Macrolides inhibit *Fusobacterium nucleatum*-induced MUC5AC induction in human airway epithelial cells

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Abstract

Fusobacterium nucleatum (Fn) is one of the most common anaerobic bacteria in periodontitis and is responsible for several extra-oral infections including respiratory tract diseases. In this study, we examined whether Fn induces mucin secretion in airway epithelial cells. We also examined the effects of macrolides on the Fn-induced mucus production compared with other antibiotics that exert anti-anaerobic activities. MUC5AC production in bronchial epithelial cells after stimulation with culture supernatants (Sup) of Fn was analyzed by performing enzyme-linked immunosorbent assay and quantitative RT-PCR. The cell-signaling pathway of Fn Sup stimulation was also analyzed by performing Western blotting. For inhibition studies, cells were treated with azithromycin, clarithromycin, clindamycin (CLDM), and metronidazole (MTZ). The Fn Sup-induced NCI-H292 cells to express MUC5AC at both the protein levels and the mRNA level in both a time- and dose-dependent manner. Macrolides inhibited Fn Sup-induced MUC5AC production, while CLDM and MTZ were less effective. Fn Sup induced the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, and this induction was suppressed by macrolides. Fn Sup-induced MUC5AC

production was blocked by the ERK pathway inhibitor U0126. Fn is likely to contribute to excessive mucin production, which suggest that periodontitis may correlate with the pathogenesis of chronic respiratory tract infection. The macrolides seem to reduce this mucin production and might represent an additional therapeutic intervention for Fn respiratory tract infections other than CLDM and MTZ.

Introduction

Although mucus secretion is useful for host protection against pathogens and irritants, mucus hypersecretion causes airway obstruction and impairment of gas exchange in chronic inflammatory lung disease including asthma, cystic fibrosis, diffuse panbronchiolitis (DPB), and COPD. Therefore, preventing mucus overproduction is beneficial for these diseases. Many factors, including bacterial infection, that contribute to mucus hypersecretion have been previously described (1, 2). However, there are few studies that focus on the relationship between oral bacterium infection and mucus hyper secretion.

Poor oral hygiene has been suggested to be a risk factor of respiratory disease (3), and several studies indicate that oral care reduces the incidence and mortality of pneumonia (4–7). However, the detailed mechanisms that described the poor oral hygiene and respiratory tract disease relationship are not fully understood.

Fusobacterium nucleatum (Fn) is a common anaerobic bacterium of periodontitis, which is also found as etiologic pathogen of respiratory anaerobic infection (8). As the important virulence factor, Fn produces high

91 amounts of butyric acid during anaerobic glycolysis. Recently, several reports
92 indicated that butyric acid plays a critical role in a variety of diseases,
93 including HIV infection (9, 10), and ulcerative colitis (11). Considering the
94 effect of oral bacterium on respiratory tract disease, the aspiration of
95 products originating from periodontal tissues has been suggested as a
96 possible mechanism of the effects on respiratory tract (12). With respect to
97 the frequency of aspiration, Marik PE et al. reported that approximately half
98 of all healthy adults aspirate small amount of oropharyngeal secretions
99 during sleep (13). On the basis of these reports, we expect that Fn, a major
100 periodontal bacterium, might have a pathogenic effect on airway epithelium
101 cells via aspiration of its products.
102 In this study, we examined the effects of Fn culture supernatant (Sup) on
103 airway epithelium cell mucus secretion. The major macromolecular
104 constituents of mucus are the mucin glycoproteins. Among mucin proteins,
105 we focused on MUC5AC, the major core protein of mucin secreted from the
106 airway surface epithelium.
107 We also examined the effects of the macrolides, azithromycin (AZM) and
108 clarithromycin CAM), on the Fn Sup-induced mucus production and

compared their effects to other antibiotics which have anti-anaerobic activities (e.g., clindamycin (CLDM) and metronidazole (MTZ)). Macrolide antibiotics have been shown to be effective for the treatment of chronic airway diseases (14, 15). The beneficial effects of macrolide therapy are not only related to its bactericidal properties, but extend to its immune-modulating/anti-inflammatory effects (16). We previously reported that macrolides inhibit MUC5AC production induced by several factors (*Pseudomonas aeruginosa* autoinducer (17), lipopolysaccharide (18), Nontypeable *Haemophilus influenza* (19), *Chlamydophila pneumonia* (20)) in human lung epithelial cells, and found that these mucin reduction by macrolides relate to several intracellular signal transduction, including extracellular signal-regulated kinase (ERK) 1/2 phosphoryration (17, 18), NFκB activation (17, 20) or AP-1 activation (19). As macrolide have no bactericidal activities against Fn, the effect on mucin production compared with CLDM and MTZ would provide insight concerning the treatment of Fn respiratory tract infections.

The aims of this study were to determine whether Fn Sup possesses stimulatory action on the production of MUC5AC, and to clarify whether

127 macrolides have different effect on Fn Sup-induced MUC5AC production as
128 compared to CLDM and MTZ.
129
130 **Materials and Methods**
131 **Fn strain and culture conditions to obtain Fn Sup**
132 A clinical isolate of Fn (strain FNU-191), maintained as a stock culture in the
133 Department of Laboratory Medicine, Nagasaki University Hospital,
134 Nagasaki, Japan, was used in this study. We identified the strain by PCR
135 amplification and sequencing analysis of the 16S rRNA gene. The
136 supernatant was obtained as described previously (10). Briefly, the Fn strain
137 was cultured on PV Brucella HK Agar (Kyokuto Pharmaceutical Industrial
138 Co., Tokyo, Japan) for 48 h in an anaerobic condition, and then scraped and
139 suspended in modified GAM broth (Nissui Pharmaceutical Industrial Co.,
140 Tokyo, Japan) and cultured in anaerobic chamber for 48 h. The supernatant
141 was then collected by centrifugation at 10,000 rpm for 50 min at 4 °C to
142 remove the bacteria and then filter-sterilized through a 0.22 μm pore
143 membrane filter (Millipore, Bedford. MA, USA). In the preliminary
144 experiments, we examined MUC5AC induction of 6 *Fusobacterium* spp.

strains including the reference strain (ATCC 10953). We found that all the supernatant of *Fusobacterium* spp. similarly induced MUC5AC production at a 1:39–1:79 dilution and inhibited MUC5AC production at a 1:4–1:19 dilution. In order to test for clinical relevancy, we selected the clinically isolated strain of Fn for the further experiments. Therefore, all the experiments in this study were performed using the F2 strain.

