

**High dose tobramycin inhibits lipopolysaccharide-induced MUC5AC production
in human lung epithelial cells**

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Key Words: high-dose tobramycin, MUC5AC, bronchial cells, *Pseudomonas aeruginosa*, lipopolysaccharide, mitogen-activated protein kinase pathway.

Abstract

Tobramycin inhalation therapy is an effective therapy for cystic fibrosis as well as severe bronchiectasis that is colonized with *Pseudomonas aeruginosa*. The mechanism responsible for the efficacy of tobramycin in the treatment of severe chronic infectious diseases has not been elucidated. We demonstrate that high-dose tobramycin decreases *MUC5AC* gene expression and protein production in NCI-H292 cell stimulated with lipopolysaccharide of *P. aeruginosa*. MUC5AC protein of NCI-H292 cell stimulated by lipopolysaccharide was analyzed by an enzyme-linked immunosorbent assay and MUC5AC expression at the mRNA level was analyzed by RT-PCR. Westernblot was performed to examine a potential role for the signaling molecules upstream of NFκB. High-dose tobramycin (500 μg/ml) decreased the level of MUC5AC protein released into the supernatant of the NCI-H292 cell line at 24 h after lipopolysaccharide stimulation ($P < 0.001$). The tobramycin treatment also inhibited MUC5AC mRNA expression at 12 h after lipopolysaccharide stimulation ($P < 0.05$) and suppressed nuclear factor-kappa B activation 60 min after lipopolysaccharide stimulation ($P < 0.001$). Tobramycin suppressed the phosphorylation of p38 MAP kinase. These results suggest that high-dose tobramycin, such as inhalation therapy, can inhibit *MUC5AC* gene expression and MUC5AC protein production in NCI-H292 cells, in part through

the mitogen-activated protein kinase pathway. Thus, the activation of TLR4 and the subsequent immune/inflammatory responses induced by chronic infections such as *P. aeruginosa* might be modulated by tobramycin. Our data may reveal one of the mechanisms responsible for the clinical effect of tobramycin inhalation therapy against severe chronic respiratory diseases due to *P. aeruginosa*.

1. Introduction

Chronic respiratory infectious diseases are difficult to treat, especially when the bronchial tissue is colonized and infected with *Pseudomonas aeruginosa* ([Eitzgerald et al., 1996], [Gibson et al., 2003], [Li et al., 1997]). The intermittent administration of inhaled tobramycin is an effective treatment for cystic fibrosis (CF) patients; tobramycin treatment leads to improved pulmonary function, a decreased density of *P. aeruginosa* in sputum, and a decreased risk of hospitalization ([Gibson et al., 2003] , [Lee et al., 2004] , [Ramsey et al., 1999] , [Ratjen et al., 2001]). Some authors reported that tobramycin inhalation therapy is also clinically effective for severe, non-cystic fibrosis bronchiectasis colonized with *P. aeruginosa* ([Bilon et al., 2006] , [Dal Nergo et al., 2008] , [Dohrman et al., 1998] , [Scheinberg et al., 2005]). It has been reported that tobramycin is effective because it can decrease the bacterial density in sputum due to its ability to directly reach the infectious source at high concentrations, even if the lung tissue is destroyed; another report indicates that tobramycin can suppress the pathogenesis of *P. aeruginosa*, but the details of the mechanism have not been elucidated ([Geller et al., 2002] , [Husson et al., 2005]).

Mucus secretion, which protects the host's mucosal surfaces against pathogens, is a component of the innate immunity system. However, mucus hyperproduction can be a

risk factor for pathogenesis because excessive mucus secretion causes airway obstruction, impairment of gas exchange, and obstructive pneumonia. The major macromolecular component of mucus is mucin protein. The *MUC5AC* gene encodes the major core protein of mucin secreted from the airway surface epithelium ([Davies et al., 1999], [Gendler et al., 1995], [Hovenberg et al., 1996]). Bacterial infection, especially *P. aeruginosa*, plays an important role in chronic respiratory infection. *P. aeruginosa* and lipopolysaccharide (LPS) stimulate mucin production ([Li et al., 1997], [Dohrman et al., 1998], [Kaneko et al., 2003], [Tamaoki et al., 1997], [Yanagihara et al., 2001]). Toll-like receptors (TLRs) play a key role in sensing microbial components and inducing innate immune responses, and LPS-induced dimerization of TLR4 is required for activation of downstream signaling pathways ([Lee et al., 2004], [Medzhitov et al., 2001]). Stimulation of TLR4 by LPS activates both myeloid differential factor 88 (MyD88) and Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon beta (TRIF)-dependent signaling pathways leading to activation of mitogen-activated protein (MAP) kinase and nuclear factor kappaB (NFκB) ([Kawai et al., 2005], [Takeyama et al., 1999]). For patients with chronic respiratory infectious disease, the control of mucus overproduction is considered beneficial for decreasing the disease severity and improving quality of life.

