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Clarithromycin inhibits type A seasonal influenza virus infection in human airway epithelial cells

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A list of nonstandard abbreviations:

Abbreviations:

APS, 3-aminopropyltrethoxy-silane; CO_2, carbon dioxide; COPD, chronic obstructive pulmonary disease; CPE, cytopathic effects; DAPI, 4',6-diamino-2-phenylindole, dihydrochloride; DF-12, DMEM-Ham's F-12; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FluA, type A seasonal human influenza; FITC, fluorescein isothiocyanate; HI, hemadsorption inhibition; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; moi, multiplicity of infection; LDH, lactate dehydrogenase ; MDCK, Madin Darby Canine Kidney; MEM, minimum essential medium; NF-κB, nuclear factor kappa-B; PBS, phosphate-buffered saline; RhoA, isoform A of the Ras-homologus; RNPs, ribonucleoproteins; rRNA, ribosomal RNA; SAα2,6Gal, sialic acid with an α2,6linkage; SNA, Sambus nigra; TCID; tissue culture infective dose; TNF, tumor necrosis factor; USG, ultroser G; V-ATPase, vascular-ATPase.

A recommended section: Inflammation, Immunopharmacology, and Asthma

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Abstract

Human influenza viruses attach to sialic acid with an α2,6linkage (SAα2,6Gal) on the airway epithelial cells, and the entry of the viruses into the cells and uncoating of the viruses require low pH of endosomes. Bafilomycin A_1, a macrolide antibiotic and the specific vascular-ATPase inhibits growth of type A and type B human influenza viruses in Madin Darby Canine Kidney cells. However, the inhibitory effects of clinically used macrolide antibiotics on influenza virus infection in human airways have not been studied. To examine the effects of clarithromycin on seasonal human influenza virus infection, cultured human tracheal epithelial cells were infected with type A influenza virus (H_3N_2). Influenza virus infection increased viral titers and the content of cytokines, including interleukin (IL)-1β and IL-6, in supernatant fluids, and viral RNA in the cells. Clarithromycin reduced viral titers and the content of cytokines in supernatant fluids, viral RNA in the cells, and the susceptibility to virus infection. Clarithromycin reduced the expression of SAα2,6Gal, a receptor for human influenza virus, on the mucosal surface of human tracheae, and the number and fluorescence intensity of acidic endosomes in the cells from which viral ribonucleoproteins enter into the cytoplasm. Furthermore, clarithromycin reduced nuclear factor-κB (NF-κB) proteins including p50 and p65 in the nuclear extracts. These results suggest that clarithromycin may inhibit seasonal human influenza virus infection by reducing SAα2,6Gal partly through the inhibition of NF-κB, and by increasing pH in endosomes in airway epithelial cells. Clarithromycin may modulate airway inflammation in influenza virus infection.

Introduction

Human influenza virus infection causes rapid onset constitutional symptoms including fever and lower respiratory tract symptoms, and also induces exacerbations of bronchial asthma and chronic obstructive pulmonary disease (COPD) in winter season (Hayden and Gwaltney, 1988; Johnston et al., 1995). To prevent exacerbations of bronchial asthma and COPD by influenza virus infection, the use of vaccination against influenza is recommended. Influenza vaccination reduces the mortality rate in elder people with COPD (Nichol et al., 1994). Clinically used anti-influenza drugs, which include neuraminidase inhibitors such as oseltamivir and zanamivir are beneficial for uncomplicated human influenza infection (Beigel and Bray, 2008). On the other hand, oseltamivir-resistant influenza A (H_1N_1) virus infection has been reported (Gooskens et al, 2009), and inhalation of zanamivir may be difficult for patients with a severe condition, and for small children. Therefore, further development of anti-influenza virus drugs is needed.

Human influenza viruses attach to sialic acid with an α2,6linkage (SAα2,6Gal) on the airway epithelial cells (Rogers and Paulson, 1983). The viruses are then delivered into the cytoplasm, and ribonucleoproteins (RNPs) of viruses, which include viral RNA, are released from acidic endosomes into the cytoplasm of the cells (Palese and Shaw, 2006). Bafilomycin A_1, a macrolide antibiotic and the specific vascular-ATPase (V-ATPase) inhibits growth of type A and type B human influenza viruses in Madin Darby Canine Kidney (MDCK) cells (Ochiai et al., 1995). Furthermore, a macrolide antibiotic erythromycin reduces the number of low pH endosomes as well as reducing ICAM-1, the receptor for rhinovirus, a major virus of common colds, and inhibits rhinovirus infection in human airway epithelial cells (Suzuki et al., 2002). However, the inhibitory effects of clinically used macrolide antibiotics on the influenza virus infection in human airway cells have not been studied.

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Increases in pro-inflammatory cytokines and monokines including interleukin (IL)-1, IL-6 and IL-8 are observed in the serum in patients and in the lung of mice infected with influenza virus (Konstantinos and Sheridan, 2001; Deng et al., 2008). These factors are suggested to be associated with the pathogenesis and severity of influenza virus infection (Deng et al., 2008). Macrolide antibiotics reduce the production of pro-inflammatory cytokines in human airways (Suzuki et al., 2002), and the reduction of these factors are suggested to relate to the clinical benefits of macrolides in diffuse panbronchiolitis and cystic fibrosis (Kudoh et al., 1998; Equi et al., 2002). Furthermore, maclorides including bafilomycin A_1, erythromycin and clarithromycin reduce the secretion of pro-inflammatory cytokines in supernatants of human airway epithelial cells after rhinovirus infection (Suzuki et al., 2001a; Suzuki et al., 2002; Jang et al., 2006). However, the effects of clarithromycin on the secretion of pro-inflammatory cytokines by human airway epithelial cells after influenza infection have not been studied.

In the present study, we examined the effects of clarithromycin on type A seasonal human influenza (FluA) virus (H_3N_2) infection in human airway epithelium. We also studied the effects of clarithromycin on the receptor expression and acidic endosomes in order to clarify the mechanisms.

Methods

Media Components

Reagents for cell culture media were obtained as follows: Dulbecco's modified Eagle's Medium (DMEM), Ham's F-12 medium, and fetal calf serum (FCS) were from GIBCO-BRL Life Technologies, Palo Alto, CA; ultroser G (USG) was from BioSepra, Cergy-Saint-Christophe, France; phosphate-buffered saline (PBS), Eagle's Minimum

Essential Medium (MEM), and trypsin was from Sigma, St. Loius, MO.