Cell culture

The NCI-H292 (Human airway epitherial) cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. The cells were grown at 37 °C with 5% CO2 in fully humidified air. For the MUC5AC production studies, cells were exposed to Fn Sup for RT-PCR, enzyme-linked immunosorbent assay (ELISA), or Western Blotting. For controls, the cells were incubated with GAM broth.

Preparations of antibiotic dilutions

AZM and CAM were provided by Pfizer (Tokyo, Japan) and Taisho-Toyama (Tokyo, Japan), respectively. Clindamycin and metronidazole were obtained from Nacalai Tesque (Kyoto, Japan). Each drug, except MTZ, was diluted in

DMSO at final concentrations of 1-100 μg/ml for the following experiments. Only MTZ was diluted in acetic acid.

ELISA

The NCI-H292 cells were plated in a 24-well plate, and MUC5AC protein was measured by performing ELISA as previously described (17–20). After Fn Sup stimulation, the culture medium was collected as the cell supernatant. This supernatant was then incubated at 40 °C in a 96-well plate until dry. The plated culture were blocked with 2% bovine serum albumin for 1 h at room temperature and were then incubated with the anti-MUC5AC antibody diluted in PBS containing 0.05% Tween 20 for 1 h. Horseradish peroxidase (HRP)-conjugated anti-goat IgG was then dispensed into each well. After 1 h, the plates were washed 3 times with PBS. Color was developed using a 3,3', 5, 5'-tetramethyl-benzine-peroxidase solution, and the reaction was stopped by the addition of 2 N H_2SO_4. Absorbance was read at 450 nm.

Inhibition of cell signaling activity

ERK inhibitor U0126, p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 and specific NFκB inhibitor caffeic acid phenethyl ester

(CAPE) were used at concentrations 10 μM (in DMSO stock solution). Cells were treated with these inhibitors 30 min before Fn Sup stimulation. Control cultures were treated with an equal volume of DMSO. All the inhibitors were purchased from Calbiochem (San Diego, California).

RT-PCR

We evaluated MUC5AC mRNA expression by RT-PCR as described previously (17–20). Total RNA was extracted from NCI-H292 cells cultured in 6 well plates using QuickGene-Mini80 and QuickGene RNA cultured cell kits (FUJIFILM Co., Tokyo, Japan), according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), and was then treated with RNaseH. To quantify the expression of the MUC5AC gene, PCR primers and Taqman probes were designed and used as reported previously (Forward primer, 5′-CAGCCACGTCCCCTTCAATA-3′; Reverse primer, 5′-ACCGCATTTGGGCATCC-3′; Taqman probe, 5′-6-FAM-CCACCTCCGAGCCCGTCACTGAG-TAMRA-3′) (21). MUC5AC was amplified for 40 cycles (15 s at 95 °C, and 30 s at 60 °C) using a

LightCycler system. To normalize MUC5AC expression, human porphobilinogen deaminase (hPBGD) was also measured using an hPBGD primer set (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Data are presented as a ratio of hPBGD.

Western blot analysis

Proteins were separated by performing reducing sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, N.J.) in a solution of 20% methanol, 25 mM Tris-HCl, 0.2 M glycine. Nonspecific binding was blocked by incubating the membranes with 10% fetal bovine serum in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature. Immunoreactive proteins were detected by incubating the membrane with rabbit anti-human ERK1/2, anti-phospho-ERK1/2, anti-human p38, anti-phospho-p38, anti-human I-κB, or anti-phospho-I-κB antibodies (each at 1:1000) overnight at 4 °C. Between each step, the membrane were washed 3 times for 15 min each with Tris-buffered saline that contained 0.1% Tween 20. Subsequently, the membranes were incubated for 1 h with anti-rabbit immunoglobulin G conjugated to HRP (1:10,000), rewashed, and developed

with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Statistical analysis

All data were expressed as the mean and standard error of the mean (SEM). Differences were examined for statistical significance by using the one-way analysis of variance for comparisons involving more than 2 groups and the Student's t test for comparisons between 2 groups. *P* values less than 0.05 were considered statistically significant.

Results

Fn Sup up-regulates MUC5AC gene and protein expression

To determine whether Fn Sup can induce mucin production in NCI-H292 cells, we evaluated MUC5AC expression at both the mRNA and the protein level after the addition of Fn Sup. Stimulation of the NCI-H292 cells with GAM broth (1:9 dilution) had small effect on MUC5AC production compared to the stimulation with RPMI medium alone. However, the amounts of MUC5AC were significantly larger in Fn-Sup stimulation at the 1:79 to 1:319 dilution compared to GAM broth stimulation. The protein level (Fig.

1A) and mRNA expression (Fig. 1B) were maximal at the 1:79 dilution. The up-regulation of MUC5AC by the addition of Fn-Sup at a 1:79 dilution occurred in a time-dependent manner, and the protein level (Fig. 2A) was maximal at 24 h after stimulation. The mRNA expression level (Fig. 2B) increased until 12 h and decreased at 18 h after stimulation. The maximal mRNA expression was obtained at 10 h after stimulation (Data not showed), thus, we analyzed all other experiments concerning MUC5AC mRNA at 10 h after stimulation.

Fn Sup phosphorylates ERK

MAPKs are important signals related to MUC5AC production. To examine the cell-signaling pathway of Fn Sup stimulation in NCI-H292 cells, we examined the phosphorylation of kinase by western blotting (Fig. 3). We analyzed kinase phosphorylation in both GAM broth and Fn Sup activated cells at 0–720 min after stimulation. Compared to GAM broth (Fig. 3A), maximal ERK phosphorylation of Fn Sup-activated cells was observed 240 min after stimulation (Fig. 3B), while ERK phosphorylation of GAM broth-activated cells was mainly observed 480 min after stimulation. We also performed an inhibition assay of the cell-signaling pathway (Fig. 4). The

ERK inhibitor U0126 effectively suppressed the MUC5AC protein production compared to the untreated cells. Although the NFκB inhibitor CAPE and the p38 MAP kinase inhibitor SB203580 also suppressed the MUC5AC production, apparent phosphorylation of IκB or p38 could not be found by Western blotting.

