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In vitro inflammatory effects of polyhexamethylene biguanide through NF-κB activation in A549 cells

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ABSTRACT

Polyhexamethylene biguanide (PHMB) is a member of the polymeric guanidine family, which is used as a biocide and preservative in industrial, medicinal, and consumer products. Some studies reported that polyhexamethylene guanidine phosphate, which is also a member of the guanidine family, induced severe inflammation and fibrosis in the lungs. However, limited studies have evaluated the pulmonary toxicity of PHMB associated with inflammatory responses. The aim of this study was to elucidate the inflammatory responses and its mechanisms induced by PHMB in lung cells. A549 cells exposed to PHMB showed decreased viability, reactive oxygen species (ROS) generation, inflammatory cytokine secretion, and nuclear factor kappa B (NF-κB) activation. The cells showed dose-dependent cytotoxicity and slight generation of ROS. PHMB triggered inflammatory cytokine secretion and NF-κB activation by modulating the degradation of IκB-α and the accumulation of nuclear p65. TNF-α plays important roles in IL-8 expression as well as NF-κB activation. Moreover, IL-8 production induced by PHMB was completely suppressed by a NF-κB inhibitor, but not by a ROS scavenger. In conclusion, we suggest that PHMB induces the inflammatory responses via the NF-κB signaling pathway.

1. Introduction

Polyhexamethylene biguanide (PHMB) is a cationic agent with low cytotoxicity and high antimicrobial activity (Müller and Kramer, 2008). PHMB is adsorbed onto the surface of the acidic and negatively charged phospholipid bilayer through the interaction of its biguanide groups with the polar headgroups of the lipids (Ikeda et al., 1984). This disrupts the organization of the phospholipid bilayer, leading to increased fluidity, permeability, and loss of integrity, followed by the death of the organism, such as bacteria, amoeba, and yeast (Barker et al., 1992; Broxton et al., 1983; Elsztein et al., 2008). However, PHMB does not interact with neutral phospholipids in cell membranes, which explains its lack of interaction with human cells and low toxicity in humans (Ikeda et al., 1984). PHMB has been widely used as a biocide and preservative in industrial (e.g. swimming pool disinfectants), medicinal (e.g. wound dressings), and consumer products (e.g. contact lens solution, wet wipes, and mouthwash). In particular, the registrant for PHMB has indicated that approximately 95% of its use is in swimming pool and spa facilities (United States Environmental Protection Agency, 2004).

There are limited reports of the adverse health effects of PHMB exposure in humans. However, PHMB is classified as being linked to acute inhalation toxicity, with an LC_{50} of 0.37 mg/L in rats according to the Organization for Economic Cooperation and Development (OECD) test guideline 403 (Scientific Committee on Consumer Safety, 2015). Moreover, PHMB has a strong intrinsic irritation potential for mucous membranes as identified by The OECD test guideline 405 for ocular irritancy (Scientific Committee on Consumer Safety, 2015). Some in vitro studies have reported that several kinds of lens solution that contain PHMB as an antimicrobial agent induced significant cytotoxic effect and inflammatory response in corneal epithelial cells (Dutot et al., 2008; Erdinest et al., 2013). Moreover, there are evidences that cationic PHMB can bind to mucins, which are negatively charged molecules (Muya et al., 2008), causing increased susceptibility to PHMB in organs with mucous membranes. The mucosae of the eye and respiratory tract have similar structures, in that they are both protected by mucous layers (Belser et al., 2012). Similar to their toxic effects on the eyes, PHMB solutions at a concentration of 20% are considered to be moderately irritating to the respiratory tract (Scientific Committee on Consumer Safety, 2015).

In Korea, polyhexamethylene guanidine phosphate (PHMG-p), which is also a member of the polymeric guanidine family, has been used as a disinfectant in humidifiers. PHMG-p is generated as its aerosol form by humidifiers, which can cause lung disease that is pathologically characterized by bronchiolitis and fibrosis. This has led to the death of many people, including pregnant women and children (Kim et al., 2014). In vivo and in vitro studies have demonstrated that PHMG-p induced NF-κB-mediated inflammatory responses such as the release of cytokines and chemokines in the lungs (Kim et al., 2016; Kim et al.,

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2015). However, the pulmonary toxicity of PHMB associated with inflammatory responses remains unclear.

The lungs are vulnerable to oxidative injury caused by a wide range of toxicants that are inhaled continuously from the air because of their unique structure, which provides an enormous surface area for ambient air and an oxygen-rich environment compared to other organs (Azad et al., 2008). A variety of inhaled toxicants often generate reactive oxygen species (ROS), which contribute to inflammatory and other pathobiological responses of the airway via the activation of kinases and transcription factors common to many response genes (Martin et al., 1997). Inflammation plays a major role in various lung injuries and diseases. The majority of inflammatory reactions are mediated by pro-inflammatory cytokines, which are regulated in part by nuclear factor kappa B (NF-κB). In non-stimulated cells, NF-κB is located in the cytosol as a dimer of p50 and p65 bound to a cytoplasmic inhibitor called IκB-α. In response to inflammatory stimuli, IκB-α dissociates from the IκB-NF-κB complex, allowing NF-κB to translocate into the nucleus where it induces gene transcription by binding to the promoter region of genes, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-8 (Schmitz, 1995). These inflammatory mediators are associated with several inflammatory lung diseases such as bronchiolitis, chronic obstructive pulmonary disease, asthma, and fibrosis (Hosseinian et al., 2015). In particular, IL-8 induces the recruitment and activation of neutrophils, which subsequently produce more ROS and contribute to local tissue destruction at sites of inflammation (Casini et al., 1997; Hammond et al., 1995).