In the present study, to confirm the clinical effect of high-dose tobramycin inhalation therapy, we investigated whether high-dose tobramycin can inhibit LPS-induced mucin secretion from airway epithelial cells activated to produce the mucin core protein, MUC5AC.

2. Materials and methods

2.1. Materials. We purchased LPS of *P. aeruginosa* serotype 10, tobramycin, and dexamethasone from Sigma (Tokyo, Japan) and the RPMI 1640 medium, penicillin-streptomycin, fetal bovine serum, and a reverse transcription (RT)-PCR kit from Invitrogen (Carlsbad, CA). Human porphobilinogen deaminase LightCycler primer set was purchased from Roche Applied Science (Heidelberg, Germany). We purchased mouse MUC5AC monoclonal antibody (clone 45M1) from Neo Markers (Fremont, CA) and goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies from Bio-Rad (Hercules, CA). Anti- extracellular signal-regulated protein kinase (ERK), anti-phospho-ERK1/2, anti-c-jun N-terminal kinase (JNK), anti-phospho-JNK, and anti-p38 MAP kinase, anti-phospho-p38 MAP kinase antibodies were obtained from Cell Signaling Technology (Danvers, MA). PD98059, SB203580, SP600125, CAPE, which is an inhibitor of ERK, p38 MAP

kinase, JNK and NFκB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture. The NCI-H292 epithelial cell line was obtained from the American Type Culture Collection (Manassas, VA) ([Imamura et al., 2004], [Ishimoto et al., 2009], [Kim et al., 2002]). The cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were grown at 37°C with 5% CO₂ in fully humidified air and were subcultured twice weekly. The cells were seeded in a 12-well plate at 5 × 10⁵ cells/well. When confluent, the cells were incubated in RPMI 1640 medium containing 0.5% fetal bovine serum for 24 h. The cells then were rinsed with serum-free RPMI 1640 medium and exposed to LPS. We used *P. aeruginosa* LPS for stimulating the cell line to exclude the bactericidal effect of tobramycin. For inhibition studies, cells were pretreated with tobramycin 30min before exposure to LPS. For controls, the cells were incubated with medium alone.

2.3. RNA extraction and reverse transcription PCR. Total RNA was prepared by using QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan). After contaminating DNA was removed with DNase (Message Clean kit; GenHunter, Nashville, TN), cDNA was constructed from 1 µg total RNA by using the

SuperScript III CellDirect cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Sequences for the MUC5AC primers are: ATC ACC GAA GGC TGC TTC TGT C (sense) and GTT GAT GCT GCA CAC TGT CCA G (antisense) (Imamura et al., 2004). PCR was performed in a total volume of 20 μ l containing 5 μ l cDNA template, 4 μ l LightCycler Taqman Master mixture (Roche Diagnostics, Basel, Switzerland), 0.2 μ M Taqman probe, and 0.5 μ M each primer. Thermal cycling was performed with an initial hold for 10 min at 95°C, followed by 30 cycles of 10 s at 95°C, 10 s at 64°C. Data was analyzed by LightCycler Software (v3.5) in the F1 mode with a fit point calculation method. Human porphobilinogen deaminase controls were used to standardize the quantification of RNA samples. We performed four times experiments.

2.4. ELISA. The NCI-H292 cells were incubated in a 12-well plate, and the level of MUC5AC protein was measured by an enzyme-linked immunosorbent assay (ELISA) (Kaneko et al., 2003). Briefly, the cells were washed with phosphate buffered saline (PBS), exposed to trypsin, and centrifuged at $600 \times g$ at 4°C for 5 min to form pellets. The pellets were resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 133 mM NaCl, 1% NP-40, 10% glycerol). The preparation then was cleared by centrifugation, and the supernatant was saved as a whole-cell lysate.

Protein concentrations in the supernatant were measured, and equal amounts of total protein were incubated at 40°C in a 96-well plate until dry. The plates then were washed three times with PBS, blocked with 2% bovine serum albumin for 1 h at room temperature, and incubated with MUC5AC antibody diluted 1:100 in PBS containing 0.05% Tween 20 for 1 h. The wells were washed three times with 400 μ l PBS, HRP-conjugated anti-goat immunoglobulin G diluted 1:10,000 in PBS containing 0.05% Tween 20 was dispensed into each well for 1 h. The plates were washed three times with 400 μ l PBS. Color was developed with 3,3',5,5'-tetramethylbenzidine-peroxidase solution, and the reaction was stopped with 1 N H₂SO₄. The absorbance was read at 450 nm. We performed four times experiments.

