

Effect of fudosteine on mucin production

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Short title : Effect of fudosteine on mucin synthesis

ABSTRACT : Fudosteine is a novel mucoactive agent, although little is known about how fudosteine decreases mucin production. In this study, we examined the effects of fudosteine on MUC5AC mucin synthesis and cellular signaling.

We used an animal model of lipopolysaccharide (LPS) induced inflammation and a bronchial epithelial cell line model of tumor necrosis factor (TNF)- α induced inflammation. Fudosteine was administered before stimulation with LPS or TNF- α . The MUC5AC mucin levels were assayed, and the expression of the MUC5AC gene was measured. Western blotting was carried out for the detection of phosphorylated epidermal growth factor receptor (p-EGFR), phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK), and phosphorylated extracellular signal-related kinase (p-ERK).

MUC5AC mucin synthesis and the expression of the MUC5AC gene were increased by LPS in rats or TNF- α in NCI-H292 cells, and these effects were inhibited by fudosteine treatment. After stimulation with LPS or TNF- α , the expression of p-EGFR, p-p38 MAPK, and p-ERK were detected. Fudosteine treatment reduced the expression levels of p-p38 MAPK and p-ERK in vivo and of p-ERK in vitro.

These results suggest fudosteine inhibits MUC5AC mucin hypersecretion by reducing MUC5AC gene expression and the effects of fudosteine are associated with the inhibition of ERK and p38 MAPK in vivo and ERK in vitro.

KEYWORDS : extracellular signal-related kinase (ERK), fudosteine, MUC5AC, p38 mitogen-activated protein kinase (MAPK)

The luminal surface of the airway is coated with mucus, which forms a protective barrier against toxins and pathogens. Mucus helps to clear particles and infectious agents from the airways, and is also an important component of the innate immune system of the lungs (1).

Fudosteine, (-)-(R)-2-amino-3-(3-hydroxypropylthio) propionic acid, was approved in Japan in 2001 for use as a new mucoactive agent with indications for chronic respiratory diseases such as bronchial asthma, chronic bronchitis, pulmonary emphysema, bronchiectasis, pulmonary tuberculosis, pneumoconiosis, atypical mycobacterial disease, and diffuse panbronchiolitis (2). In a clinical trial, fudosteine showed efficacy in improving the sense of discomfort in the chests of patients with a variety of chronic respiratory diseases (3). However, the exact mechanism by which fudosteine affects mucin secretion remains unknown.

Goblet cell hyperplasia and metaplasia are well-established hallmarks of the airways in many chronic respiratory diseases associated with mucus hypersecretion, including chronic bronchitis, bronchiectasis, cystic fibrosis, and bronchial asthma. Hypersecretion from an increased number of goblet cells is considered to contribute to mucus plugging and airway obstruction (4). Enhanced epithelial mucin expression is thought to be the rate-limiting step for goblet cell metaplasia (5). Four types of gel-forming mucins (MUC2, MUC5AC, MUC5B, and MUC19) are found in the lung. Among these, MUC5AC is the major respiratory mucin present in the secretions from goblet cells (6). MUC5AC mucin secretion is stimulated by a wide variety of stimuli, including proinflammatory cytokines such as IL-9, IL-1 β and tumor necrosis factor (TNF)- α (7, 8), lipopolysaccharide (LPS) (6), neutrophil elastase (9), epidermal growth factor receptor (EGFR) ligands (10), air pollutants (11), and bacterial products (12). However, agents that are known to inhibit MUC5AC mucin production are relatively few.

Based on these findings, we speculated that fudosteine, which is a novel mucoactive agent, would decrease mucin secretion in a process mediated by the suppression of MUC5AC gene expression. In the present study, we examined the effects of fudosteine on MUC5AC mucin synthesis and the associated signaling pathway using an animal model of LPS-induced airway inflammation and a bronchial epithelial cell line model of TNF- α induced inflammation.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats (7-weeks-old; Orient Co., Ltd., Charles River Laboratories, Korea) were used. We assigned the animals to the following five treatment groups: 1) control; 2) 1 mg/kg LPS; 3) 50 mg/kg fudosteine plus 1 mg/kg LPS; 4) 100 mg/kg fudosteine plus 1 mg/kg LPS; and 5) 200 mg/kg fudosteine plus 1 mg/kg LPS. In the LPS alone group, the rats were administered LPS (1mg/kg) in 0.3 ml saline by the intratracheal route. In the fudosteine plus LPS group, fudosteine was administered via the oral route once a day for 3 days, and then LPS was given. Rats were killed 2 days and 4 days after LPS administration (Figure 1). All of the experimental animals were treated according to the guidelines approved by the Animal Subjects Committee of The Catholic University of Korea.