Macrolides inhibits MUC5AC production by Fn Sup activated cells

To evaluate the effect of the macrolides CLDM and MTZ on Fn Sup-induced MUC5AC production, we treated cells with a 1–100 μg/ml concentration of each drug. Since CAM could not dissolved in 100 μg/ml, we examined its affects at the 1–50 μg/ml concentration. As shown in Fig. 5A and B, the macrolides significantly reduced MUC5AC protein level at the 1–100 μg/ml concentration in dose-dependent manner. CLDM significantly reduced MUC5AC protein level at the 100 μg/ml concentration, and MTZ did not reduce MUC5AC protein at any concentration. At the maximum dosage of each drug, we also examined the effect on MUC5AC mRNA expression. For controls and untreated group, the cells were also stimulated with same amount of DMSO or acetic acid contained in the drug dilutions. Since only MTZ needed to be dissolved with acetic acid, the evaluation of MTZ was

271 examined separately.

272 As shown in Fig. 6, the macrolides significantly reduced the mRNA
273 expression level of MUC5AC, while no significant reduction was found with
274 CLDM and MTZ.

275 **Macrolides down-regulate the phosphorylation of ERK in Fn Sup activated**
276 **NCI-H292 cells**

277 In order to investigate the potential role for the Fn Sup-activated
278 cell-signaling pathway of macrolides, CLDM and MTZ, we examined the
279 phosphorylation of ERK, the most significantly up-regulated signaling
280 pathway during Fn Sup induced activation. As shown in Fig. 7A, the
281 macrolides suppressed the phosphorylation of ERK compared to Fn Sup
282 stimulation alone and CLDM. MTZ did not affect the detection level
283 compared to stimulation alone (Fig. 7B).

284

285 **Discussion**

286 The present study is the first to demonstrate that the product of Fn induces
287 MUC5AC via phosphorylation of ERK1/2. We also found that macrolides
288 inhibit MUCAC production induced by the products of Fn, while CLDM and

289 MTZ were less effective.

290 Fn is a gram-negative anaerobic species of the phylum *Fusobacteria*,
291 numerically dominant in dental plaque biofilms, and important in biofilm
292 ecology and human infectious diseases (8). Fn is one of the most common oral
293 species isolated from extra-oral infections of the blood, brain, chest, lung,
294 liver, joints, abdomen, obstetrical and gynecological infections, and abscesses.
295 In addition to these infections, the products of Fn have been recently
296 reported to reactivate the latently-infected HIV-1 virus (10). Among the
297 components of its supernatant, butyric acid is thought to inhibit the catalytic
298 action of histone deacetylases and induces transcription of silenced genes
299 including the HIV-1 provirus (9). Interestingly, butyric acid contained in
300 *Fusobacterium* species has been reported to be involved in the pathogenesis
301 of ulcerative colitis by inducing cell toxicity (11). Considering these
302 discoveries, Fn is an increasingly significant pathogen with potential to have
303 societal impact on human infections. However, there are few descriptive data
304 of Fn concerning its relationship with respiratory tract diseases.

305 In the present study, we demonstrated that the product of Fn have additive
306 effects on mucin production in airway epithelial cells. Interestingly, high

concentrations of Fn Sup inhibited MUC5AC production, while relatively low concentration of Fn Sup increased MUC5AC production. This suggests that aspiration of saliva containing even low concentration of Fn products may cause hypersecretion in the associated disease. The reason why high concentration of Fn Sup inhibits MUC5AC production is not clear, however, as low dose concentration of Fn products may be found more frequently in oral contents, Fn may play a negative role in the pathogenesis of chronic respiratory tract infections via aspiration of its products.

In this study, we also demonstrated that macrolides reduces MUC5AC production induced by Fn Sup. Long-term treatment with macrolide antibiotics is considered to be effective in DPB and CF due to their anti-inflammatory effects rather than antimicrobial effects (14, 15). In addition, a multicenter, double-blind, randomized clinical trial conducted in Greece showed that intravenous CAM administration for 3 consecutive days improves the length of illness and mortality of VAP (22), which indicate the macrolide might also be beneficial for acute infections with short-term treatment. However, although CLDM and MTZ are both known to reduce cytokine production induced by certain bacterial component or products

(23–25), the existence of anti-inflammatory effects of CLDM and MTZ, similar to those of macrolides, remains uncertain. In the present study, CLDM and MTZ did not exhibit a concentration-dependent reduction of Fn Sup-induced MUC5AC production compared to macrolides. To investigate the reason for this discrepancy with the effect of CLDM and MTZ against Fn Sup-induced MUC5AC production, we examined the MAPK signal transduction pathway. Among a variety of signal transduction molecules, MAPK has been shown to play an important role in mucin production (26). In this study, Fn Sup induced the phosphorylation of ERK1/2. Enhanced MUC5AC protein production was also strongly reduced by an inhibitor of MEK (U0126). This result indicates that Fn Sup mainly up-regulates MU5AC production through MAPK transduction. However, AZM and CAM inhibited phosphorylation of ERK1/2 induced by Fn Sup, while CLDM and MTZ did not. Taken together, macrolides are effective to prevent MU5AC production by different mechanisms from CLDM or MTZ. Thus, stimulation with Fn Sup would be affected by AZM and CAM up-stream of ERK1/2.

The main limitation of our study is that modified GAM broth also had positive effect on MUC5AC production and ERK 1/2 up-regulation. However,

the effect of GAM broth alone on MUC5AC production was significantly small compared to Fn Sup stimulation. ERK 1/2 phosphorylation showed a unique patterned independent of Fn Sup treatment. Although Fn Sup induced phosphorylation of ERK 1/2 at 4 h after stimulation, GAM broth did so at 8 h. Although we could verify Fn potential for mucin production in this study, further study is needed to identify a more detailed mechanism of Fn-induced MUC5AC production that focuses on particular components of Fn products, such as butyric acid.