2.5. Western blot analysis and antibodies. Cells were harvested 30 min after LPS stimulation, washed, and homogenized at 4°C in lysis buffer (0.1% sodium dodecyl sulfate [SDS], 1% Igepal CA-630, and 0.5% sodium deoxycholate) and a protease inhibitor cocktail (Sigma, St. Louis, MO). Cell lysates (20-50 μ g) were resolved by electrophoresis on a 12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking the membrane in 10% FBS and 0.1% Tween 20 in Tris-buffered saline for 1 h at room temperature, the binding were reacted

overnight at 4°C with primary antibodies. After hybridization with secondary antibodies conjugated with horseradish peroxidase, the immuno-complexes were visualized by using an ECL western blotting detection system (GE Healthcare, Chalfont St. Giles, UK). The analysis was performed by using primary antibodies (1:1000) to phospho-ERK 1/2, ERK 1/2, phospho-p38 MAP kinase, p38 MAP kinase, and phospho JNK, JNK (Cell Signaling Technology, Danvers, MA).

2.6. NFκB transcription factor assay. Nuclear extracts from cells were obtained by using a nuclear/cytosol fractionation kit (BioVision) according to the manufacturer's protocol. Activities of NFκB p50 and p65 were investigated by using an NFκB transcription factor assay kit (Chemicon, Temecula, CA) according to the manufacturer's directions (Hasegawa et al., 2006). In short, nuclear extract (10 μg per sample) from untreated or treated cells was added to the capture probe, a double-stranded biotinylated oligonucleotide containing the consensus sequence for binding NFκB. After incubation, the sample was transferred to a streptavidin-coated 96-well plate. After washing, the bound NFκB transcription factor subunit was detected with a primary antibody. The plate was incubated with a secondary antibody, a chromogenic substrate was added to the cells, and then the absorbance of

each sample was determined with a microplate reader. All experiments were performed four times.

2.7. Inhibition of cell signaling activity. ERK inhibitor PD98059, p38 MAP kinase inhibitor SB203580 and JNK inhibitor SP600125 were used at concentrations of 10 to 50 μ M (in dimethyl sulfoxide[DMSO] stock solution). Cells were treated with these inhibitors 30 min before LPS stimulation. Control cultures were treated with an equal volume of DMSO. All experiments were performed four times.

2.8. Statistical analysis. All data were expressed as the mean and standard deviation (SD). Differences were examined for statistical significance by using the one-way analysis of variance for comparisons involving more than two groups and the Student's *t* test for comparisons between two groups. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. High-dose tobramycin inhibits MUC5AC protein production and mRNA expression induced by LPS in NCI-H292 cells. As shown in Fig. 1A, the MUC5AC protein production induced by LPS increased time dependently, and

MUC5AC production was maximal at 24h after LPS stimulation, so we performed the MUC5AC inhibition assay at 24 h after LPS stimulation. To determine the appropriate dosage of tobramycin for the inhibition of MUC5AC protein expression, we treated cells with tobramycin (1 to 2000 $\mu\text{g/ml}$). We used 10 $\mu\text{g/ml}$ dexamethasone as a positive control. As shown in Figure 1B, 500 $\mu\text{g/ml}$ tobramycin was needed at least to decrease the MUC5AC protein production. We also performed the inhibition study of mRNA 12h after LPS stimulation because the level of mRNA expression was maximal 12h after LPS stimulation (data not shown). As shown in Figure 2, 500 $\mu\text{g/ml}$ tobramycin also reduced MUC5AC expression at the mRNA level. Tobramycin had no inhibitory effect on the basal expression of MUC5AC (data not shown). Furthermore, tobramycin exposure did not significantly alter cell numbers and increase the lactate dehydrogenase (LDH) level of the supernatant.

3.2. High-dose tobramycin inhibits NF κ B activation induced by LPS in

NCI-H292 cells. The activation of NF κ B is an important factor in causing inflammation in bronchial epithelial cells. To determine whether tobramycin down-regulates NF κ B activity, we performed an assay of transcription factors. The activity levels of NF κ B p50 and p65 were increased by LPS stimulation in a

time-dependent manner, and they reached a maximum level 60 min after LPS stimulation (Figure 3A-i and ii), so we performed the inhibition assay 60 min after LPS stimulation. As shown in Figure 3B-i and ii, the activity levels of the NFκB p50 and p65 subunits were suppressed in tobramycin-treated NCI-H292 cells as compared to the LPS control cells. These results suggest that tobramycin influences p50 and p65 activity.

