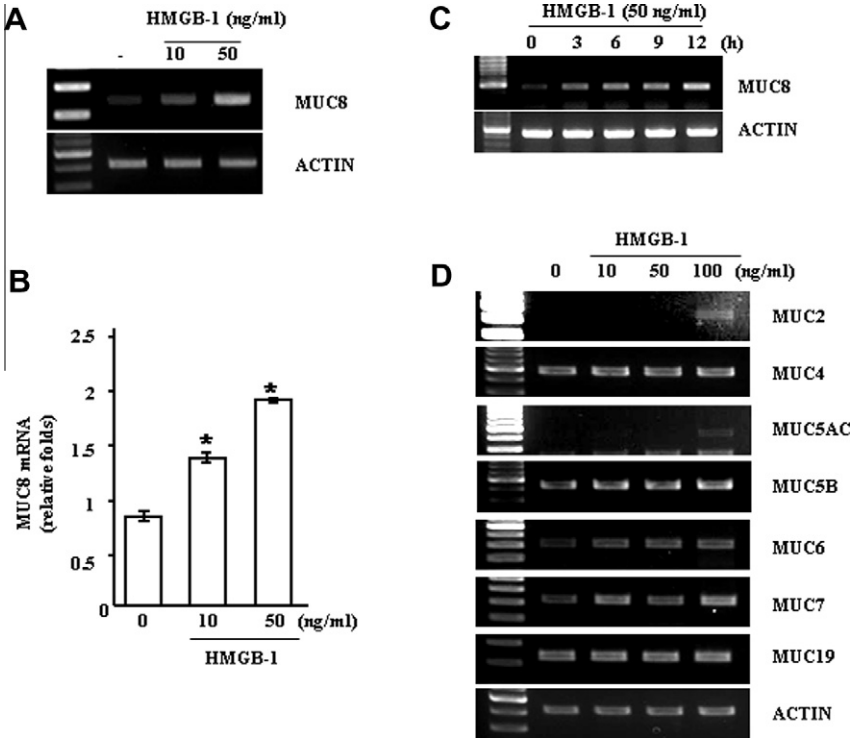

Fig. 1. Effect of HMGB1 on mucin (MUC) gene mRNA expression in NCI-H292 cells. NCI-H292 cells were treated with the indicated concentrations of HMGB1 for 12 h. The mRNA expression levels of MUC2, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, and MUC19 were determined using RT-PCR (A and D) and quantitative real-time PCR (B). NCI-H292 cells were incubated with HMGB1 (50 ng/ml) for the indicated time periods. The mRNA expression levels of MUC8 were determined using RT-PCR (C). The values in (B) are the mean ± SEM of three samples. *, $p < 0.001$ compared with the control. The data represent three independent experiments.

3.2. The JNK and PI3K/Akt signaling pathways are involved in HMGB1-induced MUC8 gene expression

Previous studies reported that HMGB1 activated the MAPK and Akt signaling pathways [20–22] and that these signaling pathways were involved in MUC gene expression [23,24]. Therefore, we investigated whether HMGB1 could activate the MAPK and Akt signaling pathways. HMGB1 increased the phosphorylation of ERK and JNK within 2 h (ERK) or 30 min (JNK), and this higher level of phosphorylation was sustained for up to 6 h (Fig. 2A). The basal level of phosphorylation of p38 MAPK was detected in untreated condition. However, the level of phosphorylation of p38 MAPK gradually declined after HMGB1 treatment (Fig. 2A). Furthermore, HMGB1 slightly induced the phosphorylation of Akt within 30 min, and the level of Akt phosphorylation increased up to 6 h (Fig. 2A). Next, we investigated whether the MAPK and PI3K/Akt signaling

