Original Article

The liver X receptor agonist T0901317 reduces the inflammation of alveolar epithelial cells induced by polyhexamethylene guanidine through inhibition of the NFκB signaling pathway

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Background: As a kind of disinfectant, polyhexamethylene guanidine (PHMG) can cause pulmonary inflammation. In addition to liver X receptors (LXRs) playing an important role in cholesterol and lipid metabolism, it has also been found to be involved in inflammation in recent years. This article explores the role of LXRs agonist T0901317 in the inflammation of alveolar epithelial cells induced by PHMG.

Methods: The A549 human alveolar basal epithelial cell line was exposed to PHMG, T0901317, or the nuclear factor (NF)κB inhibitor BAY11-7082. The cell survival rate was used to determine the cytotoxicity of PHMG and T0901317 to A549 cells. Western blot analysis was used to determine the expression of proteins related to the LXRs and the NFκB signaling pathway. Enzyme-linked immunosorbent assay (ELISA) was conducted to examine the expression of inflammatory cytokines such as interleukin (IL)-8 and interleukin (IL)-6.

Results: Incubation of A549 cells with PHMG decreased the expression of LXRs-related proteins, reduced the expression of cellular IκB, increased the expression of nuclear NFκB, and increased the levels of the inflammatory cytokineIL-8 and IL-6. However, pretreatment with the LXR agonist T0901317 partially reversed the effects of PHMG. The effects of T0901317on NFκB signaling pathway was similar to that observed with the NFκB inhibitor BAY11-7082.

Conclusions: The LXRs agonist T0901317 may reduce the inflammation of alveolar epithelial cells induced by PHMG by inhibiting the NFκB signaling pathway.

Keywords: Liver X receptors (LXRs); polyhexamethylene guanidine (PHMG); NFκB; IL-8; A549

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Introduction

Polyhexamethylene guanidine (PHMG), which belongs to the guanidine family of antiseptics, is widely used in aquaculture, shampoo, wet wipes, disinfectants, and other household products because it is colorless, odorless, soluble in water, has a wide range of disinfectant activity, and other desirable properties (1-4). Prior to 2010, most toxicity studies on PHMG focused on oral and percutaneous toxicity. The median lethal dose (LD_{50}) of oral exposure to PHMG in Sprague Dawley (SD) rats is 500–600 mg/kg, suggesting that PHMG is an acute low-toxicity substance. Therefore, the health risks and hidden dangers of guanidine cationic disinfectants, especially respiratory toxicity, has failed to attract much attention. As an excellent disinfectant, PHMG is widely used in daily life. However, in 2011, there was an incident of lung injury caused by guanidine cationic disinfectants in humidifiers in South Korea. Respiratory exposure to PHMG not only caused acute lung injury (ALI), which resulted in the death of pregnant women and children, but also caused pulmonary fibrosis in thousands of patients, affecting their quality of life and life expectancy. This incident provided a warning regarding the health risks and hazards caused by inhalation exposure of guanidine cationic disinfectants (5-7).

Since then, many scientists have performed *in vivo* and *in vitro* studies examining the mechanisms of lung injury caused by PHMG (8,9). Many studies have shown that PHMG can induce nuclear factor (NF)κB-mediated inflammation, such as the release of cytokines and chemokines in the lungs (10-12). NFκB is a key factor in the inflammatory response. When unstimulated, NFκB binds to IκB as a dimer of P50 and P65 to form a stable complex with no transcriptional activity. Under stimulation from inflammatory factors, IκB dissociates from the complex and NFκB is activated and translocates into the nucleus. It then binds to the promoter regions of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8), in the nucleus to induce transcription and translation of related genes (13).

