Macrolide antibiotics modulate ERK phosphorylation and IL-8 and GM-CSF production by human bronchial epithelial cells

Masaharu Shinkai, Gregory H. Foster, and Bruce K. Rubin

Department of Pediatrics, Wake Forest University School of Medicine, Winston-Salem, North Carolina; and Department of Medicine, Division of Infectious Diseases, University of Maryland, Baltimore, Maryland

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Shinkai, Masaharu, Gregory H. Foster, and Bruce K. Rubin. Macrolide antibiotics modulate ERK phosphorylation and IL-8 and GM-CSF production by human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 290: L75–L85, 2006. First published August 5, 2005; doi:10.1152/ajplung.00093.2005—Macrolide antibiotics decrease proinflammatory cytokine production in airway cells from subjects with chronic airway inflammation. However, in subjects with chronic obstructive pulmonary disease, short-term azithromycin (AZM) therapy causes a transient early increase in the blood neutrophil oxidative burst followed by a decrease in inflammatory markers with longer administration. We studied the effects of clarithromycin (CAM) and AZM on proinflammatory cytokine production from normal human bronchial epithelial (NHBE) cells. CAM decreased IL-8 over the first 6 h and then significantly increased interleukin (IL)-8 at 12–72 h after exposure (P < 0.0001). AZM also increased IL-8 at 24 and 48 h, and CAM increased granulocyte-macrophage colony-stimulating factor at 48 h. In the presence of LPS, both CAM and AZM dose-dependently increased IL-8 secretion over 24 h, but after 5 days of exposure to 10 μg/ml CAM there is suppression of IL-8 (P < 0.001). PD-98059, an inhibitor of MAP kinase/ERK kinase, inhibited CAM-induced IL-8 (P < 0.0001) and GM-CSF (P < 0.01) release. The p38 MAP kinase inhibitor SB-203580 increased CAM-induced IL-8 release (P < 0.001), and the c-jun NH2-terminal kinase inhibitor SP-600125 had no effect on IL-8. At 120 min and 6 h, CAM increased phospho-ERK1/2 (pERK) but not phospho-p38 or phospho-JNK. Over the first 90 min, CAM at 10 μg/ml inhibited pERK and then increased pERK in parallel with measured IL-8 secretion. After daily CAM exposure for 5 days, both IL-8 and pERK returned to baseline. The p38 MAP kinase inhibitor, SB-203580 increased ERK phosphorylation and IL-8 secretion. These results suggest that macrolide antibiotics can differentially modulate proinflammatory cytokine secretion in NHBE cells, in part through ERK.

Macrolides also attenuate the inflammatory response to lipopolysaccharide (LPS) (56). The data supporting the use of macrolides as immunomodulatory agents has recently been summarized in a book (51).

Three mitogen-activated protein kinase (MAPK) pathways, the extracellular-regulated protein kinase (ERK), c-jun NH2-terminal kinase (JNK), and p38 MAPK cascades contribute to IL-8 gene expression (22). JNK and p38 MAPK have been shown to either increase (19, 24, 66) or decrease (21, 24, 62, 66) cytokine expression and inflammatory cell apoptosis (24, 48, 66). ERK, which is activated by mitogenic stimuli, can also increase or decrease cell differentiation (24, 66), and it can induce proinflammatory cytokine expression (61). ERK and p38 MAPK increase IL-8 expression in human bronchial cells (33, 34, 37, 42, 47). In THP-1 cells, IL-8 secretion is mediated in part by transcription factors and MAPK (63). ERK can also activate NF-κB in human airway epithelial cells (13). Several studies have examined the relationship between MAPK and macrolide antibiotics. In vitro, erythromycin (EM) inhibits IL-1β-induced p38 MAPK phosphorylation in rheumatoid synovial cells (20). ERK1/2 phosphorylation is increased in the infected lung, but this is decreased after CAM therapy (25).

However, data from normal subjects suggest that exposure to macrolides can increase IL-1 and IL-2 in phagocytes (28, 30) and IL-6 in mononuclear leukocytes (9) and that EM can increase ICAM-1 expression by primary human umbilical vein endothelial cells (32).

Macrolide antibiotics may be able to modulate cytokine expression by human neutrophils, and their ability to increase or decrease cytokines is thought to depend on the presence or absence of bacteria (44, 49). It has been proposed that macrolides initially stimulate nonactivated leukocytes but suppress cytokine production in the presence of inflammatory priming (16, 43).