3.3. Inhibitors of MAP kinase and NFκB block LPS-induced MUC5AC

production. To examine the cell signaling pathway of LPS via TLR4 in NCI-H292 cells, we performed an inhibition assay of the cell signaling pathway. As shown in Figure 4, the NFκB inhibitor CAPE, the ERK inhibitor PD98059, the p38 MAP kinase inhibitor SB203580, and the JNK inhibitor SP600125 effectively suppressed the MUC5AC protein production compared with the LPS control cells. These results indicate that LPS from *P. aeruginosa* influences MUC5AC production via the MAPK pathway.

3.4. High-dose tobramycin inhibits the phosphorylation of MAP kinase in

LPS-activated NCI-H292 cells. To examine a potential role for the signaling molecules upstream of NFκB, we examined the phosphorylation of kinase, which is the extracellular signaling pathway of TLR4. Lysates of LPS-treated NCI-H292

cells were subjected to western blotting. As shown in Figure 5, ERK and p38 MAP kinase were phosphorylated in the LPS control cells and the phosphorylation of ERK, and p38 MAP kinase suppressed in tobramycin-treated cells. These results support the possibility that the inhibition of the phosphorylation of MAP kinase by tobramycin contributes to the suppression of MUC5AC.

4. Discussion

Chronic airway infectious diseases, such as diffuse panbronchiolitis, bronchiectasis, and cystic fibrosis, are characterized by mucus hypersecretion induced by inflammation of the airways ([Bilton et al., 2006], [Drobnic et al., 2005]). A large amount of sputum causes airway obstruction, atelectasis, reduced oxygenation, and reduced antibiotic permeability. MUC5AC is the major core protein of mucin secreted from bronchial epithelial cells. MUC5AC expression is up-regulated by inflammatory mediators (Takeyama et al., 2000) and cytokines ([Dabbagh et al., 1999], [Longphre et al., 1999], [Shim et al., 2001]). Chronic *P. aeruginosa* infection in the lungs often causes mucus overproduction. The supernatant of *P. aeruginosa* contains an activity

that up-regulates transcription of the mucin genes (Li et al., 1997), and LPS is present in the supernatant and can activate MUC5AC (Dohrman et al., 1998).

In cystic fibrosis patients, *P. aeruginosa* has been found to grow to high concentrations with biofilm and intravenous antibiotics can't reach the appropriate concentration to the infectious source (Singh et al., 2000), therefore, it is difficult to eradicate this organism from the airway. The intermittent administration of inhaled tobramycin is an effective treatment for cystic fibrosis patients ([Gibson et al., 2003], [Lee et al., 2004], [Ramsey et al., 1999], [Ratjen et al., 2001]). Tobramycin inhalation therapy improves pulmonary function, decreases the density of *P. aeruginosa* in sputum, and decreases the risk of hospitalization. Recently, the efficacy of tobramycin inhalation therapy to non-cystic fibrosis severe bronchiectasis colonized with *P. aeruginosa* has been reported (Scheinbeg et al., 2005). We have also observed patients who had severe bronchiectasis with *P. aeruginosa* for whom tobramycin treatment decreased the amount of sputum and improved their quality of life; the conditions of these patients did not improve with other intravenous antibiotics. In addition, some authors reported the non bacterial effect of aminoglycoside; Anderson *et al.* reported the effects of tobramycin on *P. aeruginosa* biofilm on cystic fibrosis-derived airway epithelial cells in vitro (Anderson et al., 2008). Du *et al.* reported that clinical doses of amikacin provide

effective suppression of the human CFTR-G542X stop mutation in a transgenic cystic fibrosis mouse model (Du et al., 2002). In the present study, we investigated whether tobramycin can inhibit MUC5AC expression to elucidate the relationship between the clinical effect of tobramycin and MUC5AC suppression. We treated the bronchial cell line with a high dose of tobramycin (500 µg/ml) because the concentration of inhaled tobramycin can reach very high in sputum, epithelial lining fluid, and alveolar macrophages ([Geller et al., 2002], [Touw et al., 1997]). We found that tobramycin can control the MUC5AC expression by suppressing the MAP kinase pathway. Stimulation of TLR4 by LPS activates (MyD88 signaling pathways leading to the activation of MAP kinase. Our result suggests that the anti-inflammatory effect of tobramycin involves MUC5AC as well as the other stress factors associated with the MAPK pathway; so, tobramycin may use multiple pathways to suppress inflammation. The presence of sputum decreases the bioactivity of tobramycin against the bacteria ([Hunt et al., 1995], [Landy et al., 2006]), and mucin plays an important role in the development and function of biofilm. But, few reports have addressed the effect of tobramycin on host immunity. Our results suggest that tobramycin may increase the bactericidal effect against *P. aeruginosa* by decreasing the amount of sputum via the inhibition of MUC5AC expression.