Our results provide novel evidence that *F. nucleatum* may induce mucus hypersecretion, which suggests that periodontitis may exhibit a relationship with the pathogenesis of chronic respiratory tract infection. Our study also shows that macrolides reduce this mucin production and may act as an additional therapeutic intervention unique from CLDM and MTZ.

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Figure Legends

Fig. 1. Dose-dependent effect of *Fusobacterium nucleatum* culture supernatant (FnSup) on MUC5AC expression. Confluent NCI-H292 cells were stimulated using modified GAM medium (1:9 dilution), or various concentrations of Fn Sup (dilution ratio, from 1:319 to 1:9). (A) MUC5AC protein was measured by performing enzyme-linked immunosorbent assay (ELISA) at 24 h after the addition of Fn Sup (n=3). (B) The mRNA level of MUC5AC expression at 10 h after the addition of Fn Sup was analyzed by RT-PCR (n = 3). Data are expressed as the mean and SEM for 3 experiments.

469 An asterisk and a dagger indicate P values of <0.05 and <0.01, respectively,
470 for comparison with modified GAM stimulation.
471
472 Fig. 2. Time-dependent effect of *Fusobacterium nucleatum* culture
473 supernatant (FnSup) on MUC5AC synthesis. NCI-H292 cells were
474 stimulated with modified GAM medium (1:64 dilution), or Fn Sup (dilution
475 ratio,1:64). (A) MUC5AC protein was measured by an enzyme-linked
476 immunosorbent assay (ELISA) (n = 4). (B) The mRNA level of MUC5AC
477 expression after the addition of Fn Sup was analyzed by RT-PCR (n = 3).
478 Data are expressed as the mean and SEM for the experiments. An asterisk
479 and a dagger indicate P values of <0.05 and <0.01, respectively, for
480 comparison with 0 h.
481
482 Fig. 3. Time-dependent phosphorylation of ERK 1/2, p38, and IκB after
483 modified GAM broth stimulation (control) (A), and *Fusobacterium*
484 *nucleatum* culture supernatant (FnSup) stimulation (B). Cells were treated
485 with control/Fn Sup for each time and evaluated by Western blotting.
486 ERK1/2 phosphorylation was induced 120 min after stimulation with Fn Sup,

and reached it maximum at 240 min after stimulation. The control stimulation induced ERK1/2 phosphorylation induced by the control was maximal at 480 min after stimulation. p38 and IκB phosphorylation was not evident in both stimulation. Data are representative of 3 separate experiments.

Fig.4. Effect of MAP kinase inhibitor on MUC5AC production in cells activated by *Fusobacterium nucleatum* culture supernatant (FnSup). Cells were pretreated with U0126 (ERK), SB203580 (p38 MAP kinase), and CAPE (NF κ B) 30min before Fn Sup stimulation. All the inhibitor effectively suppressed the MUC5AC protein production compared with the Fn Sup stimulation alone. Data are expressed as the mean and SEM for 4 experiments. An asterisk and a dagger indicate P values of <0.05 and <0.01, respectively, for comparison with control (modified GAM stimulation).

Fig.5. Effects of azithromycin (AZM), clarithromycin (CAM), clindamycin (CLDM), and metronidazole (MTZ) on MUC5AC production induced by *Fusobacterium nucleatum* culture supernatant (FnSup). Cells were treated

505 with 1 to 100 μg of each drug. (CAM for 1 to 50 μg/mL; As maximal dose of
506 CAM diluted in DMSO was available for 50 μg/mL) CAM and AZM
507 dose-dependently suppressed Fn Sup-induced MUC5AC production. CLDM
508 significantly suppressed Fn Sup-induced MUC5AC production only with 100
509 μg/mL, while MTZ presented no reduction of MUC5AC at any concentration.
510 Data are expressed as the mean and SEM for 4 experiments. An asterisk and
511 a dagger indicate P values of <0.05 and <0.01, respectively, for comparison
512 with Fn Sup stimulation alone.
513
514 Fig.6. Effects of azithromycin (AZM), clarithromycin (CAM), clindamycin
515 (CLDM), and metronidazole (MTZ) on MUC5AC mRNA expression induced
516 by *Fusobacterium nucleatum* culture supernatant (FnSup). Cells were
517 treated with 100 μg of each drug (CAM for 50 μg/mL). CAM and AZM
518 significantly suppressed Fn Sup-induced MUC5AC mRNA expression. Data
519 are expressed as the mean and SEM for 5 experiments (n=3 for control). An
520 asterisk and a dagger indicate P values of <0.05 and <0.01, respectively, for
521 comparison with Fn Sup stimulation alone.
522

Fig. 7. Effects of macrolides, clindamycin, (A) and metronidazole (B) on ERK phosphorylation. Cells were stimulated with *Fusobacterium nucleatum* culture supernatant (Fn Sup) concurrently with each drug of maximal concentration (50 μg/mL for CAM, 100 μg/mL for AZM, CLDM, and MTZ), and evaluated 360 min after the stimulation. Equal amounts of protein were analyzed. Macrolide inhibited the detection levels of phosphorylation of ERK when compared to stimulation alone. Data are representative of 3 separate experiments.