We evaluated the dose- and time-dependent effects of CAM and AZM on IL-8 and GM-CSF release from NHBE cells in culture. We examined the short-term (minutes to hours) and longer-term (up to 5 days) changes in the pattern of cytokine release and determined the upstream cell signaling intermediates responsible for macrolide stimulation and suppression of IL-8 and GM-CSF.

METHODS

Reagents. CAM was donated by Abbott Labs (Abbott Park, IL), and AZM was purchased from Pfizer (New York, NY). Penicillin G, gentamicin, LPS (10 mg/ml, Escherichia coli serotype 0111: B4),
dimethyl sulfoxide (DMSO), anti-β-actin, anti-mouse IgG-horseradish peroxidase (HRP), and dexamethasone (Dex) were purchased from Sigma (St. Louis, MO). SP-600125, a JNK-II inhibitor, PD-98059, a MAPK/ERK kinase (MEK), an upstream kinase of ERK1/2 inhibitor, and SB-203580, a p38 inhibitor, were purchased from Calbiochem (La Jolla, CA). Phospho- and nonphosphospecific anti-p42/p44 MAPK, anti-p38 MAPK, and anti-SAPK/JNK as well as anti-rabbit-IgG HRP antibodies were purchased from Cell Signaling Technology (Beverly, MA). Kinase inhibitors were dissolved in DMSO before use.

NHBEC cell culture. NHBEC cells (Cambrex Bio Science, Walkersville, MD) were plated at 3,500 cells/cm² in culture dishes in bronchial epithelial cell growth medium (BEGM) supplemented with 52 µg/ml bovine pituitary extract, 0.5 µg/ml hydrocortisone, 0.5 µg/ml human recombinant epidermal growth factor, 0.5 µg/ml epinephrine, 10 µg/ml transferrin, 5 µg/ml insulin, 0.1 ng/ml retinoic acid, and 6.5 ng/ml triiodothyronine without antibiotics and cultured at 37°C in a 5% CO₂ incubator. Endotoxin-free media was used (<0.005 endotoxin units/ml). We used second-passage cells for the experiments. NHBEC cells were routinely cultured on culture dishes coated with type 1 rat tail collagen (Sigma). The medium was changed at day 1 and subsequently every 48 h. The cells were grown to confluence for 6 days.

Cultures without antibiotics were then transferred to six-well or 35-mm dishes. In order for cells to be confluent they were seeded at 2 × 10⁵, 1 × 10⁶, 0.5 × 10⁶, or 0.35 × 10⁶ cells/well depending on the day of harvesting, which was determined by the duration of exposure to CAM. We chose to measure cytokine secretion at the time of cell confluence rather than normalize to the relative number of cells because cell maturation could potentially affect cell signaling and cytokine secretion. At confluence the cells are at a similar growth stage. The medium was changed every 24 h. As growth factors can stimulate proinflammatory cytokines (14), cells were grown in supplement-free BEGM for 24 h before the last drug exposure. Culture supernatants were harvested, centrifuged, and stored at −70°C until assayed. Cells were also cultured for 30 min or 2, 6, or 24 h following stimulation for Western blot analysis. The cell number was counted with a hemocytometer, and cell viability was assessed by trypan blue dye exclusion. Cell morphology was evaluated under a phase-contrast microscope (Olympus, Tokyo, Japan).

Cell stimulation and inhibition. CAM was dissolved in DMSO at a final concentration of 2 µg/ml AZM was dissolved in sterilized water at a final concentration of 1 µg/ml, and Dex was dissolved in 1:49 (DMSO/BEGM) medium at a final concentration of 5 × 10⁻⁵ M. Solutions were stored at −20°C. Penicillin G and gentamicin were dissolved in sterilized water before use. LPS was dissolved in sterile water at a final concentration of 5 µg/ml, and solution was stored at 4°C. We used LPS (10 and 100 µg/ml) as a positive stimulant for IL-8. The cells were exposed to CAM, AZM (1 and 10 µg/ml), or Dex (10⁻⁵ and 10⁻⁶ M) once daily. CAM at 10 µg/ml is the mean therapeutic concentration reported in lung tissue (46, 55). We added macrolides or other drugs to cultures at the appropriate seeding times before harvesting for each experiment. The MEK inhibitor PD-98059 (10 µM), the p38 MAPK inhibitor SB-203580 (10 µM), or the JNK inhibitor II SP-600125 (2 µM) was added 30 min before exposure to CAM.

Preliminary experiments showed that CAM, AZM, Dex, LPS, DMSO, and the inhibitors at the concentrations used had no significant effect on cell counts or cell viability for up to 48 h (data not shown).