The aim of this study is to elucidate the inflammatory responses and its mechanisms induced by PHMB in lung cells. The WST-1 assay was conducted to determine the cytotoxic effect of PHMB in A549 cells. ROS generation was measured by using the DCF-DA assay, and the ELISA assay was performed to measure the release of inflammatory cytokines such as TNF-a, IL-6, and IL-8. NF-κB activation was identified by western blot. Furthermore, various inhibitors were used to elucidate the mechanisms of PHMB-induced inflammatory responses.

2. Materials and methods

2.1. Cell culture

A549 human lung adenocarcinoma cells were obtained from Korean Cell Line Bank (Seoul, Korea), and were cultured in RPMI supplemented with 5% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in an atmosphere of 5% CO_2/95% air under saturating humidity.

2.2. Cell viability assay

The viability of A549 cells was assessed using the WST-1 assay. The cells were seeded onto 96-well plates at a density of 5×10^3 cells/well. After 24 h of culture, cells were treated with PHMB (CAS No. 32289-58-0, Scunder®MP100, purity ≥98%; Shanghai Scunder Industrial Co., Ltd., Shanghai, China) at concentrations ranging from 0 to 80 μg/mL for 1, 6, and 24 h. Ten microliters of WST-1 reagent (Roche Diagnostics, Montclair, NJ, USA) was added to each well according to the manufacturer's instructions, and the plates were incubated in 5% CO_2 at 37 °C for 2 h. Cell viability was quantified by measuring the absorbances at 440 nm and 690 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The half maximal inhibitory concentration (IC_{50}) was estimated from a 4-parameter logistic fit of percentage activity versus concentration ($r^2 \geq 0.99$ for all curves).

2.3. Intracellular reactive oxygen species production using the DCFH-DA assay

The dichlorodihydrofluorescein diacetate (DCFH-DA) method was performed to measure the production of intracellular ROS in A549 cells treated with PHMB. The cells were seeded in 48-well plates at an initial density of 7×10^4 cells/well. After 48 h, the cells were exposed to PHMB (0, 5, 10, and 20 μg/mL) for 1, 3, and 6 h. Cells were treated with 10 mM hydrogen peroxide (H_2O_2) for 30 min as a positive control. DCFH-DA was loaded for 1 h as an intracellular ROS indicator. Each well was washed twice with phosphate-buffered saline (PBS), and the cells were lysed by adding 300 μL of 0.1 N NaOH for 3 min. DCF (green fluorescence) production was measured by using a LS50B fluorimeter (Perkin-Elmer, Shelton, CT, USA) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. In addition, a Zeiss LSM 700 Laser Confocal Microscope (Thornwood, NY, USA) was used to observe the fluorescence.

2.4. Cytokine measurement using the ELISA

A549 cells were exposed to PHMB (0, 5, 10, and 20 μg/mL) for 1, 3, and 6 h, and the supernatant was collected at specified time points. BAY 11-7082 (NF-κB inhibitor; B5556; Sigma-Aldrich, St. Louis, MO, USA), WP9QY (TNF-α receptor inhibitor; sc-358755; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and *N*-acetylcysteine (NAC) (ROS antioxidant; A9165; Sigma-Aldrich) were used to establish the signaling pathway associated with PHMB-stimulated IL-8 expression. Cells were treated with BAY 11-7082 (10 and 50 μM), WP9QY (10 and 30 μM), and NAC (0.1, 1, and 20 mM) for 1 h before treatment with 20 μg/mL PHMB. Levels of TNF-α (555,212; BD Biosciences, San Diego, CA, USA), IL-6 (DY206; R&D systems, Minneapolis, MN, USA), and IL-8 (DY208; R&D systems) were quantified according to the manufacturer's protocols.

2.5. Western blot analysis

A549 cells were seeded in a 100-mm dish. When 70% confluence was reached, the cells were treated with PHMB (0, 5, 10, and 20 μg/mL) for 3 h. The cells were washed twice with PBS, and lysed with 600 μL of radioimmunoprecipitation assay buffer (786-489; G-Biosciences, St. Louis, MO, USA) followed by incubation on ice for 5 min. The cells were then collected into a 1.5-mL microtube by scraping, and were incubated on ice for 10 min. Cells were subsequently centrifuged at 13,000 ×*g* for 15 min at 4 °C. To prepare the cytoplasmic and nuclear fractions from A549 cells, cells were washed twice with PBS and 500 μL of PBS was added; cells were collected into a 1.5-mL microtube by scraping. Cells were centrifuged at 6000 rpm for 5 min at 4 °C. The supernatants were discarded and 200 μL of cytoplasmic extract buffer (10 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.3% NP-40, and protease inhibitors) was added, followed by incubation on ice for 15 min with vortexing every 5 min. The cells were then centrifuged at 3000 rpm for 5 min at 4 °C. The supernatants were transferred to a new microtube, which is used as the cytoplasmic extract. The remained pellet was resuspended in the cytoplasmic extract buffer in the absence of NP-40, followed by centrifugation at 3000 rpm for 5 min at 4 °C. The supernatant was discarded. The pellet was resuspended in the same volume of nuclear extract buffer (20 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.3% NP-40, and protease inhibitors) and incubated for 30 min on ice with vortexing every 10 min. After centrifugation at 14,000 rpm for 5 min at 4 °C, the supernatant was harvested, which was used as the nuclear extract. The samples were quantified by using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Samples were denatured by treating with a buffer containing 2% SDS, 6% 2-mercaptoethanol, 40% glycerol, 0.004% bromophenol blue, and 0.06 M Tris-HCl at 90–100 °C for 6 min, and cooling at room temperature for 5 min. Denatured total proteins (10 μg) were loaded in each lane of Mini-PROTEAN TGX gels (BIO-RAD Laboratories, Richmond, CA, USA). After subjecting to gel electrophoresis at 150 V for 40 min, the proteins were transferred onto a 0.2 mm polyvinylidene fluoride (PVDF) membrane (170-4156; BIO-RAD Laboratories) by using the Trans-Blot Turbo system (BIO-RAD Laboratories). The membrane was blocked in 5% skim milk/TBST for 1 h at room