A reduction in sputum causes improved lung function, improved accessibility of antibiotics to the small airway, and increased effects against the infection. Recently, aminoglycoside inhalation therapy is reported as the helpful therapy to prevent and treat the ventilator-associated pneumonia (VAP) ([Ghannam et al., 2009], [Mohr et al., 2007]). We consider that inhaled aminoglycoside therapy may contribute to control the ventilator-associated infection not only by eradicating the bacteria but also by decreasing the amount of sputum.

In conclusion, high-dose tobramycin inhibits the MUC5AC expression induced by *P. aeruginosa* LPS. Tobramycin seems to reduce MUC5AC production by interfering with intracellular signal transduction. Our results provide a possible explanation for the clinical efficacy of tobramycin inhalation therapy in chronic *Pseudomonas* airway infection.

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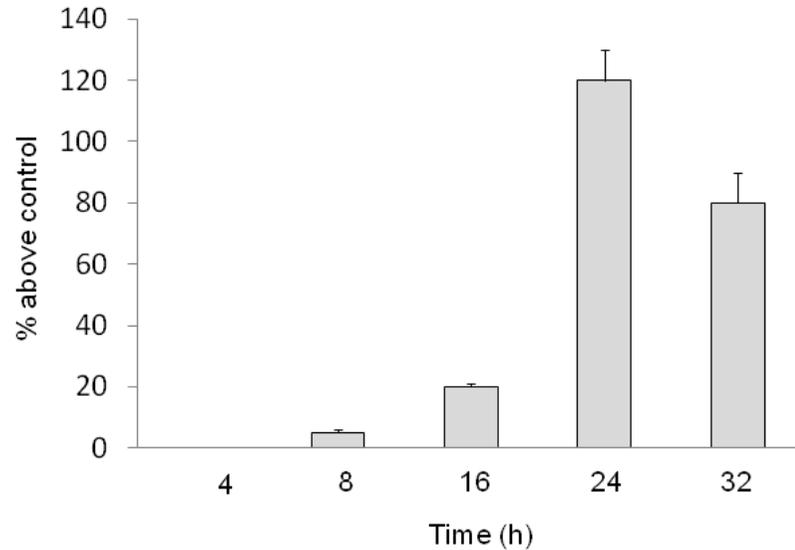
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FIGURE LEGENDS

(A)



(B)

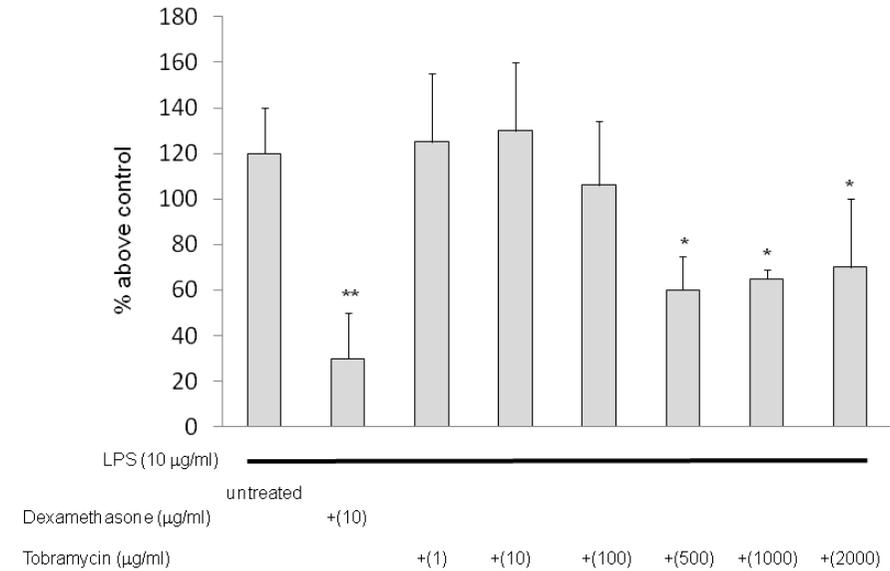


Fig. 1. The effect of LPS on MUC5AC production in NCI-H292 cells. NCI-H292 cells were stimulated with 10 µg/ml LPS of *P. aeruginosa*. The each bar shows % above control (culture medium alone). (A) MUC5AC protein level was measured by ELISA (time course). Data are expressed as the mean and \pm SD for four times experiments. (B) Cells were treated with 1, 10, 100, 500, 1000, 2000 µg/ml tobramycin. MUC5AC protein level was measured by ELISA at 24 h after the addition of LPS. Data are expressed as the mean and \pm SD for four times experiments. * $P < 0.05$, ** $P < 0.001$ compared with untreated cells (LPS alone).