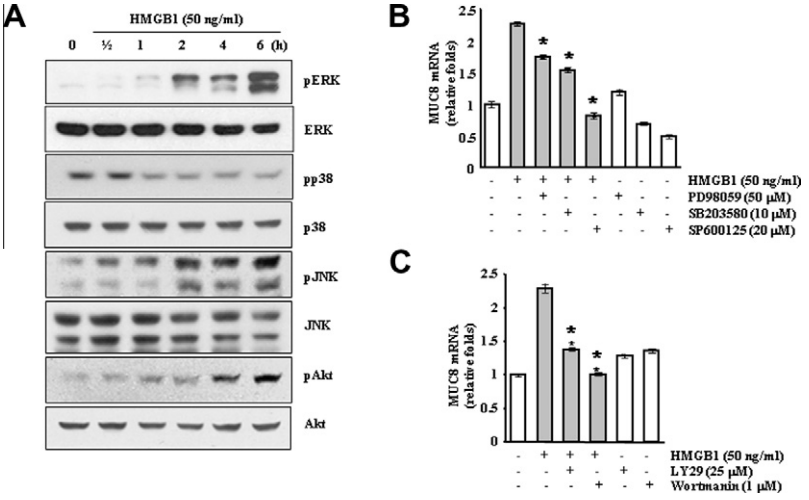

Fig. 2. HMGB1 activates the MAPK and PI3K/Akt signaling pathways. (A) NCI-H292 cells were treated with 50 ng/ml HMGB1 for the indicated time periods. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by immunoblotting using phosphorylation-specific antibodies. To ascertain that the total levels of MAPKs and Akt did not change, the blots were stripped and reprobed with the antibodies that detect total (phosphorylated and unphosphorylated) MAPKs and Akt. (B and C) NCI-H292 cells were treated with 50 ng/ml HMGB1 for 12 h in the presence or absence of MAPK inhibitors [PD98059 (ERK inhibitor), SB203580 (p38 MAPK inhibitor), and SP600125 (JNK inhibitor)] or PI3K/Akt inhibitors [LY294002 (LY29) and Wortmannin]. MUC8 mRNA expression was determined using quantitative real-time PCR. *, $p < 0.001$ compared with the HMGB1. The data represent three independent experiments.