Human Tracheal Epithelial Cell Culture

Isolation and culture of the human tracheal surface epithelial cells were performed as described previously (Suzuki et al., 2001a; Suzuki et al., 2002), with some modification (Yamaya et al., 2007). The human tracheal surface epithelial cells were plated at 5 x 10^5 viable cells/ml in plastic tubes with round bottoms (16 mm diameter and 125 mm long, Becton Dickinson) coated with human placental collagen, because attachment of the cells in plastic tubes was much better than that in glass tubes (data not shown) (Yamaya et al., 2007). Cells were cultured in 1 ml of a mixture of DMEM-Ham's F-12 (DF-12) medium (50/50, vol/vol) containing 2% ultroser G (USG) and antibiotics (Suzuki et al., 2001a; Suzuki et al., 2002; Yamaya et al., 2007). The position of plastic tubes, where cells were cultured, was fixed in an inclined stainless steel tube rack (30 cm-wide, 10 cm-high and 10 cm-deep, TE-HER TUBE RACK INCLINABLE® RF-6, Hirasawa Works Co. Ltd., Tokyo, Japan), and this tube rack was placed in a humid incubator. The positioning of plastic tubes in the tube rack made a ~ 5° angle between the long axis of the tubes and the flat bottom plate of the incubator where the tube rack was placed (Suzuki et al., 2001a; Suzuki et al., 2002; Yamaya et al., 2007). The tubes were kept stationary, and cells were immersed in 1 ml of medium and cultured at 37°C in 5% CO_2-95% air in the incubator. Because of this laid position of the plastic tubes, the cells attached and proliferated on the inner surface of the lateral wall of the tubes and the round shape of the bottom of the tubes.

The surface area of culture vessels of the plastic tubes covered by the cells became 11.6 ± 0.2 cm^2 (n=3). The opening of the tubes was loosely covered with a screw cap to make air containing CO_2 move through the slit. We confirmed the presence of a dome formation when the cells made confluent cell sheets on days 5-7 of culture using an inverted microscope (MIT-2, Olympus, Tokyo, Japan) (Suzuki et al., 2002) as

described by Widdicombe et al. (1987).

Tracheas for cell cultures were obtained after death from 40 patients (age, 68 ± 3 yr; 11 female, 29 male) without complications with bronchial asthma or COPD. The causes of death included malignant tumor other than lung cancer (n=20), acute myocardial infarction (n=4), rupture of an aortic aneurysm (n=3), sepsis (n=3), ileus (n=3), malignant lymphoma (n=2), cerebral infarction (n=2), amyotrophic lateral sclerosis (n=1), congestive heart failure (n=1), and cerebral bleeding (n=1). Of 40 patients, 15 were ex-smokers, and 25 had never smoked. This study was approved by the Tohoku University Ethics Committee.

Culture of Madin Darby Canine Kidney Cells

Madin Darby Canine Kidney epithelial cell line (MDCK) cells were also cultured in T_{25} flasks (Becton Dickinson) in MEM containing 10% FCS supplemented with 5 x 10^4 U/l penicillin, 50 mg/l streptomycin (Numazaki et al., 1987). The cells were then plated in plastic dishes (96-well plate, Becton Dickinson) or in plastic tubes with round bottoms (16 mm diameter and 125 mm length, Becton Dickinson). The opening of the tubes was loosely covered with a screw cap to make air containing CO_2 move through the slit. Cells in the plastic dishes or tubes were cultured at 37°C in 5% CO_2-95% air.

Viral Stocks

Type A seasonal human influenza (FluA) virus (H_3N_2) was prepared in our laboratory from a patient with a common cold (Numazaki et al., 1987). FluA virus was identified by the hemadsorption inhibition (HI) test using an antiserum (New York/55/2004), as described previously (Numazaki et al., 1987). MDCK cells were plated in plastic tubes with round bottoms and cultured for 7 days at 37°C in 5% CO_2-95% air to make confluent cell sheets. Then, to generate stocks of FluA virus, MDCK cells in plastic tubes were rinsed with PBS and cultured in the medium (1.1 ml)

containing 100 μl of FluA virus stock solution (10^3 $TCID_{50}$ units in 100 μl) and 1 ml of the MEM supplemented with 5 x 10^4 U/l penicillin, 50 mg/l streptomycin and 3.5 μg/ml trypsin. The opening of the tubes was loosely covered with a screw cap to make air containing CO_2 move through the slit. Cells in the tubes were cultured at 33°C in 5% CO_2-95% air after infection with FluA virus (Numazaki et al., 1987), because it has been shown that respiratory viruses such as human influenza virus and rhinovirus are well replicated and produced at 33°C in the cells (Storch, 2006). To obtain the FluA virus solution, 7 days after infection with FluA virus, MDCK cells and culture medium in the tubes were frozen in a short time in ethanol at -80°C, thawed and sonicated. The virus containing fluid was frozen in aliquots at -80°C.

Detection and Titration of Viruses

Detection and titration of influenza viruses in supernatant fluids was performed with the endpoint methods (Condit, 2006), by infecting replicate confluent MDCK cells in plastic 96-well dishes (Becton Dickinson) with serial 10-fold dilutions of virus-containing supernatant fluids as previously described (Numazaki et al., 1987; Yamaya et al., 2007). In brief, virus-containing supernatant fluids were 10-fold diluted in MEM supplemented with 5 x 10^4 U/l penicillin, 50 mg/l streptomycin and 3.5 μg/ml trypsin, and added into the replicate MDCK cells in the wells (200 μl/well) of 96-well dishes. MDCK cells in the wells were then cultured at 33°C in 5% CO_2-95% air for 7 days, and the presence of the typical cytopathic effects (CPE) of influenza virus was examined in all replicate cells as described previously (Numazaki et al., 1987; Condit, 2006). The number of wells that showed CPE of influenza virus was counted in each dilution of supernatant fluids. Then, the dilution of virus-containing supernatant fluids which showed CPE in greater than 50% of replicate wells, and the dilution of the fluids that showed CPE in less than 50% of the replicate wells were estimated. Based on these data, $TCID_{50}$ (TCID: tissue culture infective dose) was calculated with methods as

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previously described (Condit, 2006). Because the human tracheal epithelial cells were cultured in 1 ml of DF-12 medium containing 2% USG in the tubes, viral titers in supernatant fluids are expressed as $TCID_{50}$ units/ml (Numazaki et al., 1987; Condit, 2006; Yamaya et al., 2007). Furthermore, the rates were obtained by dividing the value of influenza viral titer ($TCID_{50}$ units/ml) in supernatant fluids by incubation time, and are expressed as $TCID_{50}$ units/ml/24h (Yamaya et al., 2007).

Viral Infection of the Cells

Infection of FluA virus to the human tracheal epithelial cells was performed with methods previously described (Suzuki et al., 2001a; Suzuki et al., 2002; Yamaya et al., 2007). A stock solution of FluA virus (H_3N_2, New York/55/2004) was added to the human tracheal epithelial cells in the tubes (100 μl in each tube, 1.0×10^3 $TCID_{50}$ units/100 μl). Because the number of the epithelial cells in the tubes was $2.0 \pm 0.3 \times 10^6$ of cells/tube (n=7), the multiplicity of infection (moi) was 0.5×10^{-3} $TCID_{50}$ units/cell. Because, in preliminary experiments, we found that the human tracheal epithelial cells were detached from culture vessels of the tubes when the cells were infected with 0.5×10^{-2} $TCID_{50}$ units/cell or more of influenza virus, the cells were therefore infected with 0.5×10^{-3} $TCID_{50}$ units/cell of viruses. After a 1-h incubation at 33°C in 5% CO_2-95% air, the viral solution was removed, and the epithelial cells were rinsed once with 1 ml of PBS. The cells were then fed with 1 ml of fresh DF-12 medium containing 2% USG supplemented with antibiotics. The opening of the tubes was loosely covered with a screw cap, the tubes were laid with a slant of ~ 5° and kept stationary in a humid incubator, and cells were cultured at 33°C in 5% CO_2-95% air as described previously (Numazaki et al., 1987). The supernatant fluids were stored at -80°C for the determination of viral titers.