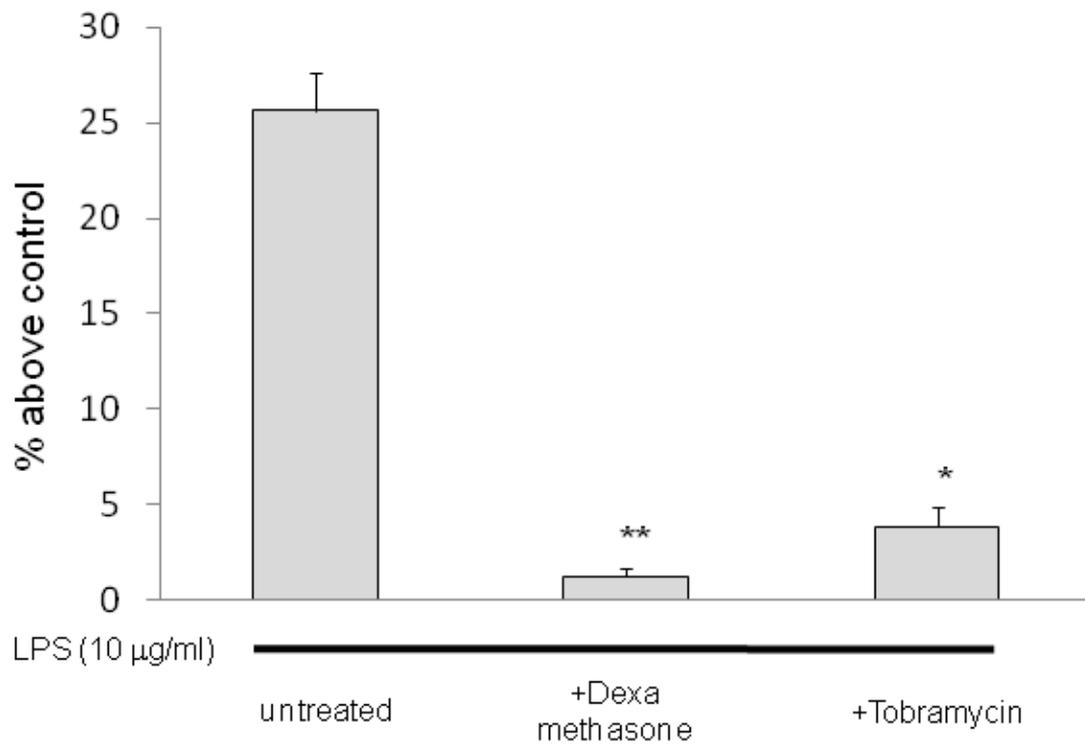


Fig. 2. MUC5AC mRNA expression at 12 h after the addition of LPS (10 µg/ml) of *P. aeruginosa* was determined by RT-PCR. Cells were pretreated with tobramycin (500 µg/ml) or dexamethasone (10 µg/ml) 30 min before LPS stimulation. The each bar shows % above control (culture medium alone). Data are expressed as the mean ± SD for four times experiments. * $P < 0.05$, ** $P < 0.001$ compared with untreated cells (LPS alone).