temperature and incubated with primary and secondary antibodies. The primary antibodies used were rabbit anti-IκB-α antibody at a 1:6000 dilution (#9242; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-p65 antibody at a 1:6000 dilution (sc-372; Santa Cruz Biotechnology), rabbit anti-β-actin antibody at a 1:4000 dilution (#4970; Cell Signaling Technology) or mouse anti-PCNA antibody at a 1:1000 dilution (sc-56; Santa Cruz Biotechnology) in blocking buffer. The secondary antibody used was horseradish peroxidase conjugates (AAC10P; Serotec, Oxford, UK) diluted 1:10,000 in blocking buffer. The membrane was incubated with ECL (170-5060; BIORAD Laboratories) for 5 min, and was developed by using an automatic X-ray film processor (JP-33; JPI Healthcare, Seoul, Korea). The ImageJ software was used for quantification. The densities of each band were normalized to those of β-actin or PCNA.

2.6. Data analysis

Sigma Plot (Jandel Scientific, San Rafael, CA, USA), Excel (Microsoft, Redmond, WA, USA), and SPSS version 21.0 (IBM SPSS Inc., Chicago, IL, USA) were used to analyze the data. The results of each assay are expressed as mean ± standard deviation. Differences between groups were assessed by one-way ANOVA followed by Duncan's post-hoc test. Statistical significance was accepted at $p < 0.05$ or 0.01.

3. Results

3.1. Effects of PHMB on viability and reactive oxygen species generation

PHMB reduced the viability of A549 cells in a dose-dependent manner depending on the exposure time (Fig. 1). When exposed to PHMB of concentrations > 10 μg/mL for 1 and 6 h, significant cytotoxicity was observed. When exposed to PHMB for 24 h, significant cytotoxicity was observed at all concentrations. The IC_{50} values of cells exposed to PHMB for 1, 6, and 24 h were 32.52, 12.49, and 5.60 μg/mL, respectively.

The fluorescent dye DCFH-DA was used to measure intracellular ROS generation induced by PHMB in A549 cells. The generation of ROS by H_2O_2 (positive control) showed a significant increase of 3.32-fold, as confirmed by the confocal images. However, significant ROS generation was induced by 20 μg/mL PHMB when exposed for 3 h (Fig. 2A). The production of ROS in A549 cells treated with 20 μg/mL PHMB for 3 h was 1.39-fold higher than the corresponding levels of the control-treated group. Similar results were observed by confocal microscopy with DCFH-DA staining (Fig. 2B).

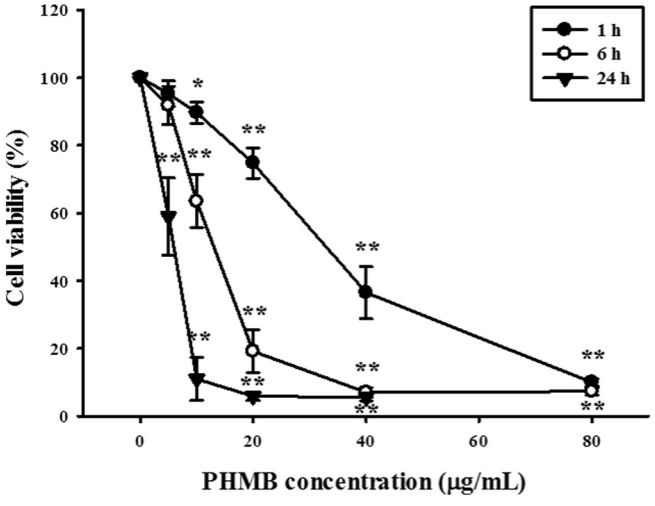

Fig. 1. Cell viability of A549 cells exposed to PHMB. The cells were incubated with PHMB (0–80 μg/mL) for 1 h (black circles), 6 h (white circles), and 24 h (black triangle). The WST-1 assay was carried out. Cell viability was expressed as a percentage of the control. Each value represents the mean ± standard deviation from double experiments in triplicate, representative of three separate experiments. Values significantly different from control: $^{*}p < 0.05$, $^{**}p < 0.01$.