The liver X receptor (LXRα and LXRβ), which belongs to the superfamily of nuclear receptors, is a ligand-dependent receptor that controls diverse pathways involved in development, reproduction, metabolism, and inflammation. LXRα is mainly distributed in the liver, intestine, kidney, spleen, lungs, adipose tissue, mononuclear macrophages, and airway smooth muscle cells, while LXRβ is expressed in almost all tissues (14). Studies have shown that the expression of LXRs is down-regulated during lung inflammation and injury caused by lipopolysaccharides (LPSs) and other stimuli. LXR activation can inhibit NFκB activity and reduce lung inflammation and lung injury induced by LPSs and other stimuli (15-17). To date, there is a paucity of research examining the role of LXRs in pulmonary inflammation and fibrosis caused by PHMG. We speculate that the expression of LXRs is down-regulated in PHMG-induced alveolar epithelial cell inflammation and the LXR agonist T0901317 may reduce PHMG-induced alveolar epithelial inflammation by restoring the expression of LXRs and inhibiting the activation of the NFκB signaling pathway. This study examined the cytotoxicity of alveolar epithelial cells after treatment with PHMG and T0901317. The expression of LXRs-related proteins, NFκB-related proteins, and the inflammatory cytokine IL-8 and IL-6 was detected by Western blot analysis and enzyme-linked immunosorbent assay (ELISA). The results demonstrated that the LXR agonist T0901317 may reduce the inflammation of alveolar epithelial cells induced by PHMG by inhibiting the NFκB signaling pathway.

We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-6501).

Methods

Cell culture

The human A549 alveolar epithelial cell line was obtained from Peking University Medical Laboratory (Beijing, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured at 37 ℃ and 5% CO_2 according to the American Type Culture Collection (ATCC) recommendations.

Cell viability assays

The survival rate of A549 cells was assessed using the Cell Counting Kit 8 (Meilun Biotech, Dalian, China). The A549 cells were plated in 96-well plates at a density of 10×10^3 cells/well and cultured for 24 hours. PHMG 0–32 μg/mL (High Polymer Bio, Shanghai, China) and T0901317 0~10 μM (Cayman chemical, Ann Arbor, Michigan, USA) were added to each well at different concentrations. After 6, 24 and 48 hours, the Cell Counting Kit 8 (CCK-8) solution (10 μL) was added to each well and

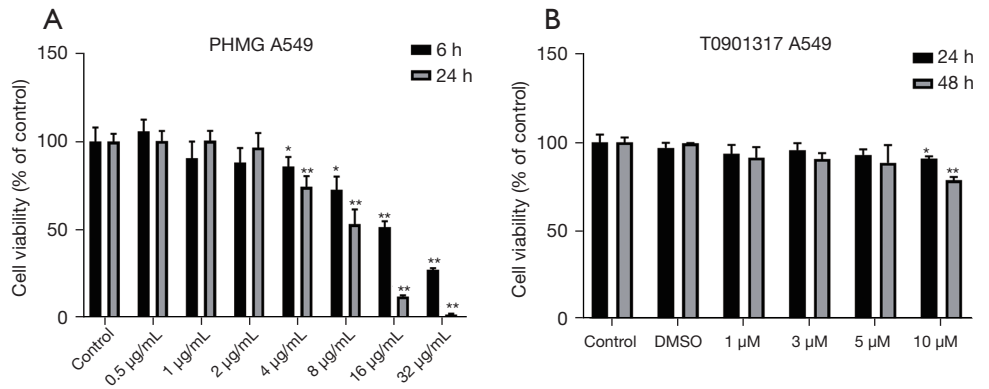

Figure 1 The cytotoxicity of PHMG and T0901317 to A549 cells. (A) A549 cells were exposed to PHMG (0–32 μg/mL) for 6 hours and 24 hours. (B) A549 cells were exposed to T0901317 (0–10 μM) for 24 and 48 hours. Cell viability was assessed using the CCK-8 assay and expressed as a percentage of the control cells (mean ± SD). *, P<0.05 compared to control; **, P<0.01 compared to control. PHMG, polyhexamethylene guanidine; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit 8; SD, standard deviation.

cells were incubated at 37 ℃ for 1 hour. The absorbance at 450 nm was detected with a microplate reader (Tecan company, Austria).