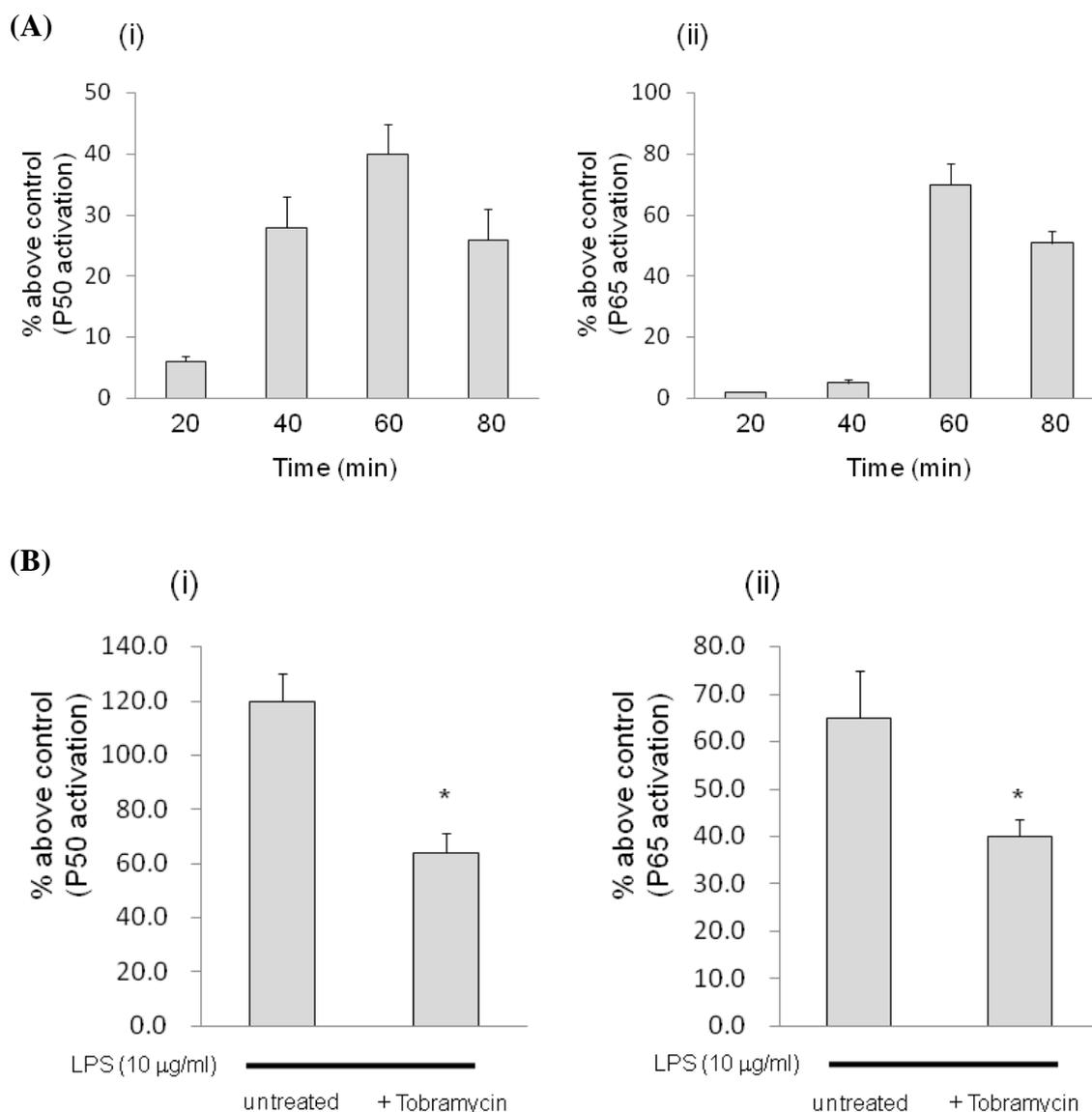


Fig. 3. Activities of NFκB p50 and p65 were investigated by using an NFκB transcription factor assay. The each bar shows % above control (culture medium alone). (A) Time-dependent effect of LPS on NFκB p50 (i) and p65 (ii) activation in NCI-H292 cells. NCI-H292 cells were stimulated with 10 μg/ml LPS of *P. aeruginosa*. Data are expressed as the mean ± SD for four times experiments. (B) Activities of NFκB p50 (i) and p65 (ii) were inhibited by 500 μg/ml tobramycin. Data are expressed as the mean ± SD for four times experiments. * $P < 0.05$ as compared with untreated cells (LPS alone).