3.2. Effects of PHMB on the regulation of inflammatory cytokines

The regulation of cytokines was assessed by the ELISA assay to evaluate the inflammatory responses induced by PHMB in A549 cells. The cells exposed to PHMB for 1, 3, and 6 h showed significantly upregulated levels of TNF-α, IL-6, and IL-8. Although the level of TNF-α was the lowest compared with the levels of IL-6 and IL-8, 20 μg/mL PHMB significantly increased the release of TNF-α at all exposure times (Fig. 3A). The levels of IL-6 and IL-8 were dose-dependently increased by PHMB (Fig. 3B and C). The level of IL-8 was the highest among the 3 cytokines

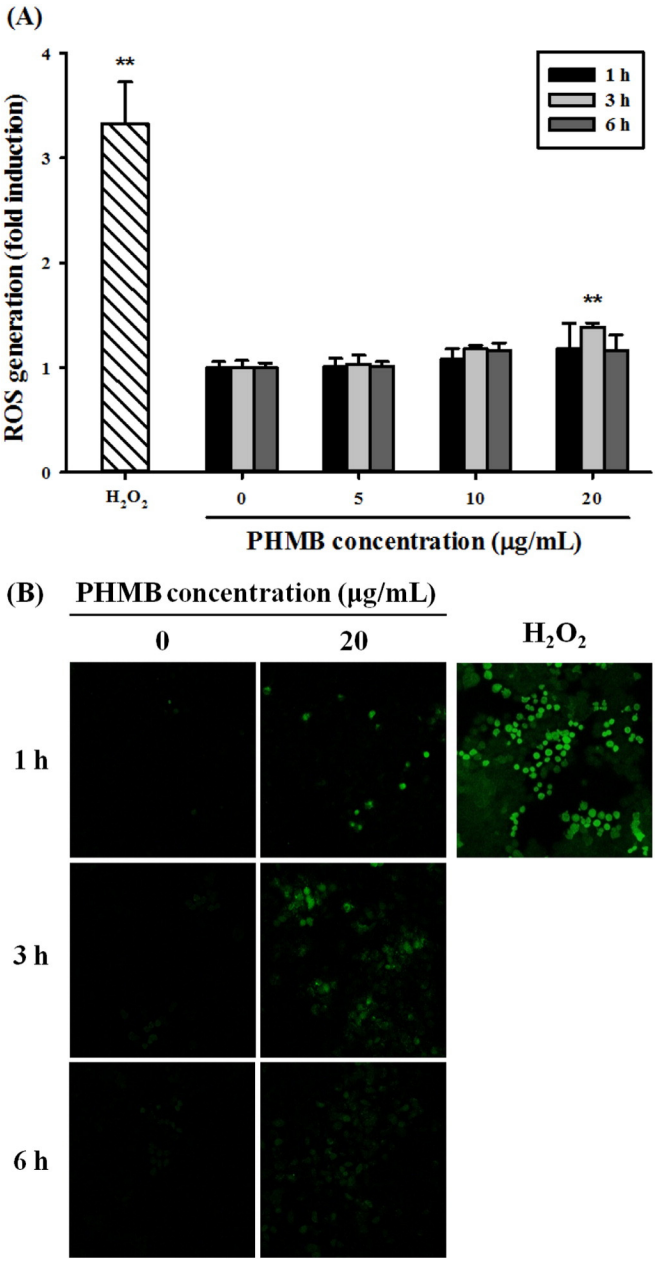

Fig. 2. Effect of PHMB on reactive oxygen species (ROS) generation in A549 cells. The cells were incubated with PHMB for 1, 3, and 6 h. Cells were treated with 10 mM hydrogen peroxide (10 mM) as a positive control for 30 min. ROS was examined by (A) fluorescence reading or (B) visualizing the reported DCF. The results are given as fold induction above the control and are expressed as mean ± standard deviation from double experiments in duplicate, representative of three separate experiments for each data point. Values significantly different from control: $^{**}p < 0.01$.

