Polyhexamethylene guanidine phosphate induces IL-6 and TNF-α expression through JNK-dependent pathway in human lung epithelial cells

Min-Seok Kim1, Jin-Young Han1,2, Sung-Hwan Kim1,2, Hyung-Young Kim1, Doin Jeon1 and Kyuhong Lee1,2

1National Center for Efficacy Evaluation of Respiratory Disease Product, Korea Institute of Toxicology, Jeongeup-si, Jeollabuk-do, 56212, Republic of Korea
2Department of human and environmental toxicology, University of Science & Technology, Daejeon, 34113, Republic of Korea

(Received April 10, 2018; Accepted April 27, 2018)

ABSTRACT — Polyhexamethylene guanidine phosphate (PHMG) is an antimicrobial biocide that causes severe lung injury accompanied with inflammation and subsequent fibrosis. Cytokines mediate the inflammatory response, leading to fibrosis in injured tissues. PHMG is known to induce the expression of various cytokines in vitro and in vivo. In the present study, we investigated the involvement of three MAPK subfamilies (JNK, p38 MAPK, and ERK) in PHMG-induced cytokine expression in A549 human lung epithelial cells. Our in vivo and in vitro data indicated that PHMG induced an increase in mRNA expression of IL-6 and TNF-α, and enhanced the phosphorylation of JNK, p38 MAPK, and ERK. Further, we investigated the involvement of MAPKs in PHMG-induced mRNA expression of IL-6 and TNF-α using JNK, p38 MAPK, and ERK inhibitors in A549 cells. Pre-treatment with the JNK inhibitor but not the p38 MAPK or ERK inhibitor, significantly attenuated the PHMG-induced mRNA expression of IL-6 and TNF-α. These results suggest that the activation of JNK is involved at least partially in the induction of IL-6 or TNF-α expression by PHMG in A549 cells.

Key words: PHMG, Inflammation, Pulmonary fibrosis, Cytokine, MAPKs

INTRODUCTION

Polyhexamethylene guanidine phosphate (PHMG) is a derivative of the polymeric guanidine family that has been widely used as an antiseptic in medicine and the food industry. It has been known to possess broad-spectrum bactericidal activity and low toxicity to humans (Rosin et al., 2001; Müller and Kramer, 2005). However, when inhaled, PHMG can cause severe lung injury, such as pulmonary fibrosis (Cheon et al., 2008; KCDC, 2011; Hong et al., 2014).

Previous studies have suggested that the inflammatory response induced by PHMG is significantly associated with the development of pulmonary fibrosis (Song et al., 2014; Kim et al., 2016, 2018). Cytokines, which are proteins secreted by all kinds of cells, act as fundamental mediators between cells (Lacy and Stow, 2011; Arango Dugue and Descoteaux, 2014). They also play a critical role in inflammatory responses underlying fibrosis induction in injured tissues (Borthwick et al., 2013; Lacy, 2015). Our previous studies showed that intratracheal instillation of PHMG in the lungs of mice increased the levels of various inflammation-related cytokines (Song et al., 2014; Lee et al., 2016; Kim et al., 2018). However, the mechanism underlying the induction of cytokine expression by PHMG is not well understood.

The mitogen-activated protein kinases (MAPKs) are implicated in the expression of inflammatory cytokines and downstream signaling events that lead to inflammation (Raingeaud et al., 1995; Cargnello and Roux, 2011; Arthur and Ley, 2013). The MAPK family consists of three subfamilies, namely: the e-Jun N-terminal kinase (JNK), the p38 mitogen-activated protein kinase (p38 MAPK), and the extracellular signal-regulated kinase (ERK) subfamilies (McCubrey et al., 2006; Kim and Choi, 2010). MAPKs are involved in the regulation
of cytokine gene expression by transmitting extracellular signals to the nucleus (Plotnikov et al., 2011). However, it has not yet been elucidated whether PHMG can induce cytokine expression via the activation of MAPKs.

Therefore, in the present study, we investigated the involvement of MAPKs in the induction of inflammatory cytokine expression by PHMG in A549 human lung epithelial cells.

MATERIALS AND METHODS

Animal experiments

C57BL/6N mice (Seven-week-old males) were purchased from Orient Bio Inc. (Seongnam, Korea). An animal room was maintained at a temperature of 22 ± 3°C, relative humidity of 50 ± 20%, light intensity of 150-300 Lux, and light/dark cycle of 12/12 hr. The air ventilation in the animal room was refreshed 10-20 times/hr. Animals were provided pelleted food (PMI Nutrition International, Richmond, IN, USA) and UV-irradiated (Steriltron SX-1; Daeyoung Inc., Seoul, Korea) and filtered (through a 1-μm filter) tap water ad libitum. Mice were used in experiments after 6 days of acclimation. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Korea Institute of Toxicology (IACUC #1704-0158).