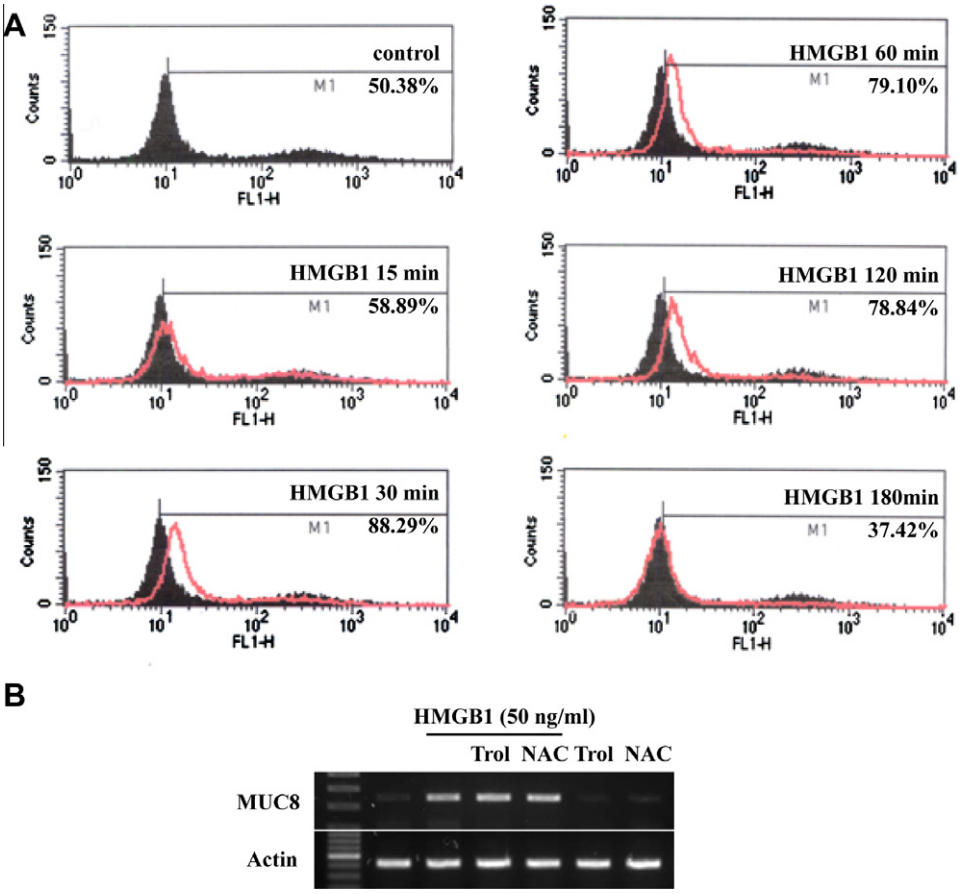

Fig. 3. HMGB1-induced MUC8 mRNA expression is independent of the ROS signaling pathway. (A) NCI-H292 cells were pretreated with a fluorescent dye, H_2DCFDA, for 30 min and then treated with 50 ng/ml HMGB1 for the indicated time periods. The H_2DCFDA intensity was quantified by flow cytometry. (B) NCI-H292 cells were pretreated with ROS scavengers, 75 μM Trolox (Trol) and 5 mM N-acetylcysteine (NAC), and then treated with 50 ng/ml HMGB1 for 12 h. MUC8 mRNA expression was determined using RT-PCR. The data represent three independent experiments.

pathways are associated with HMGB1-induced MUC8 gene expression. As shown in Fig. 2B, MAPK inhibitors [PD98059 (ERK inhibitor), SB203580 (p38 MAPK inhibitor), and SP600125 (JNK inhibitor)] reduced the level of HMGB1-induced MUC8 mRNA expression (Fig. 2B). Among these inhibitors, the JNK inhibitor markedly decreased HMGB1-induced MUC8 mRNA expression. Moreover, the PI3K/Akt inhibitors LY294002 (LY29) and Wortmannin also blocked MUC8 mRNA expression in HMGB1-treated cells (Fig. 2C). Therefore, these data suggest that HMGB1 increased MUC8 gene expression through the JNK and PI3K/Akt signaling pathways.

3.3. Effect of HMGB1-induced reactive oxygen species (ROS) on MUC8 gene expression

HMGB1 induced the production of reactive oxygen species (ROS) [25], and ROS signaling was found to be involved in MUC gene expression [26,27]. Therefore, we tested whether HMGB1 could induce ROS production in NCI-H292 cells. As shown in Fig. 3A, HMGB1 slightly increased ROS production within 15 min; the ROS production peaked at 30 min and then decreased to 180 min. Next, we wondered whether ROS production is involved in HMGB1-induced MUC8 gene expression. The ROS scavengers Trolox and NAC had no effect on MUC8 expression by HMGB1 (Fig. 3B). These data indicated that although HMGB1 increased ROS production, ROS were not associated with HMGB1-induced MUC8 expression in NCI-H292 cells.

3.4. NF-κB and CRE were not associated with MUC8 expression in HMGB1-treated NCI-H292 cells

Because HMGB1 activates NF-κB transcriptional activity [28], we determined whether NF-κB activation is involved in HMGB1-induced MUC8 gene expression in NCI-H292 cells. HMGB1 did not induce NF-κB transcriptional activity (Fig. 4A). Furthermore, the NF-κB inhibitors Bay 11-7082 (Bay) and pyrrolodine dithiocarbamate (PDTC) did not reduce MUC8 transcriptional activity in HMGB1-treated NCI-H292 cells (Fig. 4B). These results suggest that HMGB1-induced MUC8 transcriptional activity is independent of NF-κB signaling.