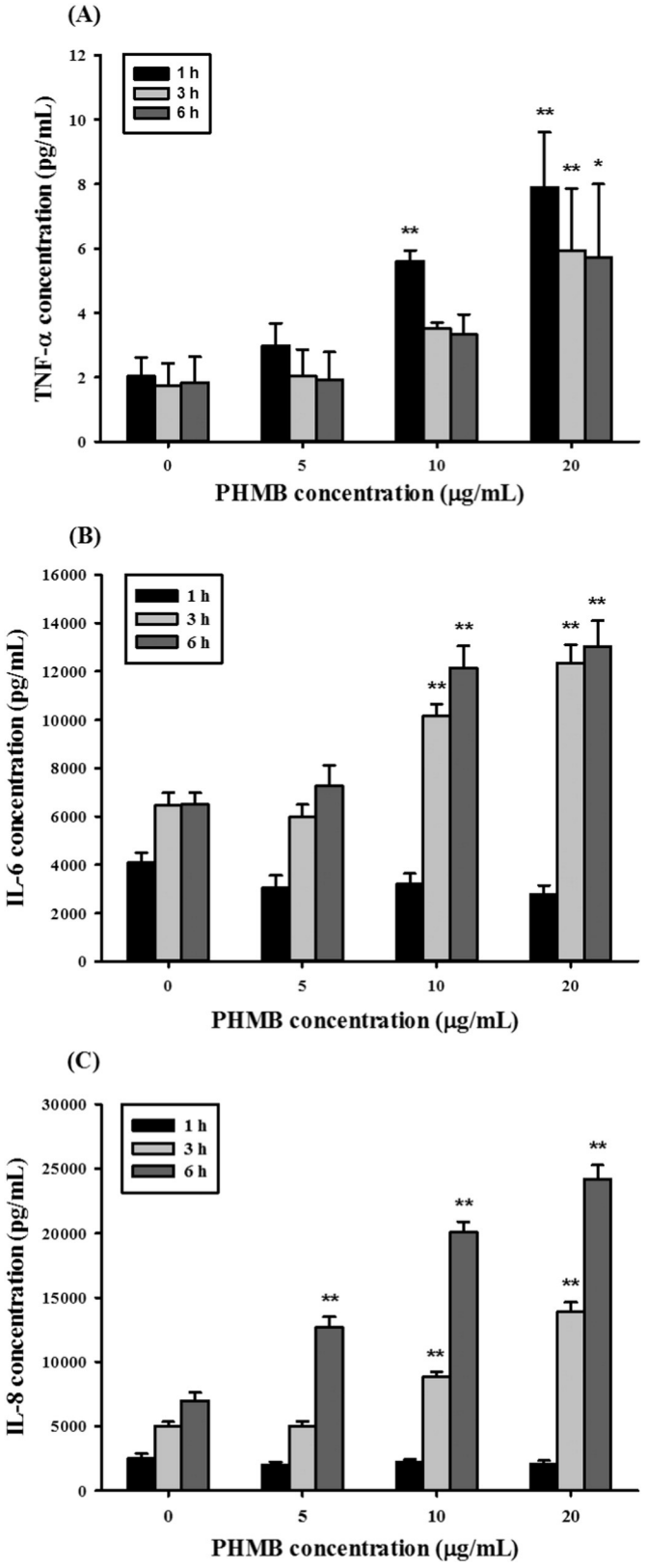

Fig. 3. Expression of cytokines in A549 cells exposed to PHMB. (A) TNF-α, (B) IL-6, and (C) IL-8 levels were measured in cells exposed to PHMB (0–20 μg/mL) for 1, 3, and 6 h. The results are expressed as mean ± standard deviation from double experiments in duplicate, representative of four separate experiments for each data point. Values significantly different from control: $^{*}p < 0.05$, $^{**}p < 0.01$.

measured. The concentration of IL-8 in A549 cells exposed to 20 μg/mL PHMB for 6 h was 24,195 pg/ml, which was the highest level among the exposure times.

3.3. Effects of PHMB on the NF-κB signaling pathway

Using western blot analysis, the expression of proteins such as IκB-α and p65 in A549 cells exposed to PHMB for 3 h was evaluated (Fig. 4A). The expression of IκB-α protein was significantly degraded at PHMB concentrations >10 μg/mL (Fig. 4B). Although the expression level of the cytosolic p65 protein did not differ among the groups, PHMB significantly induced the expression of the nuclear p65 protein in a dose-dependent manner (Fig. 4C and D). The cells pre-treated with WP9QY showed a significant increase in the expression level of IκB-α protein compared to the corresponding level in cells treated with 20 μg/mL PHMB alone. In addition, the expression level of IκB-α protein in cells pre-treated with WP9QY and the control-treated group did not differ significantly. Similarly, the cells pre-treated with WP9QY showed a significant decrease in the expression level of nuclear p65 protein compared to that in cells treated with 20 μg/mL PHMB alone. However, the expression level of nuclear p65 protein in the group pre-treated with WP9QY was significantly higher than that in the control-treated group.

BAY 11-7082 and WP9QY were used to elucidate the role of the NF-κB signaling pathway in PHMB-induced IL-8 production. Fig. 5 shows the levels of IL-8 in A549 cells exposed to PHMB in the absence or presence of inhibitors such as BAY 11-7082, WP9QY, and NAC. WP9QY partially inhibited IL-8 production stimulated by 20 μg/mL PHMB. Despite an increase in the treatment concentration of WP9QY, the levels of IL-8 in cells treated with inhibitors were significantly higher than that in the control-treated group. The pre-treatment with BAY 11-7082 significantly suppressed the levels of IL-8 induced by PHMB. In particular, the levels of IL-8 expressed in cells pre-treated with 50 μM BAY 11-7082 and the control-treated group did not differ significantly. NAC was used to elucidate the relationship between PHMB-induced production of ROS and IL-8. Fig. 5B shows the levels of IL-8 in A549 cells exposed to PHMB in the absence or presence of NAC. NAC slightly inhibited IL-8 production induced by 20 μg/mL PHMB. However, IL-8 levels in the NAC-treated groups were significantly higher than the corresponding levels in the control-treated group. The levels of IL-8 did not differ significantly in all NAC-treated groups.