Cell culture and treatment

A549 cells (a human alveolar epithelial cell carcinoma line) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in Dulbecco’s modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F-12, Gibco, Palo Alto, CA, USA) supplemented with 5% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. A459 cells were plated at 5 × 10⁵ cells/well in 6-well plates, cultured for 24 hr, and then pre-treated with or without MAPK inhibitors (Sigma-Aldrich, St. Louis, MO, USA) 1 hr prior to the addition of PHMG (Seongnam, Korea).

Cell viability

A549 cells were plated at a density of 1 × 10⁴ cells/well in 48-well plates and cultured for 24 hr, and treated with or without MAPK inhibitors for 24 hr. Cell viability was measured by the PreMix WST-1 Proliferation Assay, according to the manufacturer’s protocol (Takara Bio Inc., Shiga Japan). Premix WST-1 reagent was added after 24 hr, and incubation was continued for an additional 1 hr. Then, the absorbance was measured using a Model 680 Plate Reader at a wavelength of 490 nm (Bio-Rad Laboratories, Hercules, CA, USA).

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA from A549 cells and lung tissue of mice was isolated using the RNaseasy Mini Kit (Qiagen, Valencia, CA, USA) according to the protocol provided by the manufacturer. RNA was reverse transcribed to complementary DNA using the SuperScript™ VILO™ Master Mix (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions in a T-Gradient Thermoblock (Biometra, Gottingen, Germany). qRT-PCR was performed using the Power SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) on the StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). Sequences of the mouse gene-specific primers used were as follows: glyceraldehyde 3-phosphate dehydrogenase, GAPDH (forward, 5'-GGGTGTCGAACAGGAAAA-T'-3' and reverse, 5'-GTCATGAGCCCCTTTCTACAAT'-3'); IL-6 (forward, 5'-TTTTCTGCAAGTGCACTCATCG'-3' and reverse, 5'-GTTTTCTGCAAGTGCACTCATCG'-3'); and TNF-α (forward, 5'-ACGCGATGGATCTCAAAGC-3' and reverse, 5'-GGGCGTGGAGCACGGTAGT'-3'). Sequences of the human gene-specific primers used were as follows: glyceraldehyde 3-phosphate dehydrogenase, GAPDH (forward, 5'-ACCAGAAGACTGCTGAGG-3' and reverse, 5'-TTCAAGCTAGGATCCCTT-3'); IL-6 (forward, 5'-TGCGTGAACAGATGGATGCT-3' and reverse, 5'-TCTGCAAGCTCTGGCTT-3'); and TNF-α (forward, 5'-TCTGCAAGCTCTGGCTT-3' and reverse, 5'-GAGGACCTGGAGATGATGGA-3'). The gene expression level of each transcript was normalized to that of GAPDH. Relative gene expression was calculated using the ΔΔCt method, where Ct = threshold cycle.

Immunoblotting

A549 cells and lung tissue from mice were lysed in ice-cold RIPA Buffer (Pierce Biotechnology, Rockford, IL, USA) containing the protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), and centrifuged at 13,000 × g for 20 min at 4°C. The protein concentration was determined using the BCA Kit for protein determination. Proteins in the lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon-P membrane (Millere, Billerica, MA, USA). The membrane was blocked with 5% nonfat milk and incubated with primary antibodies against anti-MAPK (MAPK family antibody sampler kit #9926 and Phospho-MAPK Family Antibody
Sampler Kit #9910, Cell Signaling Technology, Beverly, MA, USA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, and then with horse-radish peroxidase-(HRP) conjugated secondary antibodies (Santa Cruz) for 2 hr at room temperature. Immunoreactive proteins were detected by the estimation of enhanced chemiluminescence using the SuperSignal® West Pico Stable Peroxide Solution (Thermo Scientific, Rockford, IL, USA). The intensity of bands was quantified by densitometry using ImageJ, and the results were normalized to β-actin levels.

RESULTS

Our recent study reported that intratracheal instillation of PHMG in the lungs of mice induced the expression of inflammatory cytokines such as IL-6, TNF-α, IL-1β, and CCL2 (Song et al., 2014; Lee et al., 2016; Kim et al., 2018). As shown in Fig. 1, five days after PHMG instillation in the lungs of mice, there was a significant increase in the mRNA expression of IL-6 and TNF-α. Next, we investigated whether PHMG induces the phosphorylation of the members of the MAPKs family, including JNK, p38 MAPK, and ERK, in the lungs of mice. The results showed that PHMG induced a marked increase in the phosphorylation levels of JNK, p38 MAPK, and ERK in the lungs of mice (Fig. 2).