Bronchial Epithelial Cell culture

The human pulmonary mucoepidermoid carcinoma cell line NCI-H292 was cultured in Roswell Park Memorial Institute (RPMI 1640) media that contained 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml), and HEPES (25 mM) at 37°C in a humidified 5% CO₂ water-jacketed incubator. TNF- α (recombinant human TNF- α , 50 ng/ml; R&D Systems) was used as the stimulus, as it has a potent effect on MUC5AC synthesis (13). NCI-H292 cells were also pretreated with fudosteine before stimulation with TNF- α , to examine the inhibitory effect on MUC5AC synthesis. For Western blot analysis of signaling molecules, NCI-H292 cells were pretreated with 50 μ M AG1478 (EGFR tyrosine kinase inhibitor; Calbiochem) or 40 μ M PD98059 (MEK inhibitor; Sigma Chemical Co., St. Louis, MO) 30 minutes before the addition of TNF- α .

Bronchoalveolar lavage (BAL)

Each rat was killed by CO₂ asphyxiation, and the trachea was exposed and cannulated. After the instillation of normal saline through the trachea into the lung, the BAL fluid was withdrawn. The lavage was repeated three times. The BAL fluid was centrifuged at 400 × g for 5 minutes, and the pellet was re-suspended in 1 ml of normal saline. The total cell counts in the BAL fluids were assessed with a hemocytometer. Wright-Giemsa staining was used for differential cell counting. The percentages of BAL fluid macrophages, eosinophils, lymphocytes and neutrophils were obtained by counting 400 leukocytes in randomly selected portions of the slide using light microscopy. The supernatant fluids were stored at -80°C.

CINC-1 ELISA

The concentration of CINC-1 in the BAL fluids was quantified using the rat CINC-1 ELISA set (R&D Systems, Minneapolis, MN). Samples were centrifuged at 8000 rpm for 15 min and the supernatants were assayed. Optical density (OD) measurements were obtained using an ELISA reader (BIO-TEK Instruments, Winooski, VT) at 450 nm wavelength.

Morphological analysis

Lung tissues were fixed with 4% paraformaldehyde in phosphate -buffered saline (PBS) and embedded in paraffin. Paraffin-embedded tissues were cut to 4-μm thickness using a microtome, and the deparaffinized tissue sections were subjected to alcian blue-periodic acid Schiff (AB-PAS) staining to identify goblet cells. The sections were observed and counted at 400× magnification under a light microscope. The numbers of goblet cells per tracheal ring were reported.

MUC5AC mucin ELISA

BAL fluids (50 μl aliquots) from the rat or NCI-H292 cell lysates (50 μl aliquots) were loaded into 96 -well ELISA plates with 50 μl of 2 × carbonate/bicarbonate buffer, and dried at 44°C. The plates were washed three times with PBS, and blocked with 2% bovine serum albumin (BSA) for 1 hour at room temperature. The plates were then incubated with 50 μl of mouse anti-MUC5AC antibody (1:100; Neomarker, Fremont, CA) for 1 hour. The plates were washed as described above. Mucin detection was accomplished by the addition of 100 μl/well of a 1:2500 dilution of peroxidase-conjugated goat anti-mouse IgG in PBS, followed by incubation for 1 hour. The plates were then washed as described above. The colorimetric reaction was developed by the addition of 100 μl/well of peroxidase substrate. OD measurements were obtained using

an ELISA reader (BIO-TEK Instruments) at 405 nm, with 450 nm serving as the reference wavelength. The results were expressed as percentages of the control.

MUC5AC gene analysis by RT-PCR

For RT-PCR analysis of gene expression, total RNA was isolated from the lung tissue or cultured NCI-H292 cells. The 4 µg of total RNA was reverse -transcribed with a dNTP mixture and random primers using SuperScript III (200 U/µl; Invitrogen, Carlsbad, CA). The cDNA was amplified in a thermocycler (Bio-Rad, Hercules, CA) using the following specific primer pairs: for MUC5AC (557-bp product), forward 5'-TCCGGCCTCATCTTCTCC-3' and reverse 5'-ACTTGGGCACTGGTGCTG-3'; and for β-actin (295-bp product, internal control), forward 5'-CAAGAGATGGCCACGGCTGCTTCC-3' and reverse 5'-TCCTTCTGCATCCTGTCGGCAATG-3'. The cycling conditions were 28 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C. The amplified PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

Western blot

Western blotting was performed for the detection of phosphorylated epidermal growth factor receptor (p-EGFR), phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK), and phosphorylated extracellular signal-related kinase (p-ERK). Lung tissues were disrupted using a Polytron homogenizer and centrifuged. The proteins were purified from the supernatant and the concentration was assessed by the Bradford method. Cultured NCI-H292 cells were lysed in RIPA buffer [20 mM Tris-HCL (pH 7.4), 137 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 µg/ml aprotinin, 1 mM PMSF, 0.1 mM sodium vanadate, 10 mM sodium fluoride] on ice for 20 min. The disrupted cells were centrifuged, proteins were collected from the supernatant, and the protein concentration was determined by the Bradford method. Protein samples (50 µg) were separated on a discontinuous PAGE gel, and then transferred to a nitrocellulose (NC) membrane at 80 V for 2 hours. The membrane was blocked with 5% skim milk in TBS buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl] for 1 hour, and then incubated with anti-p-EGFR (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p38 MAPK (1: 1000; Cell Signaling, Danvers, MA), and anti-p-ERK (1: 400; Santa Cruz Biotechnology) antibodies at 4°C overnight. The membrane was washed three times with washing buffer (TBS + 0.1% NP-40) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) at room

temperature for 2 hours. The target protein was detected using the ECL kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and x-ray film.