Western blot analysis

The A549 cells were cultured in 6 cm dishes until 70% confluency. Cells were then stimulated with the corresponding reagents for 24 hours, followed by 3 washes with phosphate buffered saline (PBS). The total protein, cytoplasmic protein, and nuclear protein were extracted from the cells using the Nuclear protein Extraction Kit (Solarbio, Beijing, China). Protein concentration was measured using the BCA protein concentration determination kit (Solarbio, Beijing, China). Protein samples (30 μg) were separated by electrophoresis on a 10% gel, transferred onto polyvinylidene fluoride (PVDF) membranes, and blocked with 5% skimmed milk powder for 1 hour at room temperature. Membranes were then incubated with the primary antibodies against SREBP (sc-13551, Santa Cruz Biotechnology), LXRα/β (sc-377260, Santa Cruz Biotechnology), IκB (ab32518, Abcam), or NFκB (ab32536, Abcam) overnight, followed by incubation with the secondary antibody 1 hour. The PVDF membrane was developed under a chemical imaging analyzer (Fusion FX7 Vilber Lourmat, Paris, France). The ImageJ software was used for quantification and the density of each band was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #2118, Cell Signaling Technology) or lamin B (abs131244, Absin Bioscience).

Cytokine measurement by ELISA

A549 cells were exposed to PHMG (at a final concentration 4 μg/mL), T0901317 [at a final concentration of 1 μM, diluted with 1‰ dimethyl sulfoxide (DMSO)], or the NFκB inhibitorBAY11-7082 (at a final concentration of 20 μM; MCE, Shanghai, China) for 24 hours. The supernatant was collected and the levels of IL-8 and IL-6 were quantified using ELISA (Elabscience, Wuhan, China) according to the manufacturer's protocols.

Statistical analysis

The data were expressed by mean ± standard deviation (SD). Statistical analyses were conducted using Prism 8.0.1 software (GraphPad Software, Inc., USA). Differences between multiple groups were assessed by one-way analysis of variance (ANOVA). Differences between two groups were assessed using the student's *t*-test. A P value <0.05 or <0.01 was considered statistically significant.

Results

The cytotoxicity of PHMG and T0901317 to A549 cells

Exposure of A549 cells to PHMG showed a dose-dependent cytotoxic effect. Significant cytotoxicity was observed in A549 cells at PHMG concentrations greater than 4 μg/mL after both 6 and 24 hours. PHMG had little effect on the proliferation of A549 cells at concentrations lower than 2 μg/mL (*Figure 1A*).

When A549 cells were exposed to the LXR agonist for 24 and 48 hours, cytotoxic was only observed at a concentration of 10 μM. At 5 μM or lower, T0901317 had no cytotoxic effect on A549 cells. Furthermore, DMSO had no significant cytotoxic effect on A549 cells (*Figure 1B*).

T0901317 reverses the down-regulation of LXR-related proteins in A549 cells induced by PHMG exposure

The expression of LXRα and LXRβ decreased when A549 cells were exposed to PHMG (4 μg/mL). In addition, the expression of sterol regulatory element binding protein (SREBP), a LXR downstream specific target protein, was also decreased. In cells treated with PHMG and T0901317 (the PHMG + T09 group), the expression of LXRs and SREBP was increased compared to cells treated with PHMG alone. DMSO had little effect on the expression of LXRs-related proteins (*Figure 2A,2B*).

T0901317 inhibits the activation of the NFκB signaling pathway caused by PHMG exposure

When A549 cells were exposed to PHMG (4 μg/mL), the expression of IκB decreased compared with control cells, while the expression of nuclear NFκB increased. Conversely, cells in the PHMG + T09 group showed increased expression of IκB compared to cells treated with PHMG alone, while the expression of nuclear NFκB decreased. Similarly, incubation with PHMG and the NFκB inhibitor BAY11-7082 (PHMG + BAY) reduced the expression of nuclear NFκB protein. There was little change in the expression of NFκB-related proteins in the DMSO group and the T0901317 group compared to the control group (*Figure 2C-2G*).