Fig. 1. A: effects of clarithromycin (CAM), azithromycin (AZM), and dexamethasone (Dex) on IL-8 release from NHBEC cells in culture at 24 h. Growth factors were withdrawn from the culture medium 24 h before 1 or 10 µg/ml CAM, 1 or 10 µg/ml AZM, or 10⁻⁶ or 10⁻⁵ M Dex exposure. CAM and AZM significantly increased IL-8, and Dex significantly inhibited IL-8 release. The data are shown as means ± SD. *P < 0.05, **P < 0.01 compared with control (Cont). B: dose-dependent stimulatory effect of CAM at 1 and 10 µg/ml and AZM at 1 and 10 µg/ml on IL-8 release from NHBEC cells at 48 h. NHBEC cells were cultured for 48 h with CAM and AZM, and the medium was changed daily. Growth factors were withdrawn from the culture medium 24 h before the second CAM or AZM exposure. IL-8 concentration was determined 24 h after the second macrolide exposure. Data are shown as means ± SD. ***P < 0.001 compared with control. #P < 0.001 compared with 10 µg/ml AZM. C: effect of CAM at 0.1, 1, and 10 µg/ml on GM-CSF release from NHBEC cells at 48 h. Cells were cultured for 48 h with CAM. The medium with CAM was changed daily. The concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF) was determined 24 h after the second CAM exposure. The data are shown as means ± SD. ***P < 0.001 compared with control. D: penicillin G at 5 and 50 U/ml and gentamicin at 5 and 50 µg/ml had no effect on IL-8 secretion over 24 h (P = 0.44 for penicillin G at 5 U/ml, P = 0.49 for penicillin G at 50 U/ml, P = 0.29 for gentamicin 5 at µg/ml, and P = 0.09 for gentamicin 50 µg/ml). Baseline IL-8 concentration after CAM was set at 100%. The data are shown as means ± SD.
Measurement of IL-8 and GM-CSF secretion. Cytokine immuno-reactivity was measured in culture supernatants by ELISA for IL-8 (Immunotech) and GM-CSF (R&D systems, Minneapolis, MN) with detection limit of 8 and 3 pg/ml, respectively. Optical density was measured at 450 nm on a microtiter plate reader (Spectra Max Plus; Molecular Devices, Sunnyvale, CA). Using software (Soft Max Pro version 2.0; Molecular Devices), we obtained concentrations by interpolation from standard curves, and final concentrations in each sample were calculated as the mean of the results at the sample dilution, yielding optical densities in the linear portion of the calibration curves.

Measurement of phospho-MAPK. After stimulation, culture supernatants were collected and centrifuged for 5 min at 200 g and stored at −20°C. The cells were washed twice with 2 ml of cold phosphate-buffered saline (PBS). After the supernatants were completely aspirated, the cells were lysed on ice in a modified radio immunoprecipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 10 mM Tris pH 7.5, 5 mM sodium pyrophosphate, 1 mM NaVO₄, 5 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM PMSF) for 15 min and then scraped from the dishes and collected into a tube. DNA was sheared by passing the lysate through a 27-gauge needle, and insoluble material was removed by centrifugation at 20,000 g for 15 min at 4°C. The protein concentration of the resulting supernatant was quantified by the DC protein assay (Bio-Rad, Hercules, CA), and the lysate was stored at −70°C until used. Equal amounts of protein extracts were loaded on a 12% SDS-PAGE mini gel and transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting overnight. Membranes were rinsed with distilled water, incubated for 1 h at room temperature in Tris-buffered saline (0.8% NaCl and 20 mM Tris pH 7.6) with 0.1% Tween 20 (TBS-T) with 5% nonfat dry milk to block nonspecific interactions, rinsed two times, and washed three times for 10 min with TBS-T. After washing, membranes were exposed overnight to the primary antibodies 1 μg/ml phospho (p)-44/42 MAPK (Thr202/Tyr204), pp38 MAPK (Thr180/Tyr182), or pSAPK/JNK (Thr183/Tyr185) rabbit polyclonal IgG (Cell Signaling Technology) at 4°C in TBS-T, with 5% milk. The blots were then washed and incubated at room temperature for 2 h with the anti-rabbit IgG HRP secondary antibody. Subsequently, the mem-