Morphological analysis by immunostaining

For MUC5AC immunostaining, cultured NCI-H292 cells were incubated with anti-MUC5AC monoclonal antibody (1:50; Santa Cruz Biotechnology) at 4°C overnight. The colorimetric reaction was developed with 3,3'-diaminobenzidine tetrachloride (Zymed Laboratory Inc., South San Francisco, CA).

Statistical analysis

All the data are presented as means \pm SE. Data obtained from all the experiments were analyzed by Kruskal-Wallis one-way non-parametric analysis of variance with *post hoc* evaluations using the Mann-Whitney rank sum test (SAS Institute, Cary, NC). The level of significance was considered to be $p < 0.05$.

RESULTS

Effect of fudosteine on inflammatory cells and CINC-1 levels in rat BAL fluids

The neutrophils and total inflammatory cells in the BAL fluid were significantly increased in the LPS-treated group, as compared to the control group, at both 48 hours and 96 hours after LPS instillation. Pretreatment with fudosteine significantly reduced this increase in neutrophils and total cell number, but only at the 96 hour time-point after LPS instillation (Figure 2a, 2b). In response to LPS instillation, the level of CINC-1 was increased at 48 hours. However, pretreatment with fudosteine did not change significantly the level of CINC-1 in the BAL fluids of rats (Figure 2c).

Effect of fudosteine on goblet cell numbers in rat BAL fluids

The numbers of goblet cells in the tracheal epithelium were increased at 48 hours and 96 hours after intratracheal instillation of LPS. The numbers of goblet cells in fudosteine-treated rats tended to decrease with increasing concentrations of fudosteine. A significant reduction in goblet cell number was observed at 96 hours after LPS instillation in rats that were pretreated with 200 mg/kg fudosteine (Figure 3b, 3d).

Effects of fudosteine on mucin secretion and MUC5AC gene expression

MUC5AC mucin synthesis was increased at 96 hours after LPS stimulation. Pretreatment with fudosteine could significantly reduce the increase in mucin synthesis in the 96 hours groups (Figure 3a). The MUC5AC gene expression was also increased by the instillation of LPS, which was inhibited by the pretreatment with fudosteine (Figure 3c).

Effects of fudosteine on the expression levels of EGFR, p38 MAPK, and ERK

LPS administration to the rat increased the levels of phosphorylated EGFR, p38 MAPK, and ERK. While fudosteine did not reduce the levels of p-EGFR, pretreatment with fudosteine reduced the levels of phosphorylated p38 MAPK and ERK in a dose dependent manner (Figure 4). The levels of phosphorylated EGFR, p38 MAPK, and ERK in NCI-H292 cells were also increased by TNF- α stimulation to the cultured NCI-H292 cells. Pretreatment of NCI-H292 cells with AG1478 inhibited the TNF- α induced expression of phosphorylated EGFR and p38 MAPK. However, fudosteine did not inhibit the TNF- α induced expression of phosphorylated EGFR and p38 MAPK (Figure 6a). On the other hand, pretreatment with either PD98059 or fudosteine inhibited the TNF- α -induced expression of p-ERK (Figure 6b).

Effects of fudosteine on mucin secretion in NCI-H292 cells

The level of MUC5AC mucin secreted by the NCI-H292 cells was increased by TNF- α stimulation. The levels of MUC5AC mucin in the supernatants of NCI-H292 cell cultures stimulated with TNF- α were reduced by pretreatment with 1 mM fudosteine, as confirmed by immunostaining (Figure 5a, 5b). RT-PCR analysis revealed that MUC5AC gene expression was increased by TNF- α stimulation, and that this increase was inhibited by pretreatment with fudosteine (Figure 5c).

DISCUSSION

Mucus is essential because of its role in protecting the airways. However, chronic inflammatory lung diseases, such as bronchial asthma and chronic obstructive

pulmonary disease (COPD), are often associated with excessive mucus production, especially in cases of bacterial infection. Although mucins are part of the innate immunity system, and help to clear bacteria from the lungs, excessive mucus production can have deleterious effects. Mucus plugs can lead to pulmonary obstruction and may support bacterial colonization and infection (14).

It has been reported that MUC5AC is the major mucin in human airways (6). The mechanism of regulation of MUC5AC secretion in airways is very important, and elucidation of this mechanism may suggest new therapeutic strategies for the inhibition of airway mucus hypersecretion (13).