4. Discussion

To date, PHMB is the only “chlorine free” and “not oxidizing” solution for targeting microbes in swimming pool and spa facilities. PHMB in its ionized form in aqueous solution does not easily disperse into the air since it is a non-volatile agent with a low vapor pressure of 1.32×10^{-7} Pa (Chang, 2008). The inhalation toxicity of PHMB has been overlooked owing to the low risk of exposure via inhalation. PHMB is classified as “fatal if inhaled” by the OECD test guideline 403 for acute inhalation toxicity (Scientific Committee on Consumer Safety, 2015). In addition, a total of 118 individual cases submitted to the United States Environmental Protection Agency Office of Pesticide Programs involving the use of PHMB-containing products in swimming pool were reviewed to determine the effects of exposure to PHMB (United States Environmental Protection Agency, 2004). The most common symptoms in cases of exposure via inhalation were respiratory irritation (75%) and coughing/choking (38%). The regulatory authorities have concluded PHMB as an irritating agent on the respiratory tract. However, the toxicity study of PHMB mainly focused on the eye and liver (Creppy et al., 2014; Erdinest et al., 2013). The cytotoxicity and inflammatory properties of PHMB are responsible in part for the irritation symptoms. To the best of our knowledge, this study was the first to elucidate the toxic effects of PHMB and its mechanism in lung cells.

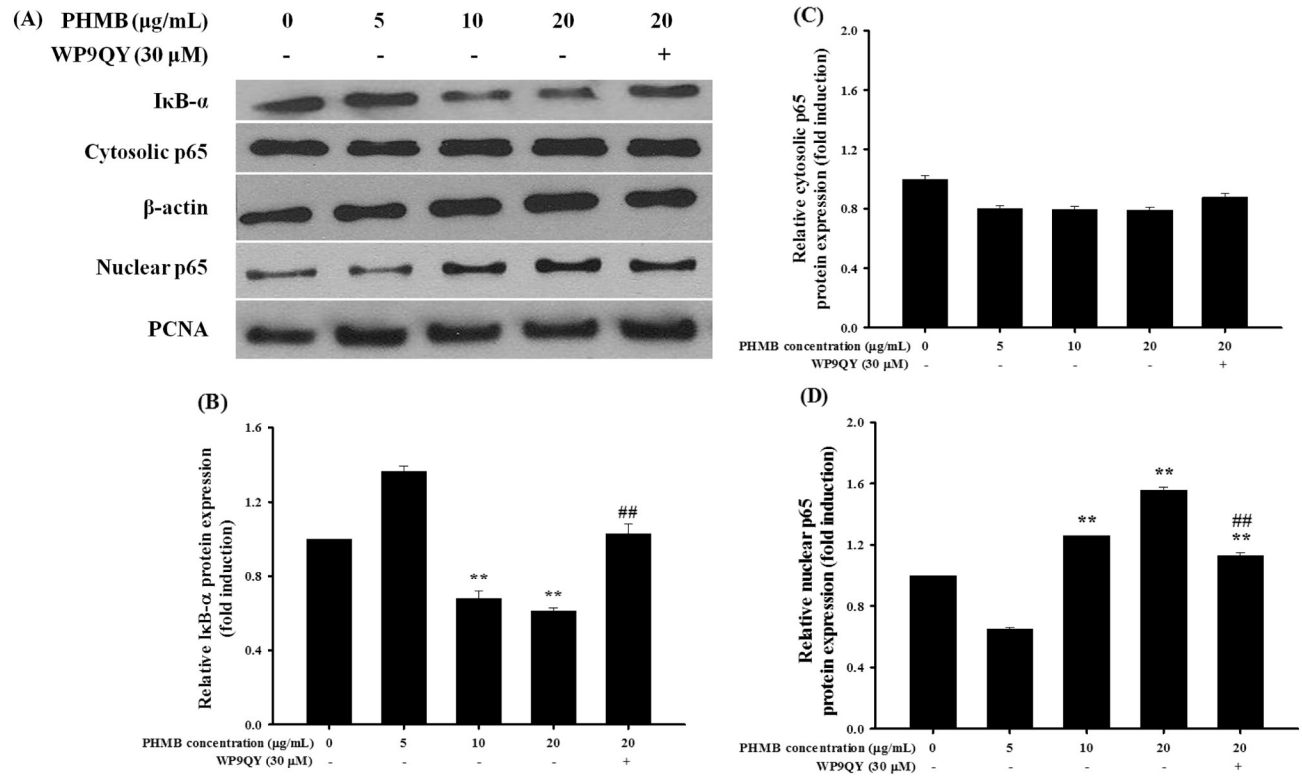

Fig. 4. Effect of PHMB on the expression of proteins associated with NF-κB signaling. A549 cells were exposed to PHMB (0–20 μg/mL) for 3 h. Inhibitory effect of WP9QY on the expression of proteins induced by 20 μg/mL PHMB. (A) Representative blots and (B), (C), and (D) bar graphs of western blot analysis. The relative abundance of each protein was normalized to that of β-actin or PCNA. All data are expressed as fold induction of the control. Values significantly different from control: $^{**}p < 0.01$; and PHMB 20 μg/mL: $^{\#\#}p < 0.01$.