T0901317 inhibits the expression of inflammatory cytokine IL-8 and IL-6 caused by PHMG exposure in A549 cell

The expression of IL-8 and IL-6 was significantly increased in A549 cells treated for 24 hours with PHMG (4 μg/mL) compared to control cells. Addition of the LXRs agonist T0901317 to cells treated with PHMG (the PHMG + T09 group) significantly reduced the expression of IL-8 and IL-6 compared to that observed in cells treated with PHMG alone. Furthermore, addition of BAY11-7082 to cells treated with PHMG significantly decreased the expression of IL-8 and IL-6 compared to cells treated with PHMG alone. DMSO and T0901317 had no effect significant effect on the expression of IL-8 and IL-6 in A549 cells (*Figure 3A,3B*).

Discussion

Since the Korean humidifier lung injury incident, many studies have been performed to understand the mechanisms by which PHMG and its related guanidine disinfectants cause lung inflammation (10,18-22) and pulmonary fibrosis (12,22-26). One study showed that exposure of mouse macrophage RAW264.7 cells to PHMG decreased IκB protein expression, increased NFκB luciferase activity, and elevated the expression of inflammatory cytokines IL-1β, IL-6, and IL-8. However, addition of the NFκB inhibitor BAY11-7082 completely abolished the increase of IL-8 caused by PHMG, suggesting that PHMG induces inflammation through the NFκB signaling pathway (10). Kim *et al.* demonstrated that exposure of A549 cells to polyhexamethylene biguanide (PHMB) decreased IκB expression, increased nuclear NFκB expression, and elevated the levels of IL-8, IL-6, and TNF-α. Adding WP9QY (TNF-α inhibitor) and N-acetylcysteine (ROS antioxidant) partially inhibited the expression of IL-8 caused by PHMB. By the way, the JNK inhibitor SP600125 could also partially inhibit the PHMG-induced expression of IL-6 and TNF-α in A549 cells (21). However, addition of the NFκB inhibitor completely inhibited the expression of IL-8, suggesting that PHMB induces inflammation through the NFκB signaling pathway (18). Our *in vitro* experiments suggested that PHMG can cause the degradation of the IκB protein in A549 cells, thereby releasing NFκB. Activated NFκB enters the nucleus from the cytoplasm and promoted the transcription and translation of downstream inflammatory genes. This investigation also demonstrated that the expression of the inflammatory cytokine IL-8 and IL-6 was increased after PHMG exposure. However, the NFκB inhibitor BAY11-7082 decreased the expression of nuclear NFκB and its downstream inflammatory cytokine IL-8 and IL-6, suggesting that PHMG can induce inflammation through the NFκB signaling pathway. Previous *in vivo* studies have shown that PHMG can cause lung inflammation and fibrosis in mice (12,23,25) and this is consistent with our preliminary animal investigations (27).

The LXRs were originally thought to be nuclear receptors that play a key role in lipid homeostasis. Studies have now shown that LXRs also have anti-inflammatory and anti-fibrotic effects *in vitro* and *in vivo* (15,16,28-31). Therefore, we speculated that LXRs might be involved in the pathogenesis of lung inflammation, and activation of the LXR signaling pathway might protect the alveolar epithelial cell inflammation caused by PHMG. Indeed, the results