The expression and activation of EGFR result in MUC5AC mucin production by airway epithelial cells in vivo and in vitro (15). An EGFR signaling cascade has been shown to be a common pathway through which many stimuli induce MUC5AC production, and MAPK is downstream of EGFR. Activation of EGFR is followed by the activation of various signaling pathways, including p38 MAPK and Ras→MEK1/2→ERK1/2, which results in the activation of various transcription factors, e.g., activator protein-1, NF-κB, and Sp1 (6). *Pseudomonas aeruginosa* upregulates MUC5AC expression following activation of the p42/44 MAPK pathway via the EGFR signaling cascade (16). ERK and p38 MAPK are essential for TNF-α induced MUC5AC gene expression (13).

LPS and TNF-α are well known stimulants of goblet cell hyperplasia and mucus hypersecretion (2, 13). Our experiments with rats and NCI-H292 cells revealed that, after stimulation with LPS or TNF-α, goblet cell hyperplasia and increased expression of MUC5AC gene occur, followed by mucus hypersecretion. The levels of phosphorylated EGFR, p38 MAPK, and ERK 1/2 were increased in both LPS stimulated rats and TNF-α stimulated NCI-H292 cells.

The therapeutic efficacy of fudosteine as a mucoactive agent has been shown in previous studies. Fudosteine showed beneficial activity against the impairment of mucociliary transport by irritant gases (17). Takahashi et al (18) have shown that isoproterenol increases the number of goblet cells in the tracheal epithelium, and that fudosteine inhibits this increase. Komatsu and colleagues have shown that fudosteine inhibits endotoxin induced goblet cell hyperplasia by inhibiting CINC-1 production and/or neutrophil migration, and that it also inhibits ovalbumin induced eosinophil infiltration (2). However, no studies have been published to date regarding the signal pathways involved in the inhibitory effects of fudosteine. In the present study, we clearly demonstrate that fudosteine inhibits mucus hypersecretion both in vivo and in vitro, and that this inhibition is related to ERK1/2 and p38 MAPK in LPS treated rats

and ERK1/2 in TNF- α treated NCI-H292 cells. To the best of our knowledge, this is the first study to reveal the signal transduction molecules involved in fudosteine inhibition of mucus hypersecretion.

Neutrophils are the predominant inflammatory cells in the airways of patients with COPD, cystic fibrosis, and acute exacerbations of asthma (6). Neutrophilic products, such as proteases and oxidants, have been evaluated for their roles in regulating mucin gene expression. Neutrophil elastase, which is a serine protease, increases MUC5AC gene expression by inducing oxidative stress (19) or releasing TGF- α , which results in EGFR activation (20). In the present study, the neutrophil counts were elevated in LPS stimulated rats and significantly lower in fudosteine pretreated rats. Therefore, the inhibitory effect of fudosteine on mucus hypersecretion may be partly due to the inhibition of neutrophil recruitment. However, in our *in vitro* experiments where neutrophils were absent, fudosteine also showed inhibitory effects on MUC5AC gene expression and mucin production. This findings suggest that fudosteine could directly suppresses mucus production by bronchial epithelial cells in the absence of neutrophils.

In the present study, fudosteine had no inhibitory effect on CINC-1, in contrast to results reported previously (2). CINC-1 is known to increase within a relatively short time (4-24 hours) after LPS stimulation, so the assay time-points of 48 hours and 96 hours used in the present study may have been too late to detect an inhibitory effect. In addition, inhibition of CINC-1 production may not be an essential target of fudosteine. We cannot rule out the possibility that CINC-1 inhibition is due to other pharmacologic effects, since fudosteine does not inhibit LPS induced CINC-1 production by rat alveolar macrophages *in vitro* (2). Therefore, although there was no inhibition of CINC-1 at 48 hours and 96 hours after LPS stimulation, fudosteine significantly inhibited LPS induced neutrophil migration and showed efficacy in reducing mucin hypersecretion in rats.

In LPS treated rats, the activation of ERK1/2 and p38 MAPK was inhibited by fudosteine. However, only the phosphorylation of ERK1/2 was blocked by fudosteine in the TNF- α treated NCI-H292 cells. We propose the following hypothesis to explain these results. In the *in vivo* setting, not only bronchial epithelial cells but also many heterogeneous cells are involved in the responses to inflammatory stimuli. The inhibitory effect of fudosteine on the phosphorylation of p38 MAPK may occur in neutrophils or other inflammatory cells. The phosphorylation of p38 MAPK in our LPS-treated rats may reflect cell signaling by both bronchial epithelial cells and neutrophils. Alvarez and colleagues have shown that p38 MAPK is activated in neutrophils upon bacterial DNA stimulation (21). Thus, the reduction in the level of p38 MAPK by

fudosteine observed in the present study may be partly attributable to the decrease in neutrophil numbers. In contrast, in the in vitro environment, only bronchial epithelial cells were involved, and the inhibitory effect of fudosteine was measured only for these cells. Therefore, we cautiously suggest that fudosteine decreases mucin secretion by blocking the activation of ERK1/2 in bronchial epithelial cells, and that p38 MAPK is implicated in neutrophils activated by LPS stimulation, which is also inhibited by fudosteine.