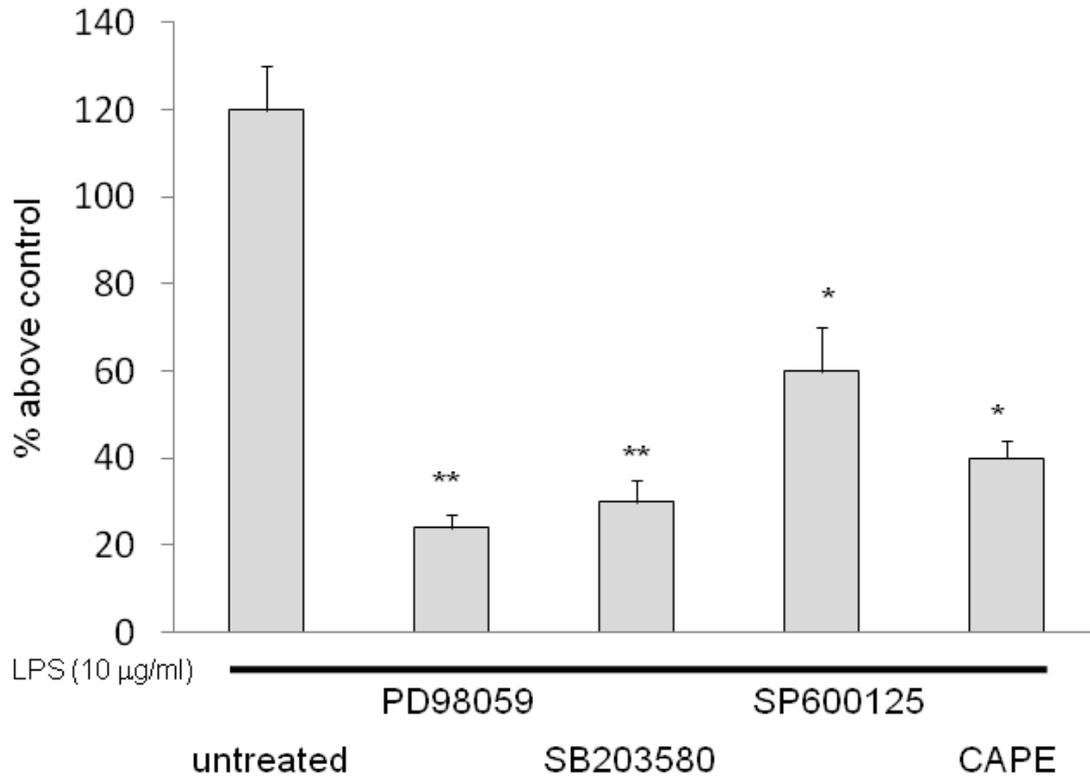


Fig. 4. Effect of MAP kinase inhibitor on MUC5AC production in cells activated by LPS. The each bar shows % above control (culture medium alone). Cells were pretreated with 50 μ M PD98059, 10 μ M SB203580, SP600125, and CAPE 30 min before LPS stimulation. Data are expressed as the mean and \pm SD for four times experiments. * P < 0.05, ** P < 0.001 compared with untreated cells (LPS alone).

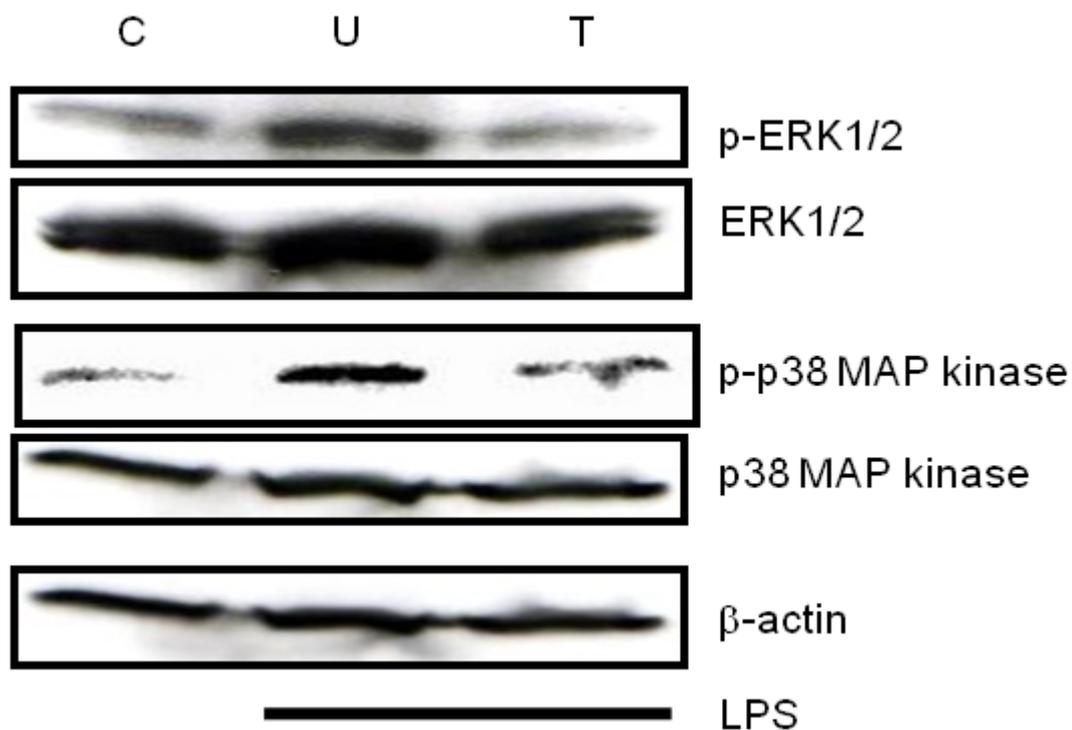


Fig. 5. Phosphorylation of ERK1/2 and p38 MAP kinase by LPS. Cells were pretreated with tobramycin (500 $\mu\text{g/ml}$) 30 min before LPS stimulation. Cells were harvested 30 min after LPS stimulation and evaluated by western blotting. Phosphorylation of ERK1/2 and p38 MAP kinase were induced in LPS-treated cells and inhibited by tobramycin. p, phospho; C, control (culture medium alone); U, untreated cells (LPS alone); T, tobramycin-treated cell.