Fig. 2. A: LPS at 10 and 100 μg/ml stimulated IL-8 release from NHBE cells in a dose-dependent manner. Growth factors were withdrawn from the culture medium 24 h before LPS stimulation, and supernatants were harvested 24 h after LPS stimulation. Data are shown as means ± SD. ***p < 0.001, ****p < 0.0001 compared with no LPS control. B: CAM and AZM stimulated IL-8 release from NHBE cells, and this effect was additive with 100 μg/ml LPS at 24 h. Growth factors were withdrawn from the culture medium 24 h before the LPS exposure. IL-8 concentration was determined 24 h after LPS exposure. In the presence of LPS, CAM, and AZM increased IL-8 secretion over 24 h dose dependently. The data are shown as means ± SD. **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with controls. ###p < 0.001 compared with AZM 10 μg/ml. C: temporal effects of up to 5 days of daily 10 μg/ml CAM, 10 μg/ml AZM, or 10−5 M Dex exposure in the presence of LPS. In order for cells to be confluent, they were initially seeded at densities calculated to reach confluence on the day of harvesting. The medium with drugs was changed daily. LPS at 50 μg/ml was added 2 days before test drug exposures and then to fresh medium daily. Growth factors were withdrawn from the culture medium 24 h before the last drug exposure, and samples were harvested 24 h after the last exposure. There was an additive stimulatory effect on LPS-induced IL-8 release by both CAM and AZM at 24 h, but after 5 days of exposure to CAM, there was suppression of IL-8 compared with control cells stimulated by LPS alone. Data are shown as means ± SE. *p < 0.05, #p < 0.05, ##p < 0.01, ***p < 0.001, ###p < 0.001, compared with control IL-8 after LPS alone.
branes were washed again and antibody binding was detected with LumiGLO chemiluminescent substrate peroxide (Cell Signaling Technology, Beverly, MA).

Membranes were stripped with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris·HCl pH 6.7) for 30 min at 60°C. The blots were washed twice with TBS-T and reprobed with anti-p44/42 MAPK, anti-p38 MAPK, or anti-SAPK/JNK antibodies followed by anti-rabbit IgG HRP secondary antibody. Blots were stripped again, and equivalent protein loading was confirmed by Western blot with anti-human β-actin antibody followed by anti-mouse IgG HRP secondary antibody and detected by ECL (Amersham, Piscataway, NJ). Western blot images were scanned and analyzed on NIH Image J software.

Statistical methods. Results are expressed as mean values ± SD or SE as appropriate. Statistical analysis of data was performed with the StatView 5 statistics package (SAS Institute, Cary, NC). Where appropriate, data were analyzed by an unpaired t-test, paired t-test, or ANOVA for categorical data. Parametric testing was conducted after confirming that raw data were normally distributed. Conventionally, P < 0.05 was considered significant.

RESULTS

Effects of CAM, AZM, and Dex on IL-8 release. To evaluate the effect of CAM, AZM, and DEX alone on IL-8 release from NHBE cells, cell supernatants were harvested 24 h after exposure to 1 or 10 μg/ml CAM, 1 or 10 μg/ml AZM, or 10−5 M or 10−6 M Dex. CAM and AZM at 10 μg/ml significantly increased IL-8 release (P < 0.01 for CAM and P < 0.05 for AZM), whereas Dex at both 10−5 M and 10−6 M significantly inhibited IL-8 release compared with basal IL-8 secretion (P < 0.01) (Fig. 1A). CAM and AZM at 1 μg/ml did not increase IL-8 secretion. We then measured IL-8 after CAM and AZM at 1 and 10 μg/ml over 48 h. The medium with CAM and AZM was changed every 24 h. IL-8 secretion was measured 24 h after the second CAM or AZM exposure. CAM and AZM at 10 μg/ml significantly increased IL-8 (P < 0.001 for CAM and P < 0.01 for AZM) (Fig. 1B).

Effect of CAM on GM-CSF release. Many of the stimuli that induce IL-8 secretion can also stimulate the secretion of other proinflammatory cytokines. Therefore, we measured the effect of CAM at 0 (control), 0.1, 1, and 10 μg/ml on GM-CSF release at 48 h. CAM at 10 μg/ml but not at 0.1 or 1 μg/ml increased GM-CSF secretion over control (P < 0.001) (Fig. 1C).

Effects of other antibiotics on IL-8 release. As a control, we measured the effect of penicillin G at 5 and 50 U/ml and gentamicin at 5 and 50 μg/ml over 24 h and demonstrated no effect on IL-8 secretion (P = 0.44 for penicillin G at 5 U/ml,
P = 0.49 for penicillin G at 50 U/ml, P = 0.28 for gentamicin at 5 μg/ml, and P = 0.09 for gentamicin at 50 μg/ml) (Fig. 1D). This is consistent with reports by other investigators (58, 59).