Another mucoactive drug, acetyl-cysteine, also shows an inhibitory effect on mucin expression (14, 22). However, there have been few studies on the signal pathways modulated by acetyl-cysteine. Takeyama and colleagues showed that N-acetyl-L-cysteine inhibited EGFR tyrosine phosphorylation induced by H₂O₂ and by the supernatant fluid of activated neutrophils but had no effect on TGF- α induced EGFR tyrosine phosphorylation (23). No data have been published to date regarding the potential involvement of ERK1/2 or p38 MAPK in this process.

In summary, we show that fudosteine inhibits MUC5AC mucin hypersecretion by reducing the expression of the MUC5AC gene. Although EGFR is a common molecule for the activation of MUC5AC gene expression, the inhibitory effect of fudosteine is not related to EGFR. With respect to the MUC5AC mucin secretion pathways, fudosteine inhibited the phosphorylation of ERK1/2 and p38 MAPK in LPS-treated rats and of ERK1/2 in TNF- α -treated NCI-H292 cells. These findings suggest that fudosteine may be useful in controlling stress-related mucus secretion states in patients with asthma, bronchiectasis or COPD.

Figure Legends

FIGURE 1. Experimental protocol for the animal model. The rats were divided into five groups. LPS (1 mg/kg) was administered in 0.3 ml saline by the intratracheal route. Different dosages of fudosteine (50, 100, and 200 mg/kg) were administered via the oral route for three days before LPS stimulation. Rats were killed 2 or 4 days after LPS administration; n, number of rats in each group.

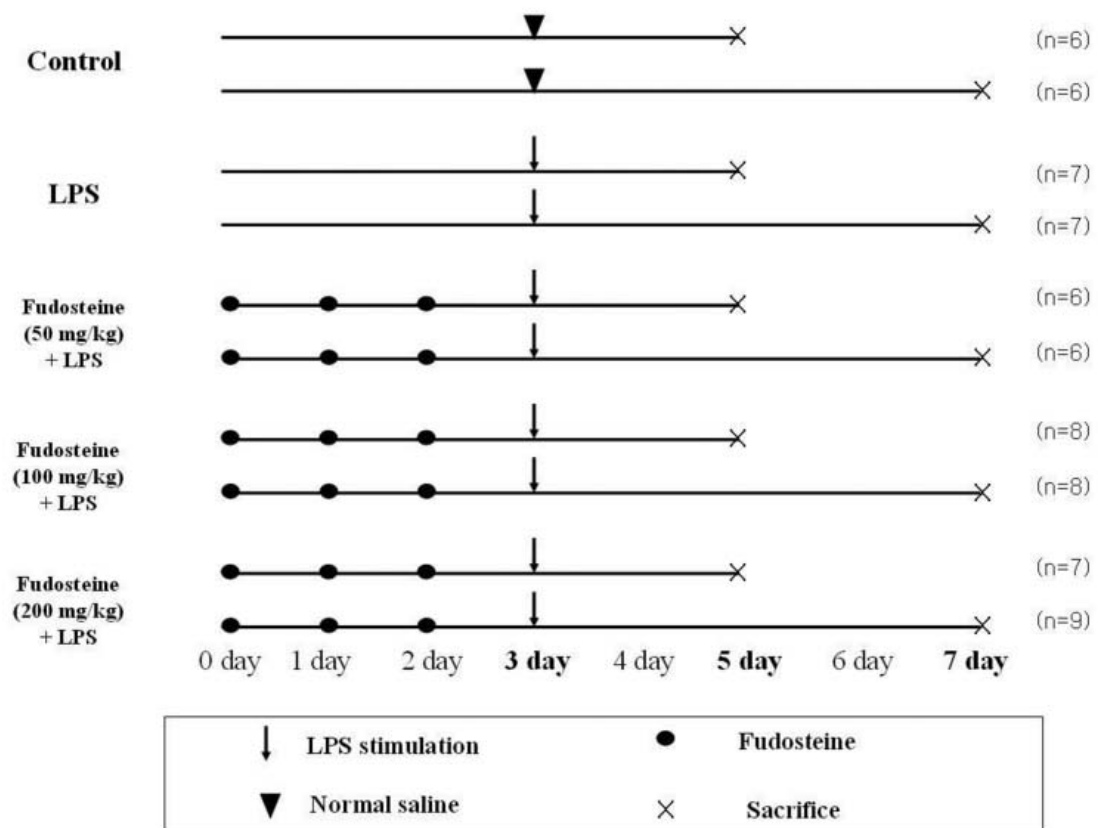


FIGURE 2. LPS administration to the rat markedly increases the numbers of inflammatory cells and neutrophils in the BAL fluids. Pre-treatment of different dosages of fudosteine (50, 100, and 200 mg/kg) result in decreased inflammatory cell (a) and neutrophil (b) numbers in the BAL fluids harvested 96 hours after the administration of LPS. In contrast, the levels of CINC-1 are not decreased by fudosteine treatment (C). The inflammatory cells were identified by Wright-Giemsa stain (d). n = 6, *p < 0.05 versus the control group; †p < 0.05 versus the LPS-treated group.