The above experiment thus demonstrated that PHMG treatment induces phosphorylation of MAPKs in the lungs of mice. The MAPKs play a critical role in the transcriptional regulation of cytokine expression (Leonard et al., 1999; Dong et al., 2002; Turner et al., 2014). A further study was performed to examine whether MAPKs are involved in the expression of cytokines by PHMG in A549 cells. In order to examine the effect of PHMG on cytokine expression in A549 cells, we evaluated the mRNA expression of IL-6 and TNF-α after treating the cells with PHMG for different time periods. As shown in Fig. 3, PHMG significantly increased the mRNA expression of IL-6 and TNF-α at 4, 8, 24, and 48 hr post treatment. Moreover, the phosphorylation of JNK, p38 MAPK, and ERK markedly increased after the PHMG stimulation of A549 cells, indicating that PHMG activated the MAPK signaling pathway in A549 cells (Fig. 4).

We investigated the MAPKs involved in the mRNA expression of IL-6 and TNF-α by PHMG in A549 cells, and tested the concentrations of MAPK inhibitors ranging from 1 nM to 10 μM on A549 cells to evaluate their non-cytotoxicity levels. The results showed that up to a concentration of 10 μM after 24 hr of treatment, the JNK inhibitor SP600125 and p38 MAPK inhibitor SB203580 did not affect cell viability; the ERK inhibitor FR180204 did not cause cytotoxicity up to a concentration of 1 μM (Fig. 5). Thus, the following experiments were performed using the inhibitors at a concentration of 100 nM or less. As shown in Fig. 6, pre-treatment of A549 cells with 100 nM of the JNK inhibitor SP600125 significantly decreased the mRNA expression of IL-6 and TNF-α by PHMG, but the p38 MAPK inhibitor SB203580 or ERK inhibitor FR180204 did not.

![Fig. 1.](image-url)

Effect of PHMG on the expression levels of IL-6 and TNF-α mRNA in the lungs of mice. Mice in the control (n = 3) and PHMG (n = 3) groups received a single intratracheal instillation of saline and 1.1 mg/kg PHMG, respectively. The mice were sacrificed on day 5 after instillation. IL-6 (A) and TNF-α (B) mRNA levels were measured by qRT-PCR. Values represent mean ± standard deviation (S.D.). Statistical differences are indicated by *P < 0.05, compared with the control group.
Fig. 2. Effect of PHMG on the activation of MAPKs in the lungs of mice. Mice in the control and PHMG groups received a single intratracheal instillation of saline and 1.1 mg/kg of PHMG, respectively. The mice were sacrificed on day 5 after instillation. (A) Lung lysates were evaluated for protein expression of phospho-JNK, JNK, phospho-p38 MAPK, p38MAPK, phospho-ERK, and ERK by western blotting. (B) Densitometry of phospho-JNK, phospho-p38 MAPK, and phospho-ERK bands was performed by ImageJ, and calculated in relation to controls after normalization with the values for \( \beta \)-actin. Values represent mean ± S.D. Statistical differences are indicated by **\( p < 0.01 \), compared with the control group. Data were analyzed using the Student’s t test.

Fig. 3. Effect of PHMG on the expression levels of \( IL-6 \) and \( TNF-\alpha \) mRNA in A549 cells. A549 cells were treated with 2.5 \( \mu \)g/mL of PHMG for the indicated times periods. \( IL-6 \) (A) and \( TNF-\alpha \) (B) mRNA levels were measured by qRT-PCR. Values represent mean ± S.D. of three replicates. Statistical differences are indicated by *\( p < 0.05 \) and **\( p < 0.01 \), compared with the control group. Data were analyzed using the Dunnett’s test.
DISCUSSION

The cytokine-mediated inflammatory response is considered to play a crucial role in the progression of PHMG-induced pulmonary fibrosis (Kim et al., 2016, 2018). Therefore, the exploration of the regulatory mechanisms underlying the inflammatory response is important to elucidate the mechanism of PHMG-induced lung injury. Kim et al. (2015) reported that PHMG exposure increases IL-8 expression by activating the nuclear factor kappa B (NF-κB) signaling pathway in murine RAW264.7 macrophages. However, the intracellular signaling mechanisms by which PHMG induced inflammatory cytokine production have not yet been clearly elucidated.