Effects of CAM and AZM on IL-8 release in the presence of LPS. As immunomodulatory drugs, CAM and AZM have generally been administered to patients who have well-established airway inflammation and infection as part of their disease. To evaluate whether CAM or AZM modulated IL-8 release in the presence of proinflammatory bacterial components, we measured IL-8 secretion from NHBE cells in the presence of bacterial endotoxin (LPS from E. coli) (53). As anticipated, there was a dose-dependent increase in IL-8 secretion after 24 h of LPS stimulation in the absence of macrolide (Fig. 2A). The addition of CAM or AZM (1 and 10 μg/ml) to LPS 100 μg/ml further increased IL-8 secretion dose dependently compared with LPS alone (Fig. 2B: P < 0.0001 for CAM 10 μg/ml, P < 0.01 for CAM 1 μg/ml, P < 0.001 for AZM 10 μg/ml, and P < 0.05 for AZM 1 μg/ml).

Macrolide administration over days to weeks attenuates the inflammatory response to LPS in vitro (56) and in subjects with chronic obstructive pulmonary disease (COPD) (45). There-

![Fig. 4. A: effect of CAM on threonine and tyrosine phosphorylation of ERK1/2, p38, and JNK. Growth factors were withdrawn from the culture medium 24 h before 0, 1, or 10 μg/ml CAM exposure. NHBE cells were exposed to CAM for 120 min. The phosphorylation of ERK, p38, and JNK was measured by Western blotting. CAM dose-dependently increased the ratio of phospho (p)-ERK1/2 / tERK1/2 at 120 min. The band intensity of pERK1/2 and tERK1/2 was calculated with NIH Image J software. B: temporal effect of 10 μg/ml CAM on pMAPK expression. Growth factors were withdrawn from the culture medium 24 h before CAM exposure. NHBE cells were treated with CAM for 30 min–24 h. CAM increased the ratio of pERK1/2 / tERK1/2 at 120 min and 6 h. The band intensity of pERK1/2 and tERK1/2 was calculated with NIH Image J. t, total.](http://ajplung.physiology.org/)
fore, we examined the temporal effects of up to 5 days of exposure to CAM, AZM, and Dex (as a control positive suppressor) in the presence of daily LPS exposure. Cells were initially seeded at densities calculated to reach confluence on the day of harvesting. CAM at 10 μg/ml, AZM at 10 μg/ml, or Dex at 10⁻³ M was added to fresh medium daily. LPS at 50 μg/ml was added 2 days before drug exposures and then to fresh medium daily. With LPS exposure CAM increased IL-8 by 751% over control at day 1 (P < 0.05), but at day 3 there were no differences between CAM-treated and control cells (P = 0.32), and at day 5 there was suppression of IL-8 by 76% below control in the presence of CAM (P < 0.001) (Fig. 2C). In the presence of LPS, AZM increased IL-8 secretion by 261% over control at 1 day (P < 0.05), but at days 3 and 5 there was no difference between AZM-treated and control cells (P = 0.74 for day 3 and P = 0.79 for day 5). Dex decreased IL-8 secretion by 60% below control at day 1, by 71% at day 3, and by 49% at day 5 (P < 0.001 for day 1, P < 0.01 for day 3, and P < 0.05 for day 5).

Effect of inhibiting ERK1/2, p38 MAPK, or JNK. Several MAPK kinase pathways are involved in the regulation of IL-8 secretion. Therefore, we examined the role of intracellular signaling pathways regulating IL-8 in NHBE cells treated with CAM by using the MEK inhibitor PD-98059 at 10 μM, p38 MAPK inhibitor SB-203580 at 10 μM, or the JNK inhibitor SP-600125 at 2 μM. We treated cells with a kinase inhibitor 30 min before adding CAM 10 μg/ml, and the cell supernatants were harvested after 24 h. Pretreatment with the MEK inhibitor decreased CAM-induced IL-8 release by 51% compared with control CAM exposure (P < 0.001) and basal IL-8 in the absence of CAM by 30% compared with untreated control cells (P < 0.0001). The amount of IL-8 secretion with the MEK inhibitor was no different with or without CAM (P = 0.19). Pretreating NHBE cells with the p38 MAPK inhibitor increased IL-8 secretion by 251% over CAM-treated controls (P < 0.001) and basal IL-8 by 29% over untreated cells. There was no change in IL-8 with the JNK1/2 inhibitor SP-600125 (Fig. 3A).

With the MEK inhibitor pretreatment for 30 min before a 48-h exposure to CAM, the CAM-induced IL-8 secretion decreased by 66% compared with cells not exposed to PD-98059 (P < 0.0001). Basal IL-8 release in the absence of CAM was also decreased by 19% with his inhibitor (P < 0.01) (Fig. 3B). In the presence of the MEK inhibitor, a 48-h exposure to CAM also decreased GM-CSF secretion by 42% (P < 0.01) (Fig. 3C), and basal GM-CSF secretion was decreased by 16%.