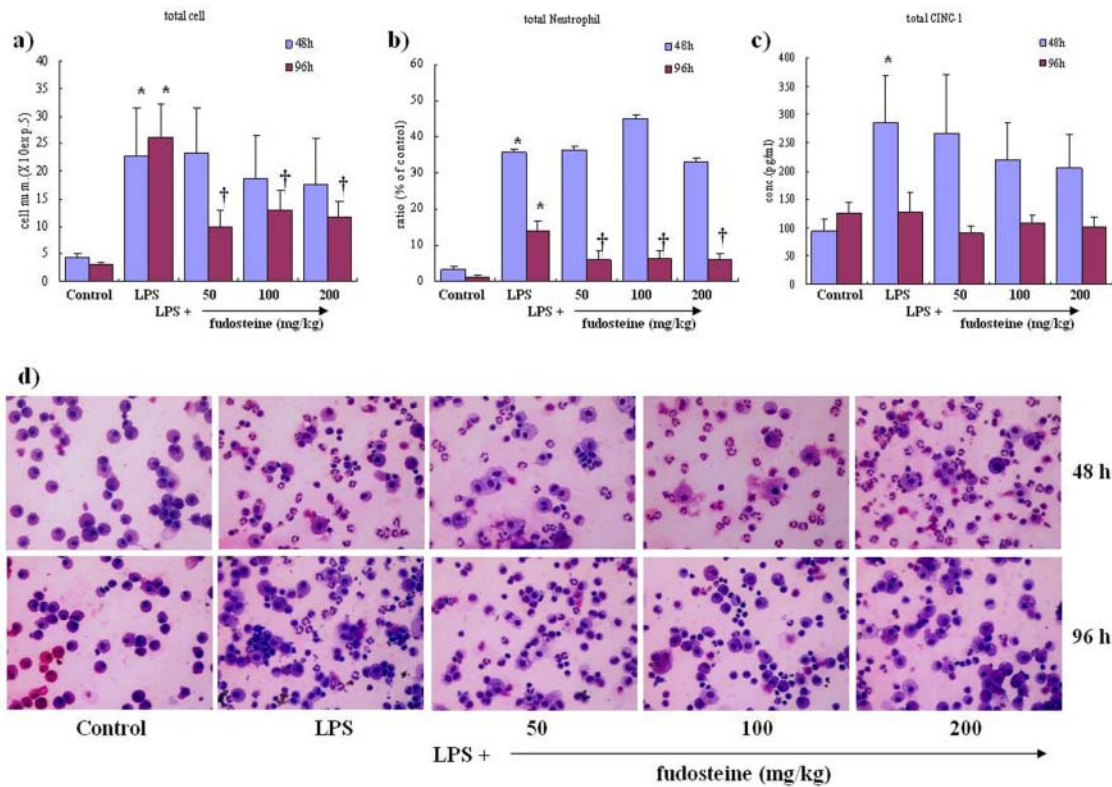


FIGURE 3. The levels of MUC5AC mucin were increased by LPS administration. This increase in MUC5AC mucin production was reduced by fudosteine (50, 100, and 200 mg/kg) when the BAL fluids were harvested 96 hours after LPS administration (a). Increased goblet cell numbers in the trachea by LPS administration was decreased by fudosteine at the concentration of 200 mg/Kg and killed at 96 hours after LPS administration (b, d). Lung sections stained with AB-PAS for mucin. RT-PCR analysis shows that LPS increases the level of MUC5AC mRNA in the rat lung. Each dosage of fudosteine (50, 100, 200 mg/kg) suppresses LPS-induced MUC5AC mRNA expression

(c). $n = 6$, $*p < 0.05$ versus the control group; $†p < 0.05$ versus the LPS-treated group.
 AB-PAS, alcian blue-periodic acid Schiff.