Treatment of the Cells with Clarithromycin

In order to examine the effects of clarithromycin on FluA virus infection, the cells were treated with clarithromycin (10 μM) (Jang et al., 2006), unless we describe other concentrations. Cells were treated with clarithromycin from 3 days before FluA virus infection until the end of the experiments after FluA virus infection (Suzuki et al., 2002). A concentration of 10 μM of clarithromycin was chosen, because a concentration of 15 μM of clarithromycin is the maximum serum concentration of macrolides in clinical use (500 mg of oral clarithromycin administration) (Honeybourne et al., 1994).

We also studied the relationship between the concentration of clarithromycin and the potency of inhibitory effects. To examine the concentration-dependent effects of clarithromycin on FluA virus infection, cells were treated with clarithromycin at concentrations ranging from 10 nM to 100 μM.

Collection of Supernatant Fluids for Measurements

We measured the time course of FluA viral release with methods as previously described (Suzuki et al., 2002; Yamaya et al., 2007). In brief, to measure viral release during the first 24 h, we used two separate cultures from the same trachea. We collected the supernatant fluids at either 1 or 24 h after influenza virus infection. Furthermore, to measure the viral titer during 1 to 3 days after virus infection, we used one culture from each trachea after collecting supernatant fluids at 1 day (24 h) after virus infection. After collecting supernatant fluids at 1 day after infection, the cells were rinsed with PBS and 1 ml of DF-12 medium containing 2% USG was replaced. Supernatant fluids were also collected at 3 days after infection. Likewise, to measure the viral titer during 3 to 5 days after FluA virus infection, after collecting supernatant fluids at 3 days after infection, the cells were rinsed with PBS and 1 ml of the fresh medium was replaced. Supernatant fluids were also collected at 5 days after virus infection. The cells were then rinsed with PBS and 1 ml of the fresh medium was replaced. Supernatant fluids were also collected at 7 days after FluA virus infection to

measure the viral titer during 5 to 7 days after virus infection.

Likewise, to examine the effects of clarithromycin on pro-inflammatory cytokines, supernatant fluids were collected just before infection, and 1 day (24h), 3 days (72h), 5 days (120h) and 7 days (144 h) after FluA virus infection.

Effects of Clarithromycin on Susceptibility to Influenza Virus Infection

The effects of clarithromycin on the susceptibility to FluA virus infection were evaluated as previously described (Subauste et al., 1995; Suzuki et al., 2002; Yamaya et al., 2007) using epithelial cells pretreated with clarithromycin (10 μM) or vehicle (ethanol, 0.1%) from 3 days before infection with FluA virus until just finishing the FluA virus infection. The epithelial cells were then exposed to serial 10-fold dilutions of FluA virus (H_3N_2) or vehicle of influenza virus (MEM) for 1 h at 33°C in 5% CO_2-95% air. Because we found in the preliminary experiments that the maximum virus titers were observed in the supernatant fluids collected for 3-5 days, the presence of FluA virus was determined in the supernatant fluids collected for 3-5 days after infection with methods described above to assess whether infection occurred at each dose of FluA virus used.

Quantification of Influenza Virus RNA

To quantify the FluA virus RNA and ribosomal RNA (rRNA) expression in the human tracheal epithelial cells before and after FluA virus infection, real-time quantitative RT-PCR using the Taqman technique (Roche Molecular Diagnostic Systems) was performed as previously described (Yamaya et al., 2007) with some modification. Each RNA sample (100 ng/10 μl of water) was mixed in 40 μl of buffer containing 100 nM forward primer (5'- AGATGAGTCTTCTAACCGAGGTCG -3'), 100 nM reverse primer (5'- TGCAAAAACATCTTCAAGTCTCTG -3') and other reagents as previously described (Spackman et al., 2002). Taqman probe influenza

virus [5'-(FAM) TCAGGCCCCCTCAAAGCCGA(TAMRA)-3'] was designed for FluA virus (Spackman et al., 2002). The fragment of RNA extracted from the human tracheal epithelial cells before or at 24 h (1 day), 72 h (3 days) 120 h (5 days) and 144 h (7 days) after infection by FluA virus was reverse transcribed into cDNA (30 min at 48°C) and amplified by PCR for 40 cycles (15 s at 95°C and 1 min at 60°C). The standard curve was obtained between the fluorescence emission signals and Cτ by means of 10-fold dilutions of the total RNA, extracted from 10^4 $TCID_{50}$ units/ml of FluA virus in the supernatants of the MDCK cells 7 days after infection with FluA virus (0.5×10^{-3} $TCID_{50}$ units/cell). Real-time quantitative RT-PCR for rRNA was also performed using the same PCR products. The standard curve was obtained between the fluorescence emission signals and Cτ by means of 10-fold dilutions of the RNA extracted from the cells. The expression of FluA virus RNA was normalized to the constitutive expression of rRNA.

Detection of SAα2,6Gal in Human Trachea

SAα2,6Gal in human trachea was detected using lectins as previously described (Shinya et al., 2006). In brief, human tracheae were cut into small pieces (10 x 10 mm), and incubated in the DF-12 medium containing 2 % USG, antibiotics, and either clarithromycin (10 μM) or vehicle of clarithromycin (ethanol, 0.1%) for 24 h at 37°C in 5% CO_2-95% air. Paraffin-embedded tissues were cut into 5-μm thick sections with a microtome, and mounted on 3-aminopropyltrethoxy-silane (APS)-coated slides (Matsunami Glass Ind., Ltd., Tokyo, Japan). Sections were incubated with 250 μl of fluorescein isothiocyanate (FITC)-labeled *Sambucus nigra* (SNA) lectin (1:100, Vector Laboratories, Burlingame, CA) overnight at 4°C. Sections were incubated with Alexa Fluor 594-conjugated streptavidin (1:250, Molecular Probes, Inc., Eugene, OR) for 2 hours at room temperature, and were counterstained with 4',6-diamino-2-phenylindole, dihydrochloride (DAPI; Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

The cover glasses were mounted on the sections and observed with a fluorescence microscope (BZ-8000; KEYENCE Co. Osaka, Japan). The excitation wave lengths were 470 nm (FITC), 560 nm (Alexa594) and 360 nm (DAPI), and the emitted light from the cells was detected through 495 nm, 595 nm and 400 nm filters, respectively. The fluorescence intensity was calculated using a fluorescence image analyzer system (Lumina Vision®, Mitani Co. Ltd., Fukui, Japan) equipped with a fluorescence microscope.