Fig. 1

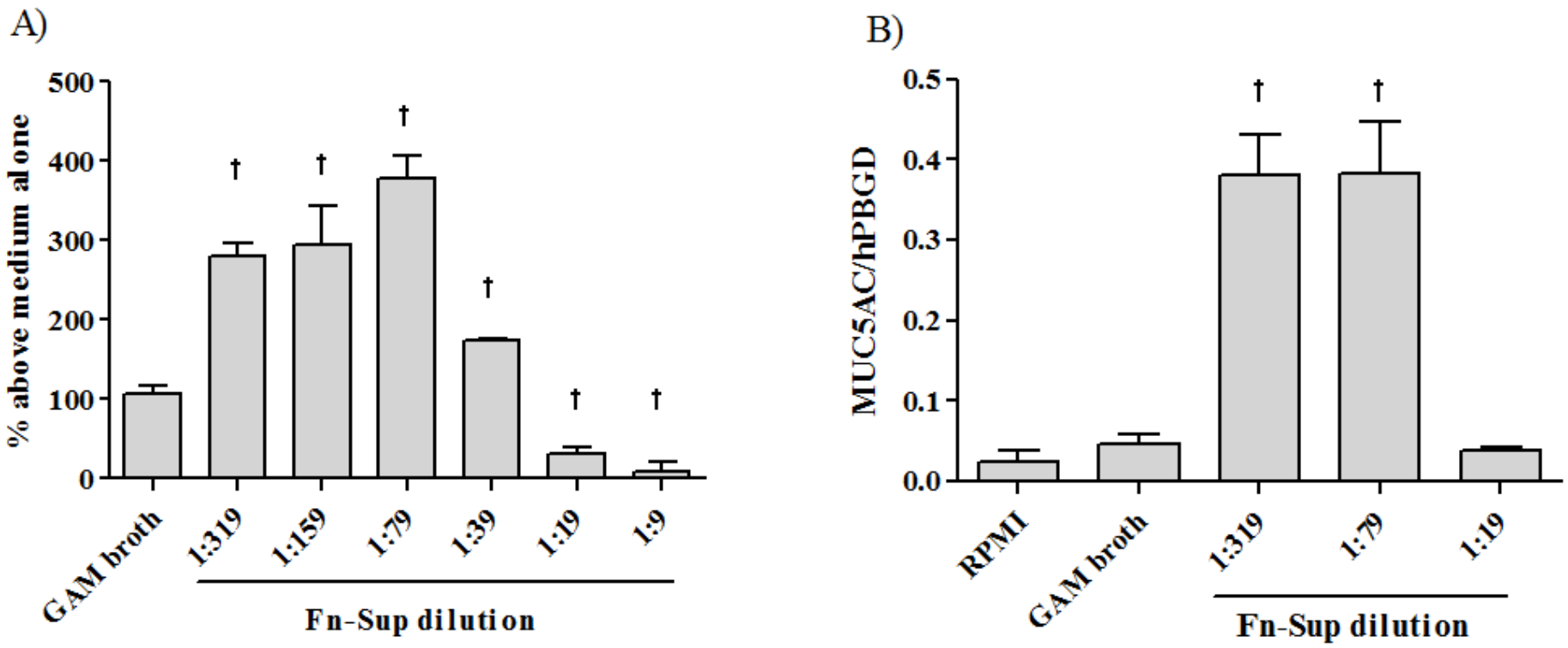

Fig. 1. Dose-dependent effect of *Fusobacterium nucleatum* culture supernatant (FnSup) on MUC5AC synthesis. Confluent NCI-H292 cells were stimulated with modified GAM medium (1:9 dilution), or various concentrations of Fn Sup (dilution ratio, from 1:319 to 1:9). (A) MUC5AC protein was measured by an enzyme-linked immunosorbent assay (ELISA) at 24 h after the addition of Fn Sup. (B) The mRNA level of MUC5AC expression at 10 h after the addition of Fn Sup was analyzed by RT-PCR. Data are expressed as the mean and SEM for three experiments. An asterisk and a dagger indicate P values of <0.05 and <0.01, respectively, for comparison with modified GAM stimulation.

Fig. 2

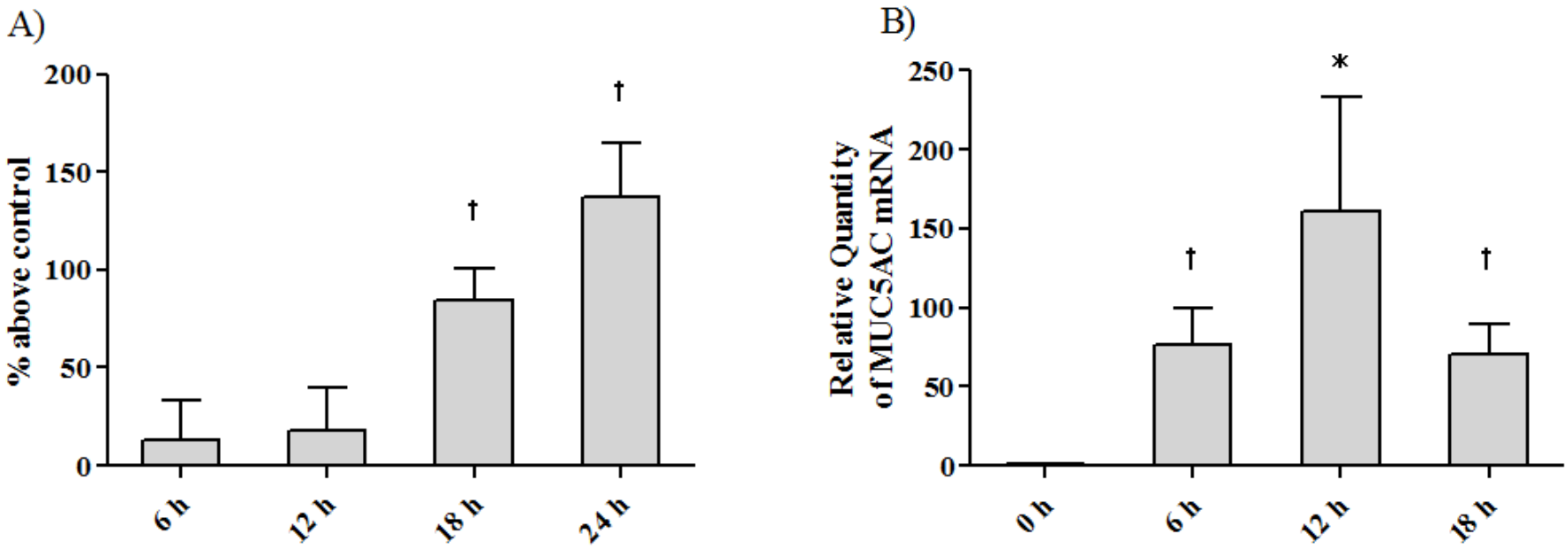

Fig. 2. Time-dependent effect of Fusobacterium nucleatum culture supernatant (FnSup) on MUC5AC synthesis. NCI-H292 cells were stimulated with modified GAM medium (1:64 dilution), or Fn Sup (dilution ratio,1:64). (A) MUC5AC protein was measured by an enzyme-linked immunosorbent assay (ELISA) (n=4). (B) The mRNA level of MUC5AC expression after the addition of Fn Sup was analyzed by RT-PCR (n=3). Data are expressed as the mean and SEM for the experiments. An asterisk and a dagger indicate P values of <0.05 and <0.01, respectively, for comparison with 0 h.