Effect of CAM on threonine and tyrosine phosphorylation of ERK1/2, p38 MAPK, and JNK. To determine whether MAPK phosphorylation was altered by CAM, cells were treated with CAM (0, 1, or 10 μg/ml) for 120 min, and the phosphorylation of ERK1/2 to pERK, p38 MAPK to pp38, and JNK to pJNK was determined by Western blotting (Fig. 4A). CAM dose-dependently increased pERK 120 min after treatment compared with controls (P < 0.001). There was no increase in phosphorylation of p38 or JNK. We also measured the effect of 10 μg/ml CAM on pERK, pp38, and pJNK at 30 min, 120 min, 6 h, or 24 h. CAM increased pERK at 120 min (P < 0.001) and 6 h (P < 0.01) but not pp38 or pJNK (Fig. 4B). Western blot experiments were repeated twice with similar results.

Temporal effects of CAM on IL-8 release and pERK. The effect of 10 μg/ml CAM on IL-8 secretion and pERK was measured over the initial 24 h of exposure (Fig. 5). There was a 22% decrease in IL-8 at 90 min (P < 0.05), 32% decrease at 4 h (P < 0.001), and a 25% decrease at 6 h (P < 0.05) compared with controls. At 9 h there was no difference in IL-8 secretion between CAM-treated and control cells, but after this, there was a 57% increase in IL-8 at 12 h (P < 0.001), 175% increase at 18 h (P < 0.0001), and a 177% increase at 24 h (P < 0.0001). These experiments were repeated four times. In association with this, 10 μg/ml CAM decreased pERK at 60 min (P < 0.0001) but not pp38 or pJNK compared with controls (Fig. 6A). CAM also decreased pERK at 90 min (P < 0.001) but then increased pERK at 120 min (P < 0.001) and at 6 h (P < 0.01) (Fig. 6, B and C).

We also examined the temporal effects of longer (up to 5 days) CAM exposure. Cells were initially seeded at densities calculated to reach confluence on the day of harvesting. CAM at 10 μg/ml was added to fresh medium daily. IL-8 increased by 104% over control at 3 days (P < 0.001), but at day 5 there were no differences between CAM-treated and control cells (P = 0.26) (Fig. 5). CAM increased pERK on day 3 (P < 0.001), but at day 5 there were no differences between CAM treated and control cells (Fig. 6D).

Figure 6E combines CAM regulated pERK data for all time periods. There was a 52% decrease in pERK at 30 min (P = 0.08), an 80% decrease at 60 min (P < 0.0001), and a 63% decrease at 90 min (P < 0.001) compared with control. CAM significantly increased pERK by 99% at 2 h (P < 0.001), 66% at 6 h (P < 0.01), and 45% at 24 h, and 53% at 3 days (P < 0.001).

Effect of the p38 MAPK inhibitor SB-203580 on phosphorylation of ERK1/2 by NHBE cells. Pretreating cells with the p38 MAPK inhibitor increased IL-8 secretion. We therefore tested whether this would increase pERK after a 6-h exposure to CAM. Both CAM and the p38 MAPK inhibitor increased pERK over control, and these appeared to be additive as shown in Fig. 7, but the differences were not significant. Western blot experiments were repeated twice with similar results.
DISCUSSION

Although the long-term use of low-dose macrolide antibiotics decreases IL-8 in the airway of subjects with DPB (52) or sinusitis and nasal polyps (65), data from healthy subjects suggest that under some conditions, these same drugs may increase the expression of proinflammatory cytokines (5, 6, 28, 30). In subjects with COPD, short-term AZM therapy causes a transient early increase in serum nitrates, nitrites, and the blood neutrophil oxidative burst followed by a decrease in inflammatory markers with longer administration (44, 45). Macrolide