Measurement of Changes in Acidic Endosomes

The distribution and the fluorescence intensity of acidic endosomes in the cells was measured with a dye, LysoSensor DND-189 (Molecular Probes), as previously described (Suzuki et al., 2002; Yamaya et al., 2007). The cells on coverslips in Petri dishes were observed with a fluorescence microscope (OLYMPUS IX70; OLYMPUS Co. Ltd., Tokyo, Japan). The excitation wave length was 443 nm, and the emitted light from the cells was detected through a 505 nm filter. The fluorescence intensity was calculated using a fluorescence image analyzer system (Lumina Vision®, Mitani Co. Ltd., Fukui, Japan) equipped with a fluorescence microscope. The effects of clarithromycin on acidic endosomes were examined from 100 sec before to 300 sec after the treatment with clarithromycin (10 μM) or vehicle (ethanol, 0.1%). Furthermore, we studied the effects of a long period of treatment with clarithromycin (10 μM, 3 days) on the distribution and the fluorescence intensity of acidic endosomes. Fluorescence intensity of acidic endosomes was measured in 100 human tracheal epithelial cells, and the mean value of fluorescence intensity was expressed as % of control value compared with the fluorescence intensity of the cells treated with vehicle of clarithromycin (ethanol, 0.1%).

Measurement of Cytokines Production

We measured IL-1β, IL-6 and IL-8 of supernatant fluids by specific enzyme-linked immunosorbent assays (ELISAs) (Suzuki et al., 2002; Yamaya et al., 2007). To demonstrate the time course of cytokines release, we expressed the rates of change in cytokines concentration in the supernatant fluids. The rates were obtained by dividing the value of cytokines concentration in supernatant fluids by incubation time, and are expressed as pg/ml/24h.

NF-kappa B assay

Nuclear extracts from human tracheal epithelial cells were prepared by using a TransFactor extraction kit (BD Bioscience/CLONTECH, CA) according to manufacturer instructions. After centrifugation at 20,000 x *g* for 5 min at 4°C, nuclear extracts were assayed for p50, p65 and c-Rel content. An equal amount of nuclear lysate was added to incubation wells precoated with the DNA-binding consensus sequence. The presence of translocated p50, p65 and c-Rel subunit was assayed by using a TransFactor Family Colorimetric Kit-NFκB (BD Bioscience/CLONTECH, CA) according to manufacturer instructions (Fiorucci et al., 2002). Plates were read at 655 nm, and results are expressed as OD.

Statistical Analysis

Results are expressed as means ± SE. Statistical analysis was performed using two-way repeated measures of analysis of variance (ANOVA). Subsequent post-hoc analysis was made using Bonferroni's method. For all analyses, values of $p<0.05$ were assumed to be significant. In the experiments using culture of human tracheal epithelial cells, n refers to the number of donors (tracheae) from which cultured epithelial cells were used.

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Results

Effects of Clarithromycin on Influenza Virus Infection of Human Tracheal Epithelial Cells

Exposing confluent human tracheal epithelial cell monolayers to type A seasonal human influenza (FluA) virus (H_3N_2, 0.5 x 10^{-3} $TCID_{50}$ units/cell) consistently led to infection. No detectable virus was revealed at 1 h after infection, while FluA virus was detected in culture supernatant fluids at 24 h, and the viral content progressively increased between 1 h and 24 h after infection (Fig. 1). Evidence of continuous viral production was obtained by demonstrating that each of supernatant fluids collected during 1 day to 3 days, 3 to 5 days, and 5 to 7 days after infection contained significant levels of FluA virus (Fig. 1). The viral titer levels in supernatant fluids increased significantly with time for the 5 days of observation ($p<0.05$ in each case by ANOVA).

Treatment of the cells with clarithromycin (10 μM) significantly decreased the viral titers of FluA virus in supernatant fluids from 1 day (24 h) after infection (Fig. 1). Furthermore, clarithromycin decreased the viral titers of FluA virus in supernatant fluids concentration-dependently and the maximum inhibitory effect was obtained at 100 μM (Fig. 2).

Treatment with clarithromycin (10 μM) did not change the concentrations of lactate dehydrogenase (LDH) in supernatant fluids throughout the experiments. The concentrations of LDH were 35 ± 3 IU/l/24h before treatment with clarithromycin, and 33 ± 3 IU/l/24h 24 h after treatment ($p>0.20$; compared with the concentrations before treatment, n=5), 34 ± 3 IU/l/24h 72 h after treatment ($p>0.20$, n=5), 34 ± 3 IU/l/24h 120 h after treatment ($p>0.20$, n=5), and 33 ± 3 IU/l/24h 144 h after treatment ($p>0.20$, n=5).

Effects of Clarithromycin on Viral RNA by PCR

Further evidence of the inhibitory effects of clarithromycin on FluA viral RNA replication in human tracheal epithelial cells was provided by real-time quantitative RT-PCR analysis. FluA viral RNA in the cells was consistently observed from 1 day (24 h) after infection, and increased with time (Fig. 3), while FluA viral RNA in the cells was not observed before infection (data not shown). Maximum FluA viral RNA in the cells was observed at 5 days (120 h) after infection (Fig. 3) (data at 144 h not shown). Clarithromycin (10 μM) decreased the FluA viral RNA from 1 day after infection (Fig. 3).

Effects of Clarithromycin on Susceptibility to FluA Virus Infection

Treatment of the cells with clarithromycin (10 μM) decreased the susceptibility of the cells to infection by FluA virus. The minimum dose of FluA virus necessary to cause infection in the cells treated with clarithromycin (10 μM, 3 days) was significantly higher than that in the cells treated with vehicle (0.1% ethanol) (Fig. 4).

Effects of Clarithromycin on SAα2,6Gal Expression

SAα2,6Gal, a receptor for human influenza, was observed as a green line or spots of green staining on the mucosal surface of human tracheal epithelium (Fig. 5A). Clarithromycin (10 μM, 24 h) reduced the number of green spots on the human tracheal epithelium (Fig. 5B), and the fluorescence intensity from the receptor (by 62 ± 7 % compared with that in vehicle of clarithromycin ($p<0.001$, n = 8, Student t-test) (Fig.5C).

Effects of Clarithromycin on the Acidification of Endosomes

Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189 (Fig. 6A and 6B). Clarithromycin (10 μM, 300 sec) significantly reduced the fluorescence intensity from acidic endosomes in the epithelial

cells (by 32 ± 2 %, $p<0.01$, n=5) (Fig. 6C and 6D). Clarithromycin also reduced the number of acidic endosomes with green fluorescence in the cells with time (data not shown).