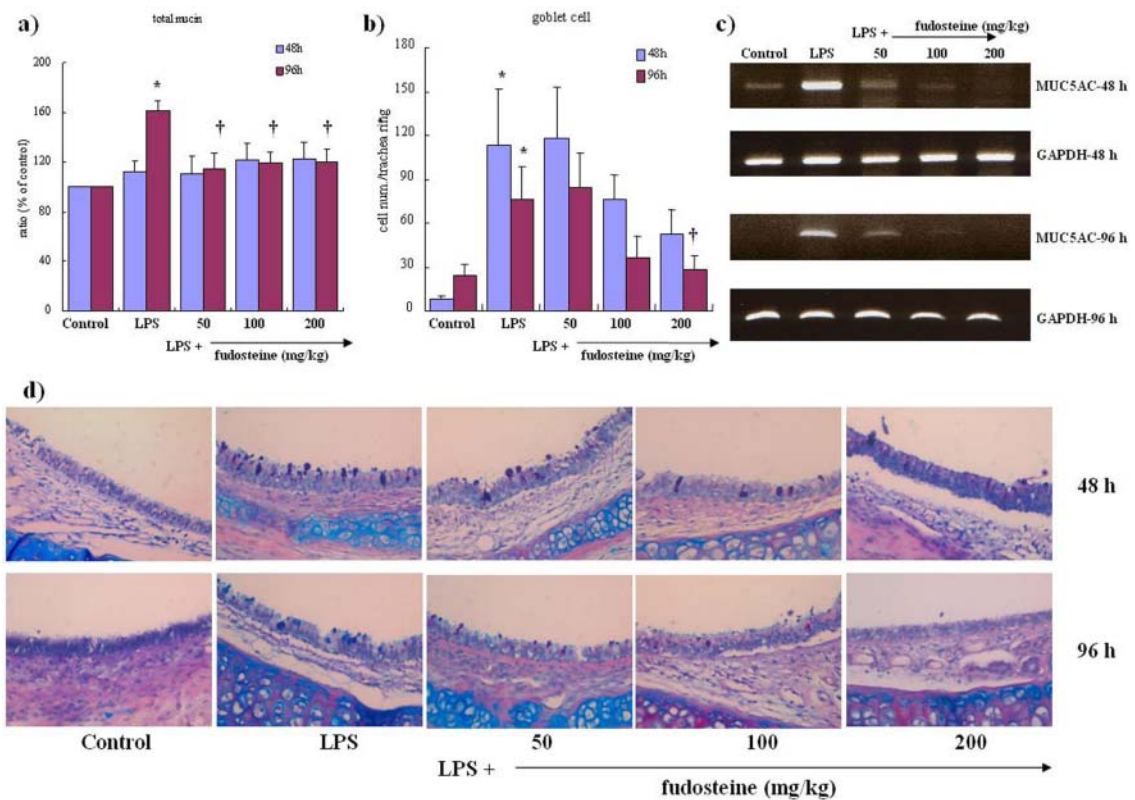


FIGURE 4. LPS administration increases the levels of phosphorylated EGFR, ERK1/2, and p38 MAPK in rat lungs. Different dosage of fudosteine (50, 100, 200 mg/kg) decreases the LPS-induced p-ERK1/2 and p-p38 MAPK levels in the rat lungs. Fudosteine did not decrease the LPS-induced expression of p-EGFR. Results were representative immunoblot of three separate experiments. p-EGFR, phosphorylated epidermal growth factor receptor; p-ERK, phosphorylated extracellular signal-related kinase; p-p38 MAPK, phosphorylated p38 mitogen-activated protein kinase.

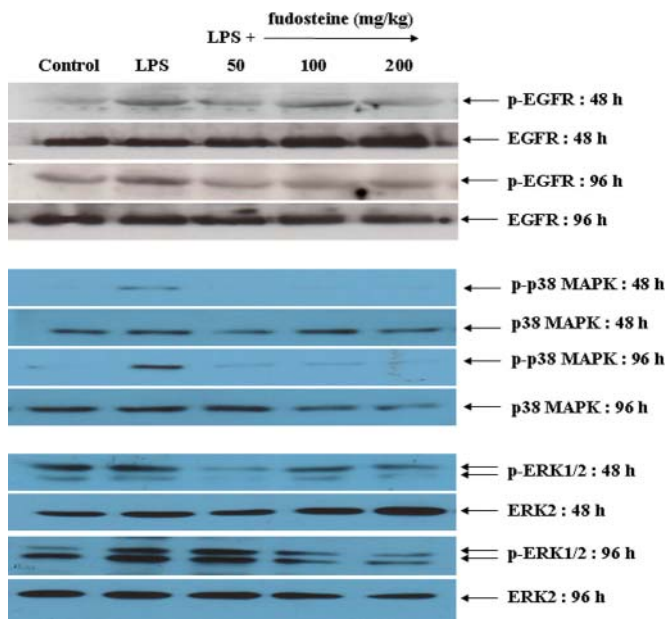


FIGURE 5. Effect of fudosteine on mucin expression in TNF- α -stimulated NCI-H292 cells. TNF- α induces MUC5AC mucin expression in NCI-H292 cells (a) and culture supernatants (b). Fudosteine suppresses TNF- α induced MUC5AC mucin secretion, albeit only at the concentration of 1 mM. RT-PCR analysis also shows that TNF- α increases MUC5AC mRNA expression in NCI-H292 cells. The TNF- α -induced increase in MUC5AC mRNA expression is inhibited by pretreatment with 1 mM fudosteine (c). n = 3, *p < 0.05 versus the control group; †p < 0.05 versus the TNF- α -treated group.