![Fig. 6. The effect of CAM at 10 μg/ml given daily on constitutive pERK. In order for cells to be confluent, they were initially seeded at densities calculated to reach confluency on the day of harvesting. The medium with CAM was changed daily. Growth factors were withdrawn from the culture medium 24 h before the last CAM exposure, and samples were harvested 24 h after the last CAM exposure at 3 and 5 days. A: pERK was decreased compared with controls at 30 and 60 min. B: pERK was decreased up to 90 min and then increased at 120 min compared with control. The band intensity was calculated with NIH Image J. Data are shown as means ± SE. pERK was increased compared with control for up to 6 h (C) and at 1 and 3 days (D) of CAM exposure, but levels returned to baseline by 5 days. The band intensity was calculated with NIH Image J software. Data are shown as means ± SE. E: pERK1/2 data for all time periods are combined (compare with Fig. 4). CAM decreased pERK at 30, 60, and 90 min, but pERK was increased compared with control at 2 h–3 days of CAM exposure, pERK returned to baseline by 5 days. Data are shown as means ± SE. **P < 0.01 and ***P < 0.001 compared with control.}
antibiotics may stimulate or decrease cytokine release from human neutrophils depending on their activation state (49). It has been hypothesized that acute neutrophil activation may facilitate the killing of microorganisms while the suppression of chronic inflammation may limit airway damage (45).

We report here that CAM increases IL-8 at 24–72 h and GM-CSF release at 48 h in NHBE cells but that after this, IL-8 secretion returns to control levels by 5 days of exposure. Another macrolide, AZM, had a similar effect on IL-8 secretion, but penicillin G and gentamicin do not affect IL-8 secretion. We also show that CAM initially decreases IL-8 during the first 9 h of exposure (Fig. 5).

Macrolide administration over days to weeks attenuates IL-8 secretion to LPS in vitro (56) and decreases inflammation in subjects with COPD (45). Therefore, we examined the temporal effects of up to 5 days of exposure to CAM or AZM in the presence of daily LPS exposure. As expected there was a dose-dependent increase in IL-8 secretion with LPS exposure in the absence of macrolides. When CAM or AZM was added to cultures, there was an additive effect on IL-8 secretion over 24 h compared with either LPS alone or with macrolide alone. However, after 5 days of exposure to CAM there was suppression of IL-8 with macrolides compared with LPS-alone control. This suggests that the biphasic, nonlinear (chaotic) response to macrolides is magnified in the presence of inflammatory stimuli like LPS.

CAM increased the levels of pERK over 3 days and PD-98059, a specific inhibitor of MEK-1 and upstream regulator of ERK (4), inhibited CAM-induced IL-8 and GM-CSF secretion. PD-98059 also significantly inhibited unstimulated IL-8 secretion at 24 h. The p38 MAPK inhibitor SB-203580 (15) increased IL-8 by 251% when cells were exposed to CAM and basal IL-8 by 29%, whereas a selective inhibitor of JNK (10) did not affect IL-8 release, and pJNK was not detected in NHBE cells treated with CAM. CAM at 10 μg/ml increased pERK at 120 min and 6 h, with a maximum at 120 min. These data suggest that CAM can acutely induce proinflammatory cytokine release from NHBE cells via the ERK signaling pathway and that the p38 MAPK pathway may downregulate IL-8 release by NHBE cells.

IL-8 secretion is also increased by activation of NF-κB (22). Some studies have shown that ERK regulates IL-8 independently of NF-κB (34), whereas others have reported that the ERK pathway can directly affect the phosphorylation of IκB-α through IKKα/β and thus activate NF-κB in human airway epithelial cells (13). ERK can also regulate the activation IκB in rat vascular smooth muscle cells isolated from the thoracic aorta (23). EM inhibits transcriptional activation of NF-κB in T cells through a calcineurin-independent signaling mechanism (7). In human monocytes and monocytic leukemia cells, CAM can suppress LPS-induced secretion of IL-8 via NF-κB (27). CAM also suppresses NF-κB DNA binding activity in LPS and IL-1β-treated human nasal epithelial and fibroblast cells (38). Abe and colleagues (1) have shown that CAM suppresses IL-8 gene transcription mainly through decreasing activator

Fig. 7. Effect of SB-203580 on phosphorylation of ERK1/2 by NHBE cells. Pretreating with the p38 MAPK inhibitor increased pERK with/without the 6-h exposure to CAM by NHBE cells.

Fig. 8. Hypothesis: diagram of possible mechanisms for macrolide-induced cytokine secretion modulation. Macrolides may initially decrease proinflammatory cytokines by inhibiting ERK signaling and then increase proinflammatory cytokines through signal transduction. This biphasic response could be due to signaling pathway cross talk, time-dependent changes in receptor expression or receptor binding (e.g., tolerance), posttranslational degradation of mRNA, or temporal changes in mRNA expression.