Likewise, a long period of treatment with clarithromycin (10 μM, 3 days) reduced the number of acidic endosomes with green fluorescence in the cells (Fig. 6B), and the fluorescence intensity from acidic endosomes in the epithelial cells compared with that in the cells treated with vehicle (0.1 % ethanol) (by 69 ± 4 %, $p<0.01$, n=5) (Fig. 6D). The inhibitory effects of a long period of treatment with clarithromycin (3 days) on the fluorescence intensity were concentration-dependent (Table 1).

Effects of Clarithromycin on Cytokines

The secretion of IL-1β, IL-6 and IL-8 all increased after FluA virus infection (Fig. 7), and maximum secretion was observed at 5 days after the infection (data not shown). Treatment with clarithromycin (10 μM) reduced the concentrations of IL-1β, IL-6 and IL-8 5 days after FluA virus infection as well as baseline concentrations of these cytokines before FluA virus infection (Fig. 7). In contrast, ultraviolet-irradiated FluA virus did not increase IL-1β, IL-6 and IL-8 (Fig. 7).

Effects of Clarithromycin on NF-kappa B

Clarithromycin (10 μM, 3 days) significantly reduced the amount of p50, p65 and c-Rel of NF-κB in the nuclear extracts in the cells before FluA virus infection (Fig. 8). On the other hand, the amount of p50, p65 and c-Rel of NF-κB in the nuclear extracts increased 5 days after FluA virus infection (Fig. 8), and clarithromycin (10 μM) also significantly reduced the amount of p50, p65 and c-Rel of NF-κB induced by FluA virus infection (Fig. 8).

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Discussion

In the present study, we have shown that viral titers in supernatant fluids and RNA of type A seasonal human influenza (FluA) virus in human tracheal epithelial cells increased with time, and clarithromycin reduced viral titers in supernatant fluids concentration-dependently, RNA replication of FluA virus in the cells, and the susceptibility to FluA virus infection. The surface epithelium of human tracheae expressed sialic acid with an α2,6linkage (SAα2,6Gal), a receptor for human influenza virus (Rogers and Paulson, 1983), stained with FITC-labeled *Sambucus nigra* lectin as shown by Shinya et al. (2006). Treatment of human tracheae with clarithromycin reduced the expression of the receptor on the epithelium. These findings suggest that clarithromycin might inhibit FluA virus infection, partly through the reduced expression of the receptor for human influenza virus in the human tracheal epithelium. Clarithromycin also reduced the number of acidic endosomes from which viral ribonucleoproteins (RNPs) containing RNA of FluA virus enter into the cytoplasm, and reduced the fluorescence intensity from acidic endosomes. These findings suggest that the reduction of acidic endosomes might also relate to the inhibition of FluA virus infection by clarithromycin. Furthermore, clarithromycin reduced concentrations of cytokines, including IL-1β, IL-6 and IL-8 in supernatant fluids. Clarithromycin may also modulate airway inflammation induced by FluA virus infection.

Human seasonal influenza viruses and classical H_1N_1 swine influenza viruses bind to SAα2,6Gal, and most avian and equine viruses bind to SAα2,3Gal (Rogers and Paulson, 1983). An expression of SAα2,6Gal was observed in epithelial cells in the nasal mucosa, pharynx, tracheae and bronchi (Couceiro et al., 1993; Shinya et al., 2006). In contrast, SAα2,3Gal was reported not to express on the tracheal epithelial cells (Couceiro et al., 1993), while recent reports demonstrated its expression on ciliated cells in the human tracheae (Matrosovich et al., 2004). SAα2,3Gal is also expressed in

nonciliated cuboidal bronchiolar cells and type II cells lining the alveolar wall (Shinya et al., 2006). In this study, human tracheal epithelial cells expressed SAα2,6Gal, but they did not express SAα2,3Gal (data not shown). These findings are consistent with those in previous reports (Couceiro et al., 1993; Shinya et al., 2006). In the present study, immunohistochemistry demonstrated the reduced expression of SAα2,6Gal in human tracheal epithelial cells after treatment with clarithromycin. Furthermore, the minimum dose of FluA virus necessary to cause infection in the cells treated with clarithromycin was significantly higher than that in the cells treated with vehicle of clarithromycin, showing the reduced susceptibility to FluA virus infection. These effects are similar to those of erythromycin on the reduced expression of ICAM-1, a receptor for rhinovirus, and on inhibition of the rhinovirus infection (Suzuki et al., 2002). These effects are also similar to those of clarithromycin on the reduced expression of activated RhoA (isoform A of the Ras-homologus), one of receptors for respiratory syncytial virus, and on inhibition of respiratory syncytial virus infection (Asada et al., 2009). Clarithromycin might reduce the amount of FluA virus virions attached to the epithelial cells through the reduced expression of SAα2,6Gal in the cells.

The mechanisms for the reduction of SAα2,6Gal expression by clarithromycin are uncertain. However, tumor necrosis factor (TNF)-α, one of inflammatory mediators in airways, increases the expression glycosyltransferase and sulfotransferase responsible for biosynthesis of sialylated epitopes in the bronchial mucosa (Delmotte et al., 2002), through the activation of NF-κB (Chen et al., 2008). α2,6-Sialic acid in glycoproteins also increases in inflamed mouse serum in response to turpentine oil (Yasukawa et al., 2005). On the other hand, erythromycin, one of macrolides, reduces the increased activation of NF-κB by rhinovirus infection as well as the baseline NF-κB activity before rhinovirus infection (Suzuki et al., 2002). In the present study, clarithromycin reduced NF-κB proteins including p50, p65 and c-Rel in the cells, and reduced the expression of SAα2,6Gal, a receptor for human influenza virus, on the mucosal surface

of human tracheae before influenza virus infection. Clarithromycin also reduced NF-κB proteins induced by influenza virus infection. These findings are consistent with those reported previously (Delmotte et al., 2002; Suzuki et al., 2002; Yasukawa et al., 2005; Chen et al., 2008). Clarithromycin might reduce the expression of SAα2,6Gal on the cells partly through the reduction of NF-κB activation.

Treatment of the cells with clarithromycin did not change the concentrations of LDH in supernatant fluids throughout the experiments. These findings suggest that clarithromycin did not have toxic effects on the epithelial cells, and toxic effects of clarithromycin were not responsible for the effects described for clarithromycin in the present study.

After attachment of influenza virus to the receptor, viruses enter the airway epithelial cells and are internalized by endocytic compartments via four internalization mechanisms, including clarthrin-coated pits; caveola; nonclathrin, noncaveolae pathway; and macropinocytosis (Palese and Shaw, 2006). Of these mechanisms, a nonclathrin, noncaveolae-mediated internalization pathway depends on low pH (Sieczkarski and Whittaker, 2003). Furthermore, after binding on the cell surface, the virus is internalized by receptor-mediated endocytosis, and the low pH in the endosome triggers fusion of the viral and endosomal membranes. The viruses then release their RNPs containing viral RNA, into the cytoplasm, resulting in the next processes of viral replication (White et al., 1983; Palese and Shaw, 2006). As shown previously (Suzuki et al., 2002) and in this study, macrolide antibiotics including erythromycin and clarithromycin reduced the number of acidic endosomes and the fluorescence intensity from acidic endosomes. Furthermore, clarithromycin reduced FluA viral RNA in human tracheal epithelial cells and viral titers of FluA in supernatant fluids. These findings are consistent with the reports that bafilomycin A_1 reduces the number of acidic endosomes (Ochiai et al., 1995; Suzuki et al., 2001a) in the epithelial cells, and reduces the growth of influenza virus in MDCK cells (Ochiai et al., 1995). Increased pH in

acidic endosomes might relate to the inhibition of entry of the viral RNPs into the cytoplasm, and might inhibit FluA virus infection in this study.