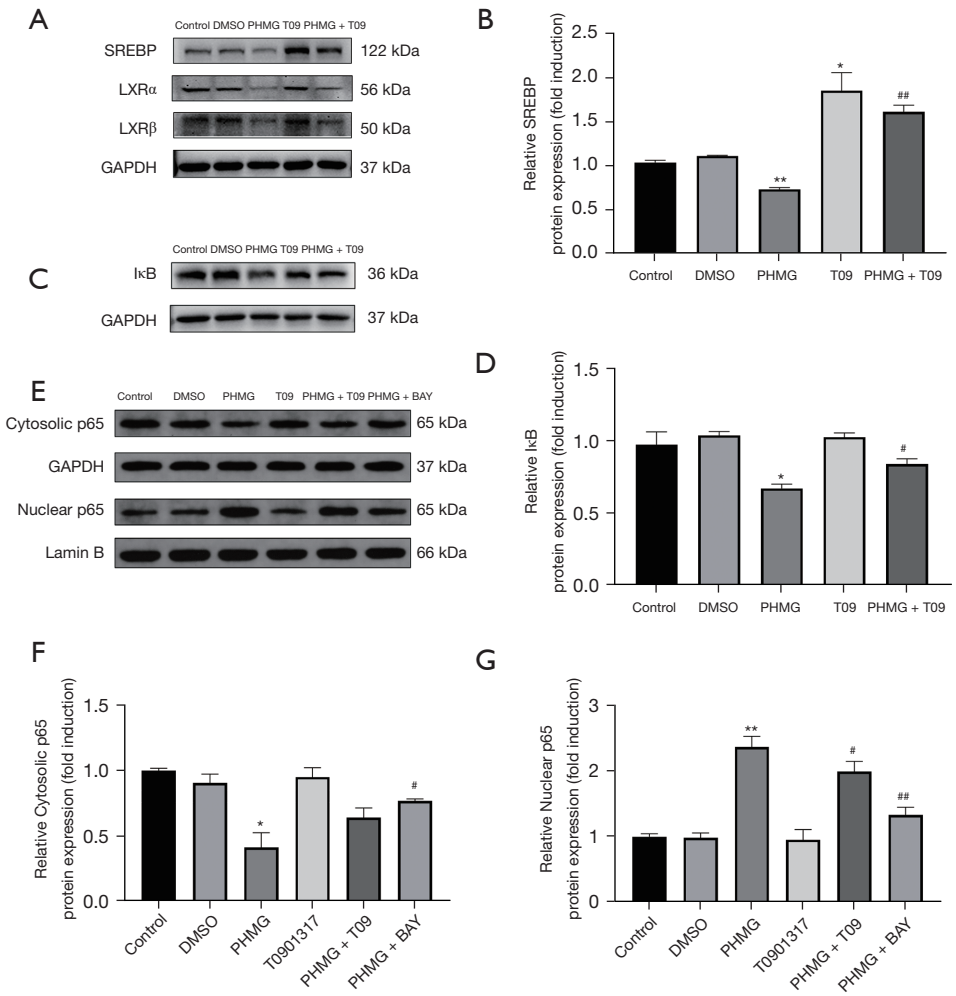

Figure 2 The effects of PHMG and T0901317 on LXRs and NFκB related proteins. A549 cells were exposed to PHMG (4 μg/mL), T0901317 (1 μM), and BAY11-782 (20 μM) for 24 hours. Protein expression is shown by a representative Western blot (A,C,E) and the corresponding bar graph (B,D,F,G). The relative abundance of each protein was normalized to that of GAPDH or lamin B. *, P<0.05 and **, P<0.01 compared to control cells; #, P<0.05 and ##, P<0.01 compared to PHMG-treated cells. DMSO, dimethyl sulfoxide; PHMG, polyhexamethylene guanidine; LXR, liver X receptor; NF, nuclear factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; T09, T0901317; BAY, BAY11-7082.

of this study showed that PHMG exposure decreased the expression of LXRα and LXRβ in A549 alveolar epithelial cells. Interestingly, pretreatment with the LXRs agonist T0901317 restored the expression of LXRα and LXRβ in PHMG-exposed alveolar epithelial cells. These results demonstrated that in the alveolar epithelial cells treated with PHMG, the NFκB signaling pathway is activated, and the expression of the downstream inflammatory cytokine IL-8 and IL-6 are increased. Pretreatment with T0901317 inhibited the NFκB signaling pathway and reduced the expression of downstream IL-8 and IL-6 expression. Indeed, the effects of T0901317 on inflammatory factors were similar to that observed with NFκB inhibitors, suggesting that T0901317 may reduce PHMG-induced inflammation of alveolar epithelial cells by inhibiting the NFκB signaling pathway. A previous study showed that LXR agonists in human umbilical vein endothelial cells (HUVECs) not only dose-dependently inhibited the lysophosphatidylcholine (LPC)-induced increase in IL-8, but also reversed the induction of LPC-mediated NFκB activation. Moreover, LXR agonists also inhibited the binding of NFκB to the IL-8 promoter induced by LPC in HUVECs, suggesting