PHMB induced significant cytotoxicity in various mammalian cell lines as well as microorganisms. An IC_{50} value of 164 μg/mL was reported in murine fibroblast L929 exposed to PHMB for 30 min (Müller and Kramer, 2008). Using the MTT assay, PHMB treatment for 3 h demonstrated IC_{50} values of 80, 160, and 160 μg/mL in HepG2 cells, Neuro-2A cells, and Caco-2 cells, respectively (Creppy et al., 2014). In this study, an IC_{50} value of 32.52 μg/mL was observed in A549 cells exposed to PHMB for 1 h using the WT-1 assay (Fig. 1). It has been shown that contact lens solution containing PHMB significantly decreased the viability of human conjunctival cells via the activation of P2X7 cell death receptor (Dutot et al., 2008). P2X7 purinoreceptors facilitate the rapid influx of extracellular CA^{2+}, which has been proven to generate ROS (Lew et al., 1984). High levels of ROS are known to promote cytotoxicity, whereas a non-toxic level of ROS directly activates NF-κB (Schreck et al., 1991) and indirectly modulates stimuli such as TNF-α, phorbol ester, and IL-1β (Zhou et al., 2010). A549 cells did not show significant increase in ROS when exposed to PHMB, except for cells exposed to 20 μg/mL PHMB for 3 h, (Fig. 2). These results were not consistent with the observed concentration and exposure time of PHMB that induced significant cytotoxicity. Moreover, the levels of ROS induced by PHMB were markedly lower than that induced by PHMG-p. In previous study, ROS generation in cells exposed to PHMG-p was 4.01-fold higher than that of the control (Kim et al., 2015); however, in this study a 1.39-fold increase was noted in cells exposed to PHMB. Creppy et al. (2014) reported that PHMB did not induce significant lipid peroxidation, nor did it induce hydroxylation of DNA. In addition, yeast strains exposed to PHMB for 72 h did not show signs of oxidative stress, including mobilization of intracellular thiol groups and peroxidation of the membrane phospholipids (Elsztein et al., 2011). Taken together, we assume that PHMB induces a non-toxic level of ROS, which is not relevant to the cytotoxicity of PHMB in A549 cells.

According to the OECD test guideline 412 for subacute inhalation toxicity, the exposure to PHMB via inhalation for 28 days resulted in histopathological changes related to inflammation in the lungs of rats (Scientific Committee on Consumer Safety, 2015). Similarly, A549 cells exposed to PHMB produced significantly levels of TNF-α, IL-6, and IL-8 in a dose-dependent manner (Fig. 3). TNF-α is a multifunctional pro-inflammatory cytokine that has a central role in the pathogenesis of a variety of human inflammatory disorders. IκB kinase, which is activated by TNF-α, phosphorylates IκB and triggers its degradation, resulting in the liberation of NF-κB and subsequently induces a myriad of gene expression involved in inflammatory responses, including IL-6 and IL-8 (Miyamoto et al., 1994; Schmitz, 1995). In particular, the activation of the NF-κB signaling pathway was a critical mechanism of the inflammatory responses induced by PHMG-p (Kim et al., 2015). In this study, we demonstrated that PHMB stimulated the activation of the NF-κB signaling pathway through the degradation of IκB-α and accumulation of nuclear p65 (Fig. 4). Moreover, the activation of NF-κB by PHMB was inhibited by a TNF-α receptor inhibitor WP9QY, indicating that TNF-α plays an important role in PHMB-induced NF-κB activation.

IL-6 and IL-8 contribute to local inflammatory reactions by amplifying the recruitment of leukocytes and neutrophils (Hammond et al., 1995; Romano et al., 1997), thus implicating the pathogenesis of lung diseases associated with inflammation such as asthma (Ordoñez et al., 2000), chronic obstructive pulmonary disease (Gao and Zhan, 2012), fibrosis (Radomska-Leśniewska et al., 2010), and bronchiolitis (Kim et al., 2005). In particular, the overexpression of IL-8 was associated with the pathological symptoms of the toxicity of PHMG-p on the lungs. Song et al. (2014) reported that neutrophils were recruited to the inflamed site in mice exposed to PHMG-p. The macrophage exposed to PHMG-p showed abundant IL-8 expression (Kim et al., 2015). The expression of IL-8 is mainly regulated via the NF-κB signaling pathway. Many toxicants such as bleomycin, diesel exhaust particles, cigarette smoking, asbestos, and PHMG-p have been reported to induce the production and secretion of IL-8 mediated by NF-κB activation (Gon et al., 2000; Kim et al., 2015; Luster and Simeonova, 1998; Nishikawa et al., 1999; Takizawa et al., 1999). BAY 11-7082, which selectively and irreversibly inhibits NF-κB activation by blocking the phosphorylation of IκB-α