Various inflammatory factors are suggested be associated with not only the pathogenesis and severity of influenza virus infection (Deng et al., 2008), but also exacerbations of bronchial asthma and COPD in the influenza virus infection (Seemungal et al., 2001). Increases in pro-inflammatory cytokines and monokines including interleukin IL-1, IL-6 and IL-8 are observed in the serum in patients and in the lung of mice infected with influenza virus (Konstantinos and Sheridan, 2001; Deng et al., 2008). Macrolide antibiotics reduce the production of pro-inflammatory cytokines in human airways (Takizawa et al., 1995), after infection of rhinovirus and respiratory syncytial virus (Suzuki et al., 2002; Jang et al., 2006; Asada et al., 2009). Erythromycin and clarithromycin reduce the frequencies of exacerbations and hospitalization in COPD patients (Suzuki et al., 2001b; Seemungal et al., 2008; Yamaya et al., 2008), and the frequencies of common colds in COPD patients (Suzuki et al., 2001b). Erythromycin reduces the mortality rate by pneumonia in mice after influenza virus infection (Sato et al., 1998). Although the clinical benefits of macrolides in influenza virus infection are still uncertain, reduction of pro-inflammatory cytokines by clarithromycin may modulate influenza virus infection-induced inflammation and severity of the disease, and may be associated with COPD exacerbations.

In this study, we examined the inhibitory effects of clarithromycin on the expression of FluA virus receptor and on acidic endosomes in the airway epithelial cells. However, there are many other interactions between epithelial cells and influenza virus components such as neuraminidase which have important roles in virus infection (Palese and Shaw, 2006). Further studies are needed to clarify the mechanisms of clarithromycin.

In summary, we demonstrated that clarithromycin, a clinically used macrolide, reduces FluA viral titers and cytokines secretion in supernatant fluids, FluA virus RNA

replication in the cells, and susceptibility of the cells to infection by the virus. Clarithromycin also reduced the expression of SAα2,6Gal, a receptor for human influenza, on the mucosal surface of human tracheae, and reduced the number of acidic endosomes from which viral RNPs enter into the cytoplasm. These findings suggest that a clinically used macrolide antibiotic clarithromycin may inhibit type A seasonal human influenza virus infection via reducing its receptor on the airway epithelial cells and via reducing entry of viral RNPs, which contain viral RNA, into the cytoplasm. Clarithromycin may also inhibit airway inflammation induced by influenza virus infection. Macrolide antibiotics may modulate the severity of influenza infection.

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Footnotes

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Conflict of interest: None

Legends for Figures

Fig. 1. The time course of viral release in supernatant fluids of human tracheal epithelial cells obtained at different times after exposure to 0.5 x 10^{-3} $TCID_{50}$ units/cell of type A seasonal human influenza virus in the presence of clarithromycin (10 μM) (closed circles) or vehicle of clarithromycin (0.1% ethanol) (open circles). The rates of change in virus concentration in the supernatant fluids are expressed as $TCID_{50}$ units/ml/24h. Results are means ± SE from 5 different tracheae. Significant differences from viral infection alone are indicated by *p<0.05.

Fig. 2. Concentration-response effects of clarithromycin on the viral release in supernatant fluids collected during 3 to 5 days after infection. The cells were treated with clarithromycin or vehicle (Control; 0.1% ethanol) from 3 days before type A seasonal human influenza virus infection until the end of the experiments after influenza virus infection. The rates of change in virus concentration in the supernatant fluids are expressed as $TCID_{50}$ units/ml/24h. Results are means ± SE from 5 different tracheae. Significant differences from vehicle alone (Control) are indicated by *p<0.05 and **p<0.01.

Fig. 3. Replication of viral RNA in human tracheal epithelial cells after infection with type A seasonal human influenza virus in the presence of clarithromycin (10 μM) (FluA + CAM) or vehicle (0.1% ethanol) (Control; FluA) as detected by real-time quantitative RT-PCR. Results are expressed as the relative amount of RNA expression (%) compared with those of maximal influenza viral RNA at day 5 (120 h) in the cells treated with vehicle, and reported as means ± SE from 5 samples. Significant differences from treatment with a vehicle (FluA) at each time are indicated by *p<0.05 and **p<0.01.

Fig. 4. The minimum dose of influenza virus necessary to cause infection in the human tracheal epithelial cells treated with either clarithromycin (10 μM, 3 days) or vehicle (control, 0.1% ethanol). The minimum dose of influenza virus necessary to cause infection is expressed as $TCID_{50}$ units/ml. Results are means ± SE from 7 different tracheae. Significant differences from vehicle alone (control) are indicated by $*p<0.05$.

Fig. 5. A and B: The expression of SAα2,6Gal (arrows), a receptor for human influenza virus, on the mucosal surface of the human tracheal epithelium treated with clarithromycin (10 μM, 24 h) (B) or vehicle of clarithromycin (0.1% ethanol, 24 h) (A, Control). Data are representative of three different experiments. (Bar = 100 μm)

C: The fluorescence intensity of SAα2,6Gal on the mucosal surface of the human tracheal epithelium treated with clarithromycin (10 μM, 24 h) or vehicle of clarithromycin (0.1% ethanol, 24 h). Results are expressed as relative fluorescence intensity (%) compared with those treated with vehicle, and reported as means ± SE from 8 samples. Significant differences from control values are indicated by $***p<0.001$.

Fig. 6. A and B: Changes in the distribution of acidic endosomes with green fluorescence in human tracheal epithelial cells 3 days after treatment with clarithromycin (CAM, 10 μM) or vehicle of clarithromycin (0.1% ethanol, Control). Data are representative of 5 different experiments. (magnification; x 400)

C: Time course changes in the intensity of green fluorescence from acidic endosomes in human tracheal epithelial cells after treatment with either

clarithromycin (10 μM, open circles) or vehicle (0.1% ethanol, closed circles). Inhibitors were administrated at time 0.

D: The fluorescence intensity of acidic endosomes 300 sec and 3 days (72 h) after the addition of clarithromycin (CAM, 10 μM) or vehicle of clarithromycin (Control, 0.1% ethanol). Results are expressed as relative fluorescence intensity (%) compared with those treated with vehicle for each period, and reported as means ± SE from 5 samples. Significant differences from control values are indicated by $**p<0.01$.