A previous study reported that protein kinase A (PKA)-cAMP-response element-binding protein (CREB) signaling is associated with IL-1β-induced MUC8 expression in NCI-H292 cells [24]. Therefore, we determined whether PKA-CREB signaling is involved in HMGB1-induced MUC8 expression because PKA signaling is a major pathway upstream of CREB. HMGB1 had no effect on cAMP-response element (CRE)-transcriptional activity, and a PKA inhibitor, H89, did not reduce MUC8 transcriptional activity in HMGB1-treated cells (Fig. 4C and D). Therefore, these data suggest that HMGB1-induced MUC8 expression is independent of NF-κB and PKA-CREB signaling.

These results suggest that HMGB1 induces MUC8 expression through the activation of the JNK and PI3K/Akt signaling pathways and that the induction of MUC8 expression by HMGB1 is independent of ROS, NF-κB, and PKA-CREB signaling in NCI-H292 cells.

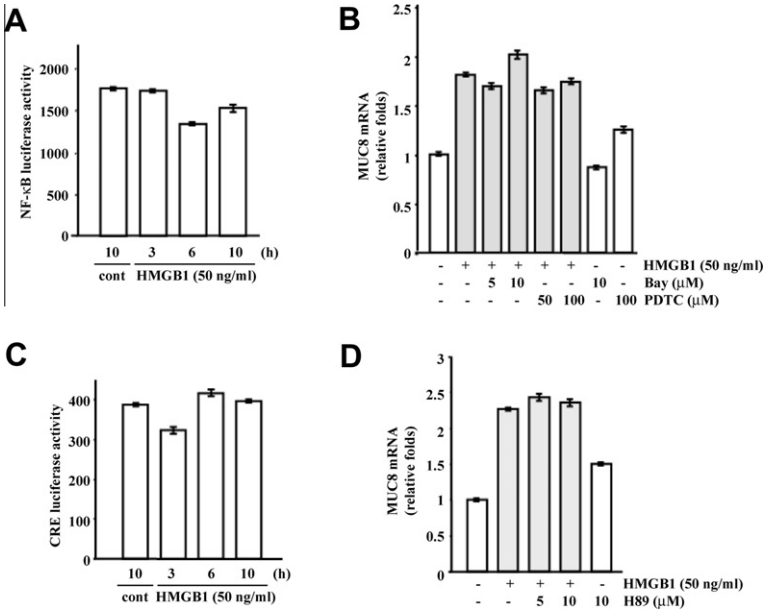

Fig. 4. Effect of NF-κB and CRE signaling on HMGB1-induced MUC8 expression. (A) NCI-H292 cells were transiently transfected with a NF-κB-luciferase construct and then treated with 50 ng/ml HMGB1 for the indicated time periods. After treatment, the cells were lysed and then assayed for luciferase activity. (B) NCI-H292 cells were pretreated with the indicated concentrations of the NF-κB inhibitors Bay 11-7082 (Bay) and pyrrolodine dithiocarbamate (PDTC) for 30 min and then stimulated with 50 ng/ml HMGB1 for 12 h. MUC8 mRNA expression was determined using quantitative real-time PCR. (C) NCI-H292 cells were transiently transfected with a CRE–luciferase construct and then simulated with 50 ng/ml HMGB1 for the indicated time periods. After treatment, the cells were lysed and then assayed for luciferase activity. (D) NCI-H292 cells were pretreated with the indicated concentrations of a PKA inhibitor, H89, for 30 min and then stimulated with 50 ng/ml HMGB1 for 12 h. MUC8 mRNA expression was determined using quantitative real-time PCR. The values in B and D are the mean ± SEM of three samples ($n = 3$). The data represent three independent experiments.