Fig. 3.

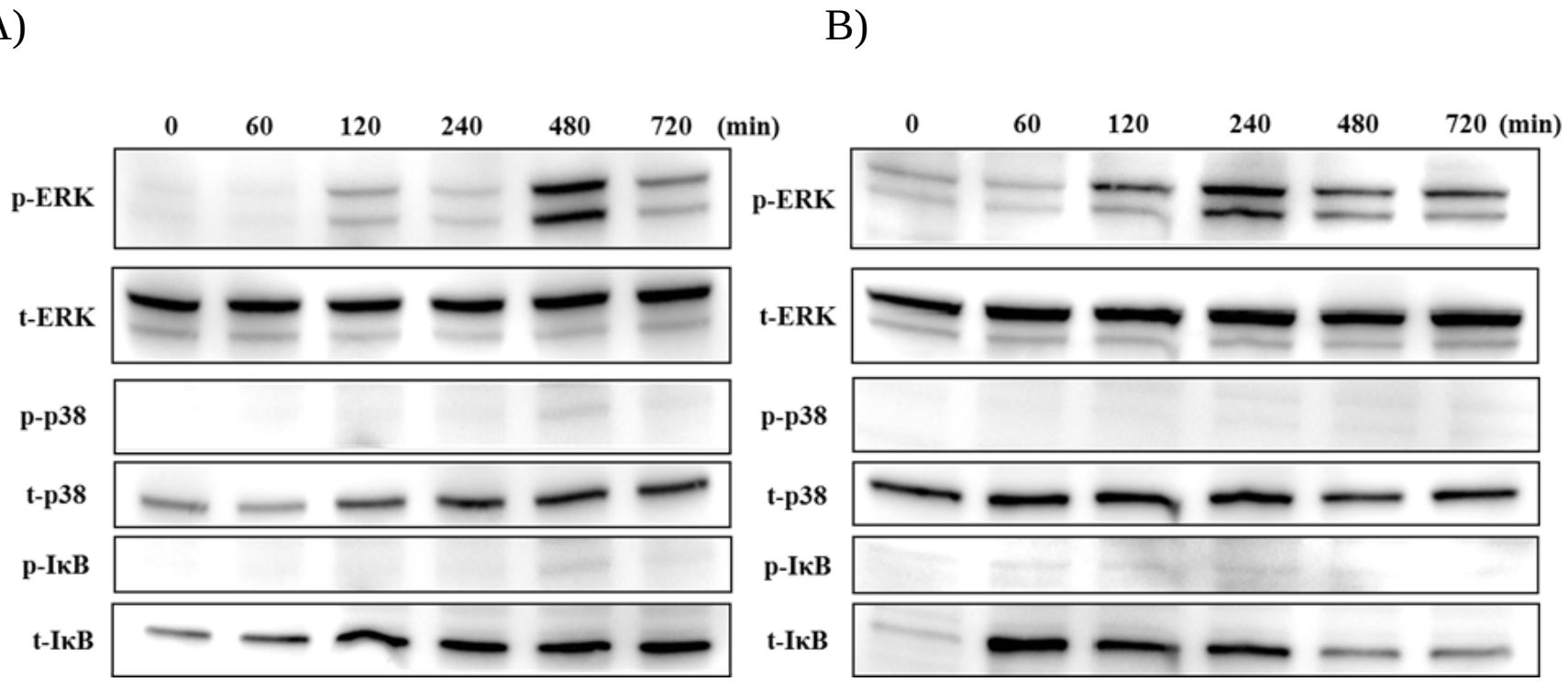

Fig. 3. Time-dependent phosphorylation of ERK 1/2, p38, and IκB after modified GAM broth stimulation (control) (A), and *Fusobacterium nucleatum* culture supernatant (FnSup) stimulation (B). Cells were treated with control/ Fn Sup for each time and evaluated by Western blotting. ERK1/2 phosphorylation was induced 120 min after stimulation with Fn Sup, and maximal at 240 min after stimulation. The control stimulation induced ERK1/2 phosphorylation induced by the control was maximal at 480 min after stimulation. p38 and IκB phosphorylation was not evident in both stimulation. Data are representative of three separate experiments.

Fig. 4.

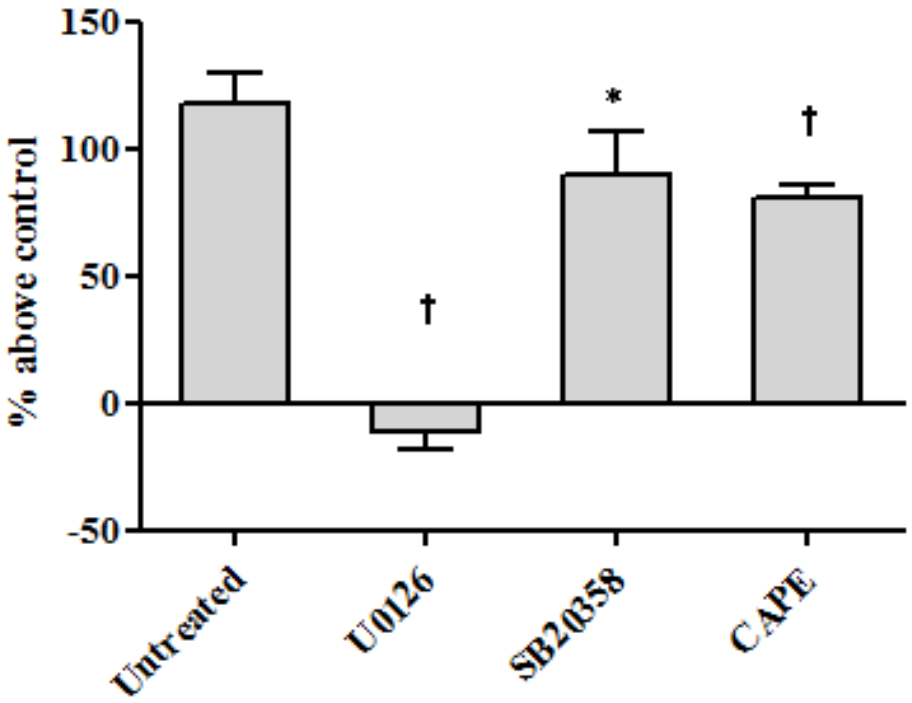

Fig. 4. Effect of MAP kinase inhibitor on MUC5AC production in cells activated by Fusobacterium nucleatum culture supernatant (FnSup). Cells were pretreated with U0126 (ERK), SB203580 (p38 MAP kinase), PD98059 (ERK1/2), and CAPE (NFκB) 30min before Fn Sup stimulation. All the inhibitor effectively suppressed the MUC5AC protein production compared with the Fn Sup stimulation alone. Data are expressed as the mean and SEM for four experiments. An asterisk and a dagger indicate P values of <0.05 and <0.01, respectively, for comparison with control (modified GAM stimulation).