Fig. 7. The release of cytokines into supernatant fluids of human tracheal epithelial cells before and 5 days after type A seasonal human influenza virus infection in the presence of clarithromycin (FluA + CAM, 10 μM) or vehicle of clarithromycin (0.1% ethanol, FluA), and after UV-inactivated influenza virus infection (UV + FluA). The rates of change in cytokines concentration in the supernatant fluids are expressed as pg/ml/24h. Results are means ± SE from 5 different tracheae. Significant differences from values before influenza virus infection (time 0) in the presence of vehicle of clarithromycin (0.1% ethanol) are indicated by $*p<0.05$ and $**p<0.01$. Significant differences from influenza virus infection alone (FluA) 5 days after infection are indicated by $+p<0.05$.

Fig. 8. Amount of p50 (A), p65 (B) and c-Rel (C) in nuclear extracts in human tracheal epithelial cells treated with clarithromycin (CAM, 10 μM) or vehicle (C, 0.1% ethanol) for 3 days before type A seasonal human influenza (FluA) virus infection, and the amount of p50, p65 and c-Rel in the cells 5 days (120 h) after infection with either FluA virus or vehicle of FluA virus (minimum essential medium) in the presence of clarithromycin (10 μM, FluA + CAM) or vehicle of clarithromycin (0.1 % ethanol, FluA) from 3 days before FluA virus infection

until the end of the experiments after FluA virus infection. Results are expressed as OD, and are means ± SE from 5 different tracheae. Significant differences from control values (C) before FluA virus infection are indicated by *p<0.05 and **p<0.01. Significant differences from FluA virus infection alone (FluA) are indicated by ++p<0.01.

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TABLE 1

Concentration-response effects of clarithromycin on the fluorescence intensity from acidic endosomes.

Cells were treated with clarithromycin (CAM) for 3 days. Results are expressed as relative fluorescence intensity (%) compared with those treated with vehicle. Significant differences from control values are indicated by $p<0.05$ and $p<0.01$.

Condition	Fluorescence intensity (% pretreatment) (n=5, means ± SE)	*p* value
Control	100 ± 2	-
CAM (0.01 μM)	99 ± 2	$p>0.50$
CAM (0.1 μM)	82 ± 9	$p>0.20$
CAM (1.0 μM)	54 ± 5	$p<0.05$
CAM (10 μM)	31 ± 4	$p<0.01$