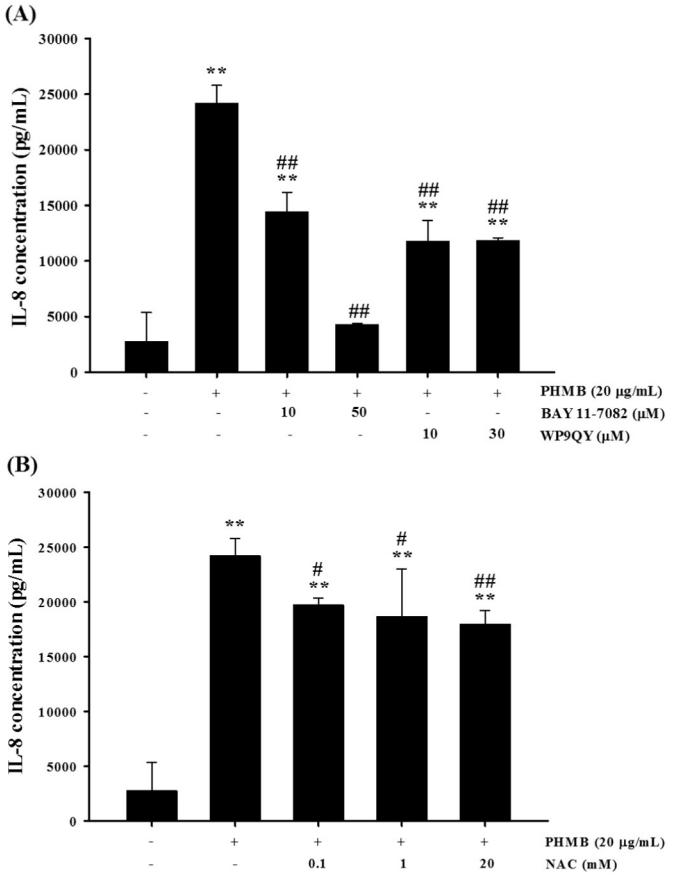

Fig. 5. Effects of inhibitors on IL-8 production induced by PHMB. A549 cells were exposed to 20 μg/mL PHMB for 6 h. (A) BAY 11-7082 (10 and 50 μM), WP9QY (10 and 30 μM), and (B) *N*-acetylcysteine (0.1, 1, and 20 mM) were used in the IL-8 ELISA assay. All results are expressed as mean ± standard deviation from double experiments in duplicate, representative of three separate experiments for each data point. Values are significantly different from control: $^{**}p < 0.01$; and PHMB 20 μg/mL: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

(Pierce et al., 1997), was used to establish the relationship between PHMB-induced IL-8 expression and the NF-κB signaling pathway. WP9QY was also used to elucidate the role of TNF-α induced by PHMB in NF-κB activation. The pre-treatment of BAY 11-7082 completely suppressed IL-8 production induced by PHMB (Fig. 5A), indicating that PHMB-induced IL-8 expression is mediated by NF-κB activation. In addition, TNF-α was partially involved in PHMB-induced IL-8 expression. Although NF-κB can be activated by numerous conditions and agents (Thanos and Maniatis, 1995), these results demonstrate that TNF-α plays an important role in IL-8 expression via NF-κB activation.

A non-toxic level of ROS can act as a second messenger to regulate IL-8 expression via NF-κB activation. Thus, NAC was used to identify the role of ROS in NF-κB-mediated IL-8 expression. However, the inhibitory effect of NAC on IL-8 expression was negligible (Fig. 5B). Although NAC is a typical scavenger of ROS, it has been reported that NAC inhibits the phosphorylation of IκB kinase (Oka et al., 2000). In addition, Hayakawa et al. (2003) showed that NAC directly binds to TNF receptors and inhibits TNF-mediated signaling. Our results showed that NAC slightly inhibited the IL-8 expression in A549 cells exposed to PHMB either by blocking the TNF signaling pathway or scavenging ROS. Taken together, the role of ROS generated by PHMB on the cytotoxic and inflammatory responses in lung cells remains unclear. Further studies are necessary to elucidate the relationship between ROS and the toxicity of PHMB.

Our results showed that PHMB induced dose-dependent cytotoxicity and inflammatory responses in lung cells. However, the in vitro test conditions in the present study did not mimic the exposure conditions of PHMB in humans. The first reason being, the relevance of A549 cell line in normal biology of respiratory epithelium, which is debatable. In comparison with normal epithelial cells, the A549 alveolar type II cells have a different genetic background and are functionally deficient in tight junctions (Roggen et al., 2006). In addition, respiratory epithelium is directly exposed to chemicals in the form of vapor, aerosol, and particles; however, this study was performed using only A549 cell line under submerged conditions, which can be mimicked more realistically in vivo by exposing epithelial cells at the air-liquid interface. Although in vitro models using A549 cell line under submerged conditions are widely used to study the inflammatory effects of various chemicals in the lungs, the inflammatory effects of PHMB using various types of mono/co-cultured lung cells under air/liquid interface conditions need to be verified. The second reason is the gap between test concentrations of PHMB and its intake in real life, which could not be measured during the study. The recommended dose for general water treatment is 4–8 μg/mL of PHMB (United States Environmental Protection Agency, 2004). However, considering its low vapor pressure, the respiratory tract is considered to be exposed to a very small amount of PHMB. The essence of toxicity testing is not just to check how safe a test substance is, but also to characterize the possible toxic effects it can produce. PHMG-p, a disinfectant used in humidifiers, is released in its aerosol form by the humidifiers, and can penetrate deep into the lungs, causing severe damage to the lung architecture. In addition, the silver nanoparticles stabilized with PHMB have been approved for legal sale in the territory of the Russian Federation as an active ingredient of pesticides for the treatment of various crop diseases (Gusev et al., 2016). Although PHMG-stabilized silver nanoparticles are linked with low toxicity in humans and animals, sufficient aggregative stability, and good adhesion to plant seeds and leaves, a major concern is the penetrating property of nanoparticles upon their unexpected exposure to the respiratory tract because they penetrate deep into the air spaces of the lungs. Therefore, the present study is meaningful for reporting the potential toxic effects of PHMB.

In conclusion, the present study clarified the inflammatory responses and its mechanism induced by PHMB. PHMB stimulated pro-inflammatory cytokine secretion and NF-κB activation. In addition, TNF-α induced by PHMB plays an important role in the activation of the NF-κB signaling pathway, which mediates IL-8 expression in lung cells. Although PHMB generated significant levels of ROS, we assumed that ROS was not relevant to the cytotoxicity and inflammatory responses induced by PHMB. Our results show the potential hazard of PHMB exposure.

Transparency document

The Transparency document associated with this article can be found, in online version.

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