Fig. 5.

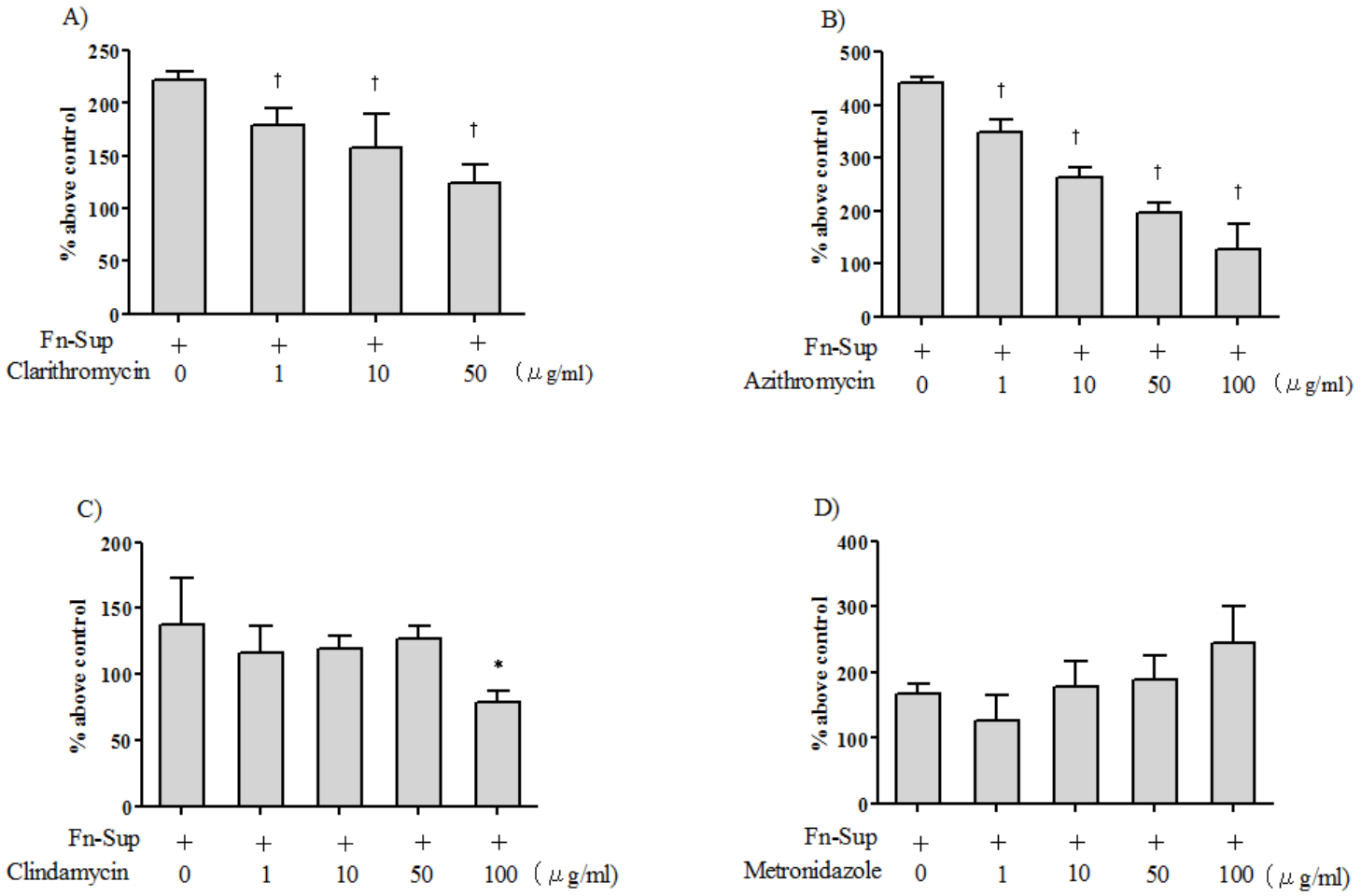

Fig.5. Effects of azithromycin (AZM), clarithromycin (CAM), clindamycin (CLDM), and metronidazole (MTZ) on MUC5AC production induced by *Fusobacterium nucleatum* culture supernatant (FnSup). Cells were treated with 1 to 100μg of each drugs. (CAM for 1 to 50μg/mL ; As maximal dose of CAM diluted in medium was available for 50μg/mL) CAM and AZM dose-dependently suppressed Fn Sup-induced MUC5AC production. CLDM significantly suppressed Fn Sup-induced MUC5AC production only with 100 μg/mL, while MTZ had no reduction at any concentration. Data are expressed as the mean and SEM for four experiments. An asterisk and a dagger indicate P values of <0.05 and <0.01, respectively, for comparison with Fn Sup stimulation alone.