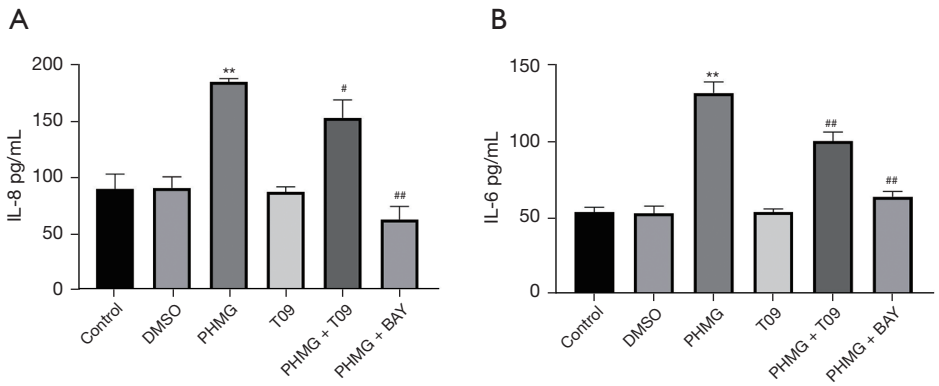

Figure 3 The expression of inflammatory cytokine IL-8 (A) and IL-6 (B) in A549 cells treated with PHMG (4 μg/mL), T0901317 (1 μM), and BAY11-782 (20 μM). The data are expressed as mean ± SD. **, P<0.01 compared to control cells; #, P<0.05 and ##, P<0.01 compared to cells treated with PHMG. IL-8, interleukin-8; IL-6, interleukin-6; PHMG, polyhexamethylene guanidine; DMSO, dimethyl sulfoxide; T09, T0901317; BAY, BAY11-7082; SD, standard deviation.

that the inhibitory effect of LXR-mediated inflammation was partly related to the NFκB pathway (28). Other investigations using rat models have also reported that ALI induced by LPSs decreased the expression of LXRα/β, increased the expression of inflammatory cytokines IL-1β, IL-6, and TNF-α, and elevated the expression of NFκB. The LXRs were activated after treatment with T0901317 and the expression of NFκB decreased, as did the expression of inflammatory cytokines, such as IL-1β. This suggested that the mechanism by which T0901317 reduces the inflammatory response in ALI rats may be related to the inhibition of NFκB activation (15). Another report showed that intraperitoneal injection of high-dose paraquat resulted in decreased expression of LXR-related proteins, lung inflammation, and ALI in mice. IκB expression was decreased, expression of TNF-α and IL-1β was increased, as was the expression of nuclear NFκB. Furthermore, antioxidant enzyme activity was reduced. The pro-apoptotic gene Bax was up-regulated and the apoptotic gene Bcl-2 was down-regulated. After intraperitoneal injection of the LXRs agonist T0901317, the expression of LXR-related proteins was restored, and all the above changes were reversed by inhibiting the NFκB and JNK/p38 MAPK signaling pathways, and exerting an anti-inflammatory, antioxidant, and anti-apoptotic effect (16).

To the best of our knowledge, this current investigation is the first to report the effects of PHMG on the expression of LXR-related proteins in A549 cells, and the effects of the LXR agonist T0901317 on the inflammation of alveolar epithelial cells induced by PHMG. There were some limitations to this study. First, the experiments were performed *in vitro*, and no *in vivo* studies were performed. Then, the role of oxidative stress in this process has not been discussed. In addition, no experiments related to cell morphology detection were conducted. Finally, although the results suggested that the activation of the LXRs might reduce PHMG-induced inflammation of A549 cells by inhibiting the NFκB signaling pathway, the role of LXRα or LXRβ in this process remains to be elucidated and further researches warranted.

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Footnote

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org/10.21037/atm-21-6501). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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