Normal bronchial epithelial cell

MAPK

ERK

MEK

Protein

(basal cytokine production)

IL-8

GM-CSF

With a single exposure to CAM

With daily CAM exposure ≥ 5 days

Possible explanations:
1. Crosstalk through cell signaling with p38 MAPK contra-regulating ERK
2. Tolerance with decreased receptor binding or suppression of receptor expression
3. Suppression of proinflammatory gene transcription or mRNA degradation

To baseline

ERK

Nonlinear feedback or cross-talk

To baseline
protein-1 binding in TNF-α-treated BET-1A cells, but this inhibition requires a CAM exposure of 96 h, with the degree of inhibition by CAM less than that of corticosteroids, suggesting that CAM does not directly interact with the IL-8 promoter. It is also reported that a 72-h exposure to EM is required for inhibition of NF-κB activation induced by phorbol myristate acetate in BET-1A cells (18). Thus macrolide antibiotics do not appear to require direct interaction with transcription factors, and some immunomodulatory effects may be mediated directly through kinase signaling.

EM, CAM, and roxithromycin (10−5 and 10−6 M) have all been shown to inhibit LPS-stimulated IL-8 secretion from human nasal epithelial cells isolated from nasal polyps (57); CAM (5 μg/ml) and EM (1 μg/ml) also have been shown to inhibit IL-8 release from human eosinophils isolated from atopic subjects (29). EM inhibits PMA stimulated IL-8 from the human bronchial epithelial cell line BET-1A (17), and CAM (10 μg/ml) inhibits TNF-α-stimulated IL-8 release from BET-1A cells (1).

Although it is established that macrolide antibiotics decrease IL-8 secretion and inhibit nuclear transcription factors in vitro, most published studies have primarily made measurements at discrete time points rather than temporally. However, in healthy volunteer subjects, EM at 500 mg increases neutrophil migration within 2 h followed by a return to normal levels at 96 h (6). Macrolide antibiotics may modulate cytokine expression by human neutrophils, and their ability to increase or decrease cytokines appears to be dependent on the presence or absence of bacteria (49). It has been proposed that macrolides will initially stimulate nonactivated leukocytes but suppress cytokine production in the presence of inflammatory priming (16, 43). We have shown that CAM decreases IL-8 secretion in vitro over the first 6 h of exposure in association with a decrease in pERK but increases proinflammatory cytokine release and pERK after 6 h of exposure. The time lag between the changes in MAPK (Fig. 6E) and IL-8 protein levels (Fig. 5) is consistent with studies showing that silica will increase MAPK to a maximum at 30 min, whereas IL-8 release occurs 4–8 h later (41).

We show that after 5 days, IL-8 secretion returns to control levels. ERK1/2 phosphorylation due to *Pseudomonas aeruginosa* infection is inhibited in murine lung cell homogenates after CAM is given at 10 mg·kg−1·day−1 for 7 days (25). The inhibition or activation of ERK signaling is regulated by cyclic adenosine monophosphate (cAMP) (54), and macrolides can induce AMP and cAMP-responsive binding proteins (2, 8). The increase in intracellular cAMP in primed leukocytes was associated with macrolide inhibition of IL-8 and IL-6 generation. However, the anti-inflammatory cytokine IL-10 is increased in a transcription-dependent manner (44).

Our data suggest that CAM initially decreases proinflammatory cytokine secretion through ERK inhibition, but ERK signaling is then increased perhaps via cross talk among cell-signaling transduction pathways (3) or through negative feedback (64). It has recently been reported that p42/44 and p38 MAPK cross talk may play a crucial role in cell differentiation (60) and in the immune response (36). Consistent with this, we show that a p38 inhibitor increases pERK and basal IL-8 secretion. A hypothesis explaining the nonlinear (or polyphasic) cytokine secretory response to macrolides and cell signaling in NHBE cells is diagramed in Fig. 8.

It is reported that constitutively active ERK signaling may decrease NF-κB-driven transcription, in part, through inhibition of p38 MAPK by modulating TATA-binding protein phosphorylation (12). Thus constitutive ERK activation might decrease proinflammatory cytokine production with time or by cross talk among signal transduction pathways with p38 MAPK contraregulating ERK. It is also possible that decreased receptor binding or suppression of receptor expression might induce tolerance (68). Finally, as macrolides accumulate in epithelial cells (31) they may suppress gene transcription (18) or increase posttranscriptional cytokine mRNA degradation (34).

The immune and inflammatory response represents a complex nonlinear interaction with a driving tendency toward homeostatic balance between defensive immune activation and self-protective immune and inflammatory damping. The polyphasic response to the macrolides that we describe is consistent with their immunoregulatory role in boosting the initial inflammatory response followed by a homeostatic damping of this response. This is in contradistinction to the monophasic immunosuppressive effects of dexamethasone as shown in our experimental system.

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L84 MACROLIDES, CYTOKINES, AND ERK