Fig. 6

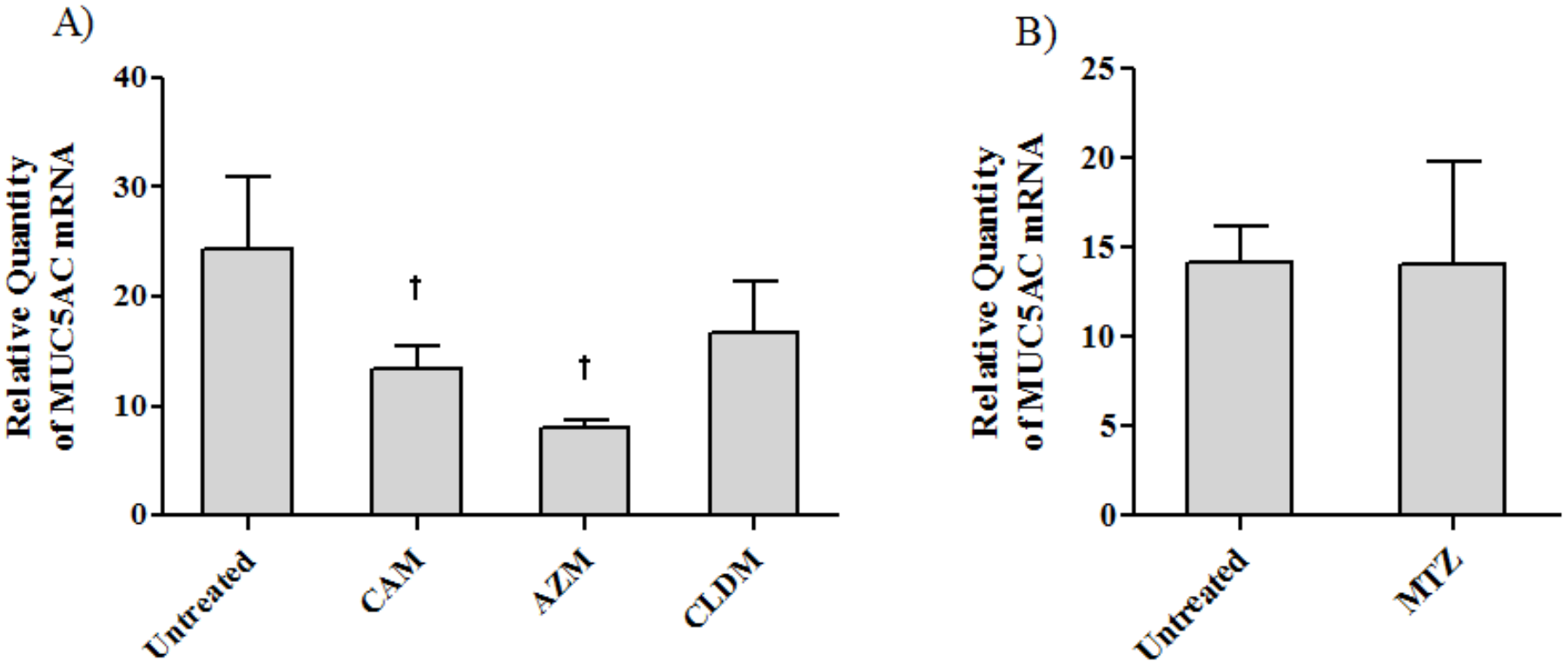

Fig.6. Effects of azithromycin (AZM), clarithromycin (CAM), clindamycin (CLDM), and metronidazole (MTZ) on MUC5AC mRNA expression induced by Fusobacterium nucleatum culture supernatant (FnSup). Cells were treated with 100μg of each drugs. (CAM for 50μg/mL) CAM and AZM significantly suppressed Fn Sup-induced MUC5AC mRNA expression. Data are expressed as the mean and SEM for five experiments (n=3 for control). An asterisk and a dagger indicate P values of <0.05 and <0.01, respectively, for comparison with Fn Sup stimulation alone.

Fig. 7

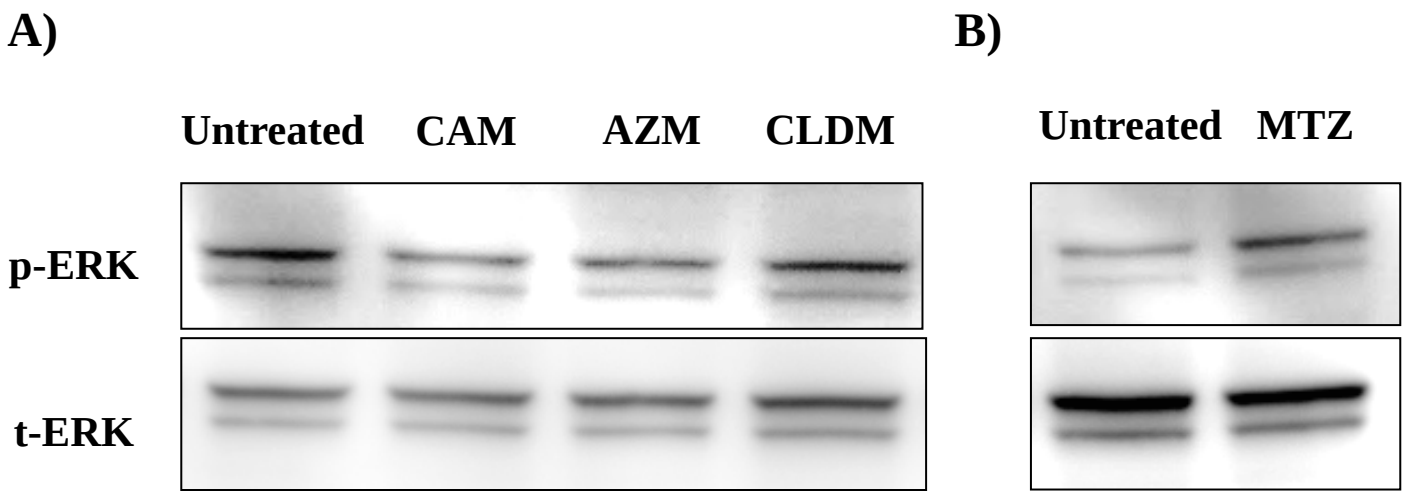

Fig.7. Effects of macrolides, clindamycin, (A) and metronidazole (B) on ERK phosphorylation. Cells were stimulated with Fusobacterium nucleatum culture supernatant (Fn Sup) concurrently with each drug of maximal concentration (50μg/mL for CAM, 100μg/mL for AZM, CLDM, and MTZ), and evaluated 360 min after the stimulation. Equal amounts of protein were analyzed. Macrolide inhibited the detection levels of phosphor-ERK when compared to stimulation alone. Data are representative of three separate experiments.