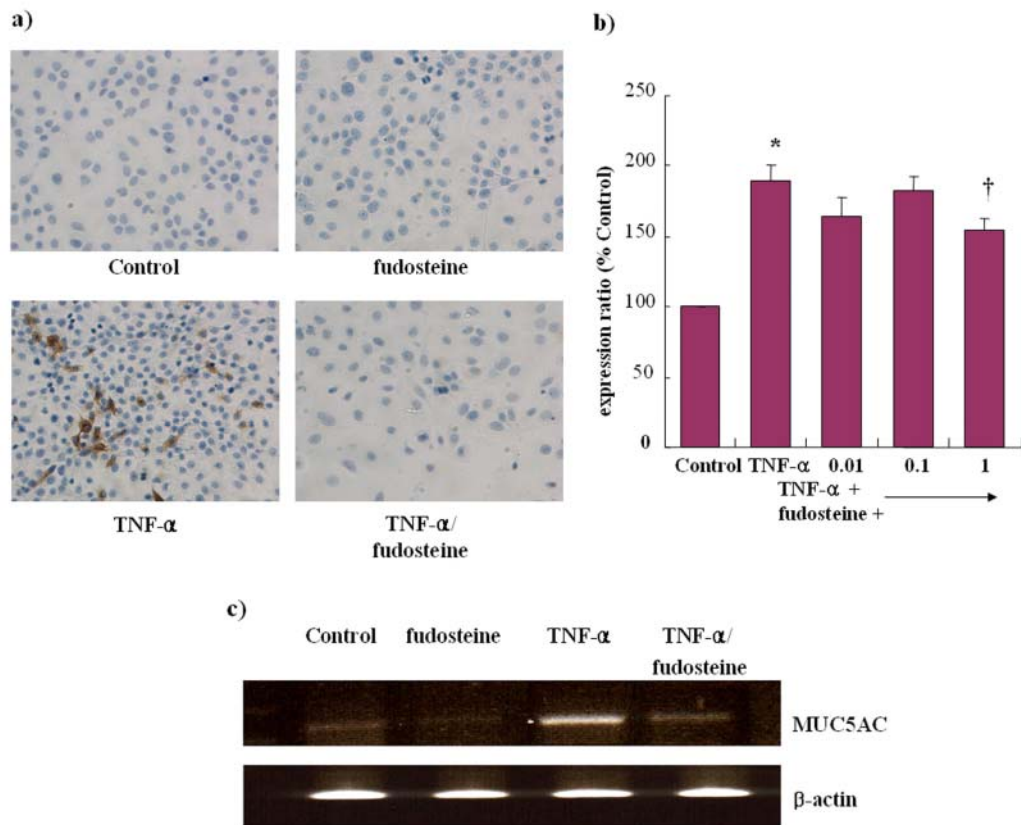
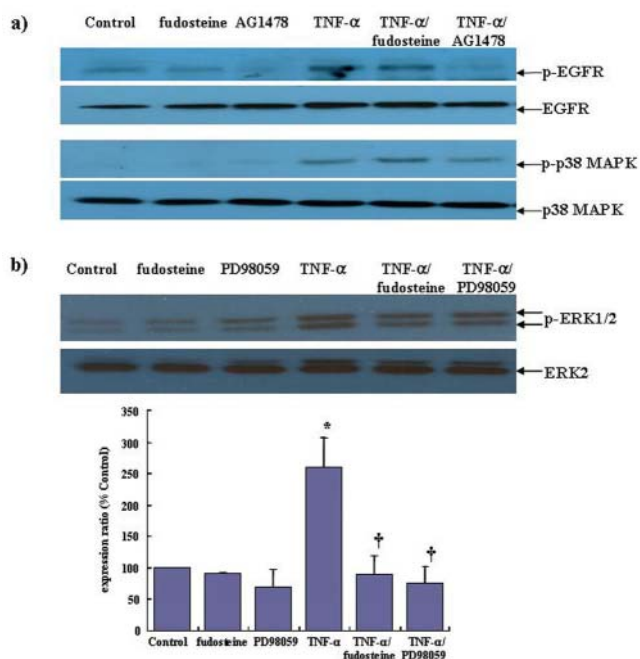


FIGURE 6. Western blotting for phosphorylated EGFR, p38 MAPK and ERK1/2 in NCI-H292 cells. TNF- α treatment results in the phosphorylation of EGFR, p38 MAPK, and ERK1/2. Fudosteine reduces the expression of p-ERK 1/2 (b), but not that of EGFR or p38 MAPK (a) during the stimulation with TNF- α . A selective inhibitor of EGFR tyrosine kinase, AG1478 (10 μ M) inhibited the phosphorylation of EGFR and a selective MEK inhibitor, PD98059 (30 μ M), inhibited the ERK1/2 phosphorylation during TNF- α stimulation. Results were representative immunoblot of three separate experiments. * $p < 0.05$ versus the control group, [†] $p < 0.05$ versus the TNF- α treated group.



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