Recent studies have demonstrated the elevated expression of various inflammatory-related cytokines and chemokines such as IL-6, TNF-α, IL-1β, CCL2, and CCL17 in the lungs of PHMG-treated mice (Song et al., 2014; Lee et al., 2016; Kim et al., 2018). Our in vivo data have also showed that the mRNA expression of IL-6 and

Fig. 4. Effect of PHMG on the activation of MAPKs in A549 cells. A549 cells were treated with 2.5 μg/mL of PHMG for the indicated times. (A) The protein expression of phospho-JNK, JNK, phospho-p38 MAPK, p38MAPK, phospho-ERK, and ERK was evaluated in the lysates by western blotting. (B) Densitometry of phospho-JNK, phospho-p38 MAPK, and phospho-ERK bands was performed by ImageJ, and calculated in relation to controls after normalization with the values for β-actin. Values represent mean ± S.D. of three replicates. Statistical differences are indicated by *p < 0.05 and **p < 0.01, compared with the control group. Data were analyzed using the Dunnett’s test.

Fig. 5. The viability of A549 cells after treatment with MAPK inhibitors. A549 cells were treated with 1 nM, 10 nM, 100 nM, 1 μM or 10 μM of MAPK inhibitors for 24 hr. Cell viability was measured by WST-1 assay. Values represent mean ± S.D. of three replicates. Statistical differences are indicated by *p < 0.05 and **p < 0.01, compared with the control group. Data were analyzed using the Dunnett’s test.
TNF-α was increased in the lungs of PHMG-treated mice (Fig. 1). The cytokines IL-6 and TNF-α are elevated in most, if not all, inflammatory states, and are known to be induced by various stimuli in the lungs (Hao et al., 2000; Rittirsch et al., 2008; Guo et al., 2016; Xu et al., 2016). MAPKs are a large family of proline-directed, serine/threonine kinases that are activated via dual phosphorylation of certain amino acid residues through multiple signal transduction pathways (Lee et al., 1994; Raingeaud et al., 1995; Son et al., 2011). The activation of MAPKs is involved in a number of biological processes, including cell proliferation, differentiation, and death, inflammation, and responses to environmental stresses (Zhang and Liu, 2002; Winter-Vann and Johnson, 2007). In addition, MAPKs play a crucial role in regulating the expression of proinflammatory cytokines (Leonard et al., 1999; Dong et al., 2002; Turner et al., 2014). Our in vivo data revealed an increase in the levels of IL-6 and TNF-α mRNA, along with enhanced phosphorylation of JNK, p38 MAPK, and ERK in the lungs of PHMG-treated mice (Figs. 1 and 2). Thus, it is possible that MAPK signaling pathways may be involved in the induction of cytokine expression by PHMG.

Alveolar epithelial type II (ATII) cells act as defenders and are a key component of lung tissues; however, they are also the targets of many lung diseases, including acute respiratory distress syndrome, ventilator-induced lung injury, and pulmonary fibrosis (Mao et al., 2015). The A549 human lung epithelial cell line is the most popular model of ATII cells, which provides a platform for studying pulmonary host defense by secreting numerous cytokines that modulate the functions of inflammatory cells (Nogee et al., 1993; Wang et al., 2007). In the present study, we investigated the involvement of MAPKs in PHMG-induced cytokine expression in A549 cells. We examined the effect of PHMG on the mRNA expression of IL-6 and TNF-α in A549 cells. We found that PHMG significantly increased the mRNA expression of IL-6 and TNF-α at several time points after culturing (Fig. 3). We also found that PHMG enhanced the phosphorylation of JNK, p38 MAPK, and ERK in these cells (Fig. 4). Next, to investigate the involvement of MAPKs in PHMG-induced expression of IL-6 and TNF-α, we used MAPK inhibitors. We found that the pre-treatment of A549 cells with the JNK inhibitor, SP600125, but not the p38 MAPK inhibitor, SB203580, or ERK inhibitor, FR180204, significantly decreased the levels of IL-6 and TNF-α mRNA following PHMG treatment (Fig. 6).

JNKs are generally associated with cellular responses to diverse stress conditions and are also involved in the expression of various inflammatory cytokines, including IL-6 and TNF-α (Sugden and Clerk, 1998; Das et al., 2009; Kim and Choi, 2010; Laviola et al., 2013; Oh et al., 2016). Zhang et al. (2014) noted that suppression of the JNK signaling pathway inhibited TNF-α and IL-6 expression in lipopolysaccharide (LPS)-stimulated astro-
cytes. Lou et al. (2014) also showed that the JNK inhibitor dramatically reduced the oleic acid (OA)-induced increase of TNF-α transcription in RAW 264.7 cells. These results suggest that PHMG-induced IL-6 or TNF-α expression in human alveolar epithelial cells is partly mediated through the activation of the JNK signaling pathway. This study may provide insights into the mechanism underlying the PHMG-induced inflammatory response.

ACKNOWLEDGMENTS

This study was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea [HI16C0062] and by Korea Institute of Toxicology, Republic of Korea [KK-1803].

Conflict of interest—— The authors declare that there is no conflict of interest.

REFERENCES