4. Discussion

In this study, we showed that HMGB1 markedly increased MUC8 expression through the activation of JNK and PI3K/Akt signaling. Although HMGB1 markedly increased ROS production, ROS signaling was not involved in HMGB1-induced MUC8 expression. Furthermore, the NF-κB and CRE signaling pathways also had no effect on MUC8 expression in HMGB1-treated NCI-H292 cells. Our data indicate that HMGB1 could modulate the inflammatory response via the regulation of MUC8 expression in human epithelial NCI-H292 cells.

Several papers reported that MUC8 expression is regulated by inflammatory mediators. For example, Yoon et al. reported that tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β), or their combination induced MUC8 mRNA expression in passage-2 normal human nasal epithelial cells [6]. Seong et al. also reported that a mixture of inflammatory mediators induced MUC8 expression in cultured normal human nasal epithelial cells [7]. Furthermore, IL-1β also increased MUC8 expression in human airway epithelial cells [24]. IL-1β induces MUC8 expression through the activation of the ERK, p90 ribosomal S6 protein kinase 1 (RSK1), and CRE signaling pathways in human airway epithelial cells [24]. In our studies, like IL-1β, HMGB1-induced MUC8 expression via ERK, p38, and JNK activation (Fig. 2). However, CRE activation and NF-κB activation had no effect on HMGB1-induced MUC8 expression (Fig. 4). The promoter of MUC8 has not yet been fully identified. To identify the transcriptional factors that are associated with HMGB1-induced MUC8 expression, we need to conduct further experiments. However, we did demonstrate that HMGB1-induced MUC8 expression may be independent of NF-κB and CRE activation.

HMGB1 binds to DNA in a sequence-independent manner, and this binding has multiple functions. The major role of intracellular HMGB1 is to modify the DNA structure to facilitate transcription, replication, and repair [29]. A recent study has shown that HMGB1 is released from activated innate immune cells and functions as a proinflammatory mediator in endotoxemia, sepsis, arthritis, and local inflammation [13–15]. HMGB1 is released in a delayed manner from activated macrophages and, monocytes in response to stimulation with exogenous bacterial endotoxin and endogenous proinflammatory cytokines, such as TNF, IL-1, and interferon-γ [30–32]. HMGB1 also causes the delayed and biphasic release of TNF in human monocytes, thereby prolonging the inflammatory response [13]. *In vivo,* the intratracheal administration of HMGB1 to C3H/HeJ mice caused acute inflammatory lung injury with pulmonary production of IL-1β and TNF [33]. In the present study, our results showed that HMGB1 might significantly contribute to the production of mucins in human airway epithelial cells via the up-regulation of MUC8 gene expression. Our data support those of previous reports detailing the proinflammatory function of HMGB1.

HMGB1 activates intracellular signaling via receptor for advanced glycation end products (RAGE). The binding of RAGE to HMGB1 leads to the activation of NF-κB and MAPK [21,34,35]. The expression of RAGE could be regulated by several stimuli, such as TNF-α [36], angiotensin II [37], and HMGB1 [38]. NF-κB bound to the promoter of the RAGE gene and then increased RAGE expression. Thus, the activation of NF-κB participated in a positive feedback loop. HMGB1-induced RAGE expression could amplify intracellular signaling and then induce the inflammatory response. We also tested whether HMGB1 could induce RAGE-1 expression in human airway epithelial cells. However, HMGB1 did not induce RAGE expression in our study (data not shown).

Because HMGB1 had no effect on NF-κB transcriptional activity (Fig. 4), the RAGE levels may be not changed in human airway epithelial cells. However, we could not exclude the possibility that there exist HMGB1–RAGE signaling pathways because human airway epithelial cells express RAGE.

Taken together, our results suggest that HMGB1 increased MUC 8 expression through the activation of the JNK and PI3K/Akt signal pathways in human airway epithelial cells. It is possible that HMGB1 may be an important determinant of the clinical response in individuals with inflammatory diseases such as chronic rhinosinusitis, chronic bronchitis, and asthma.

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