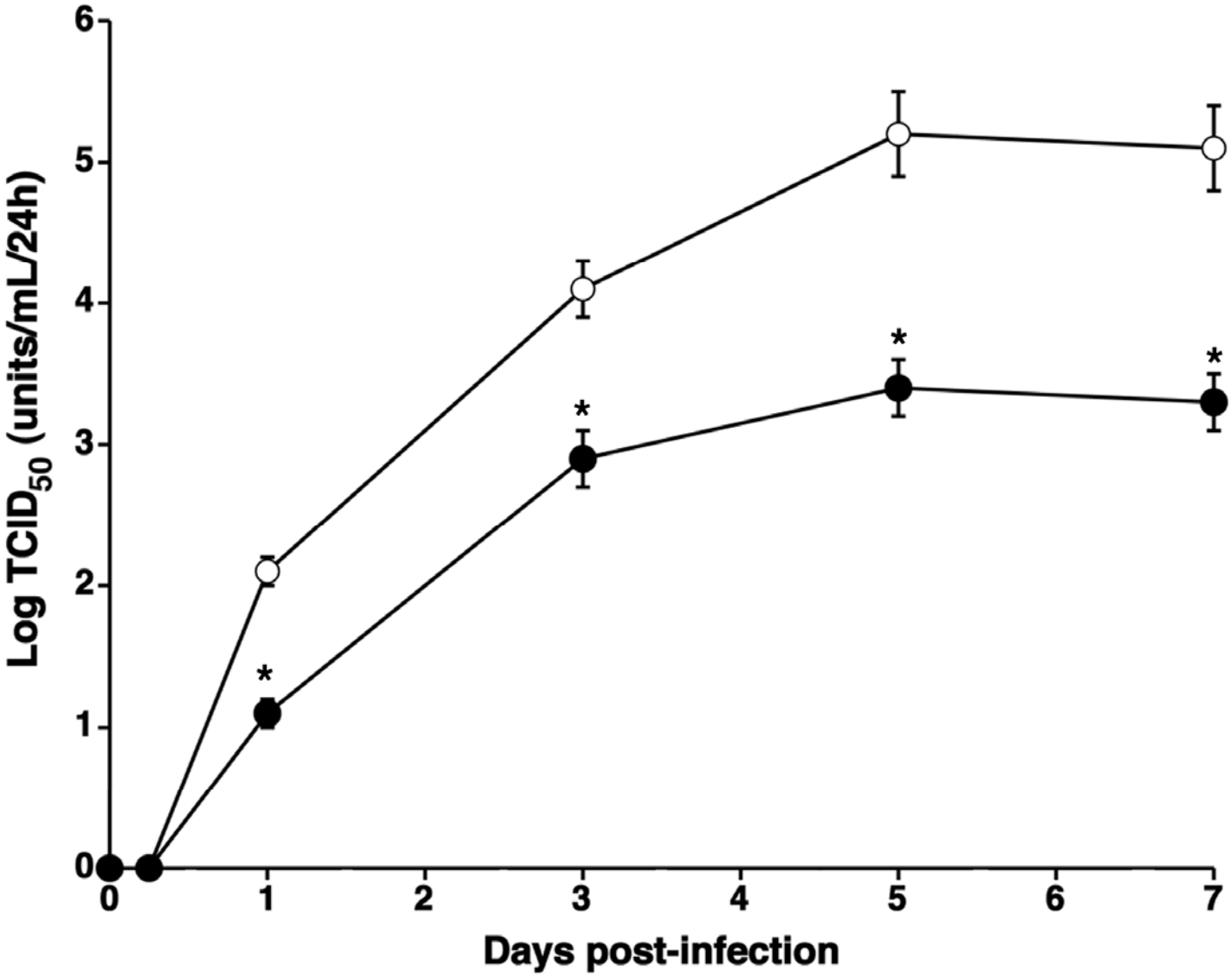

Fig 1

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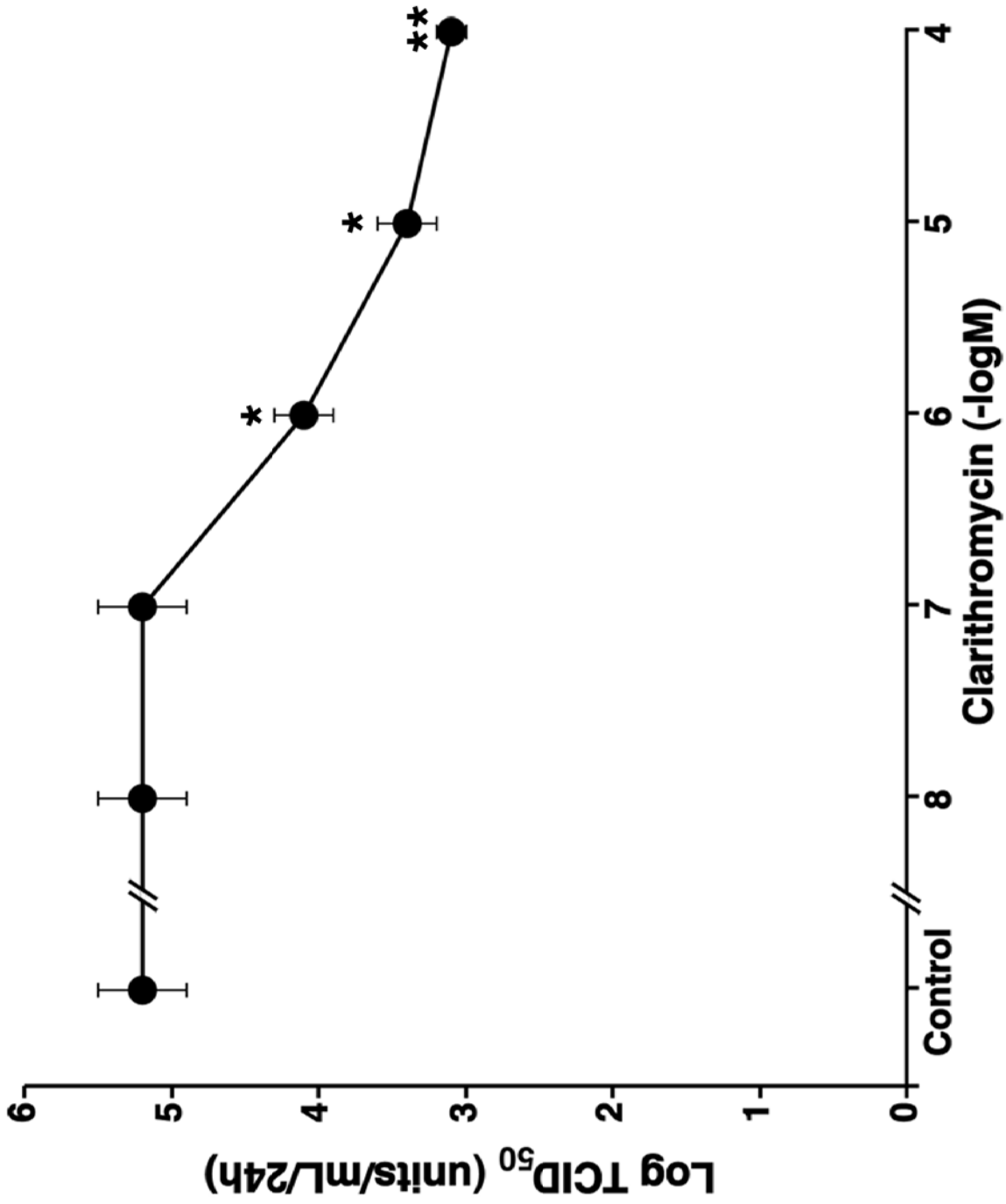

Fig 2

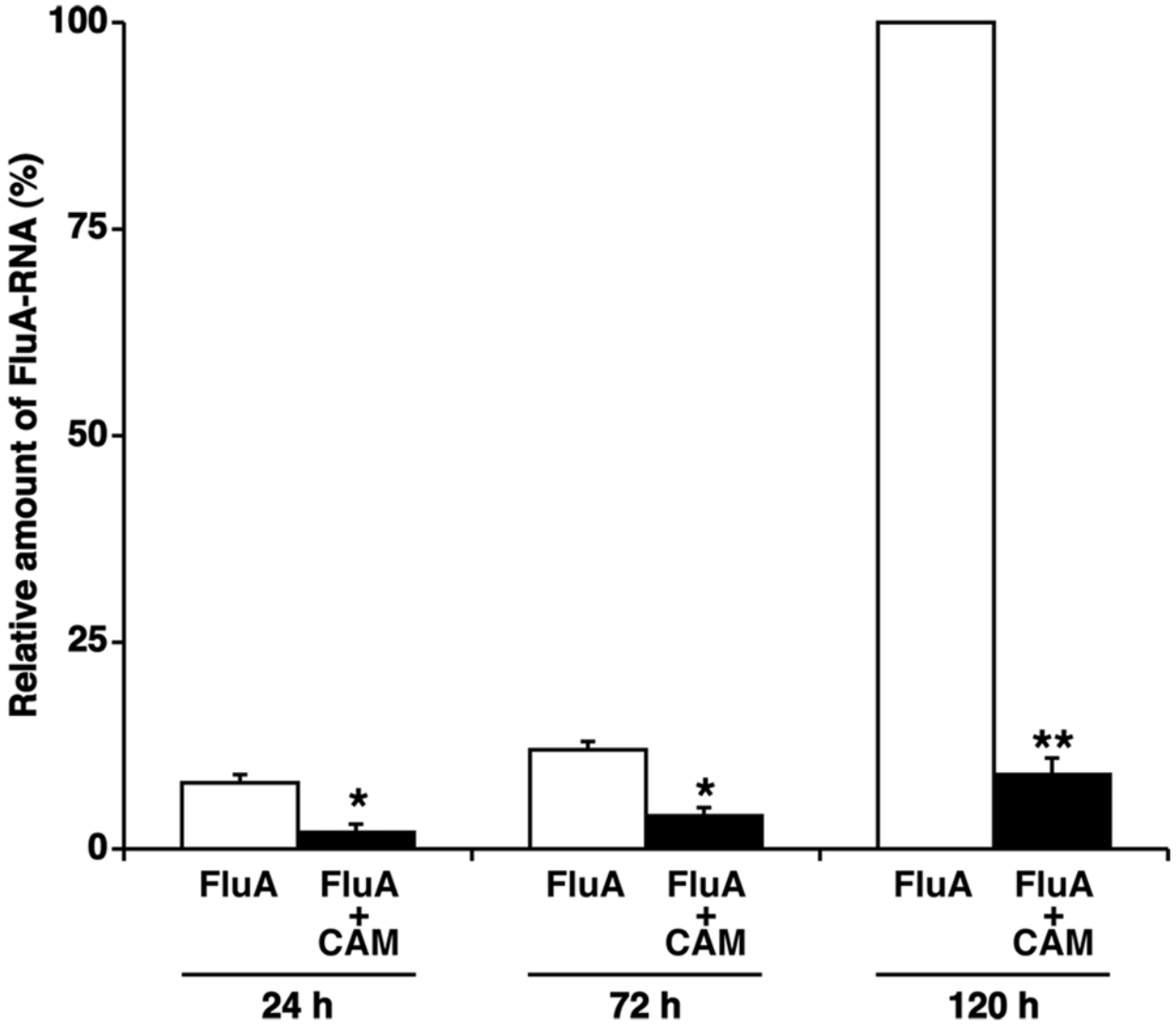

Fig 3

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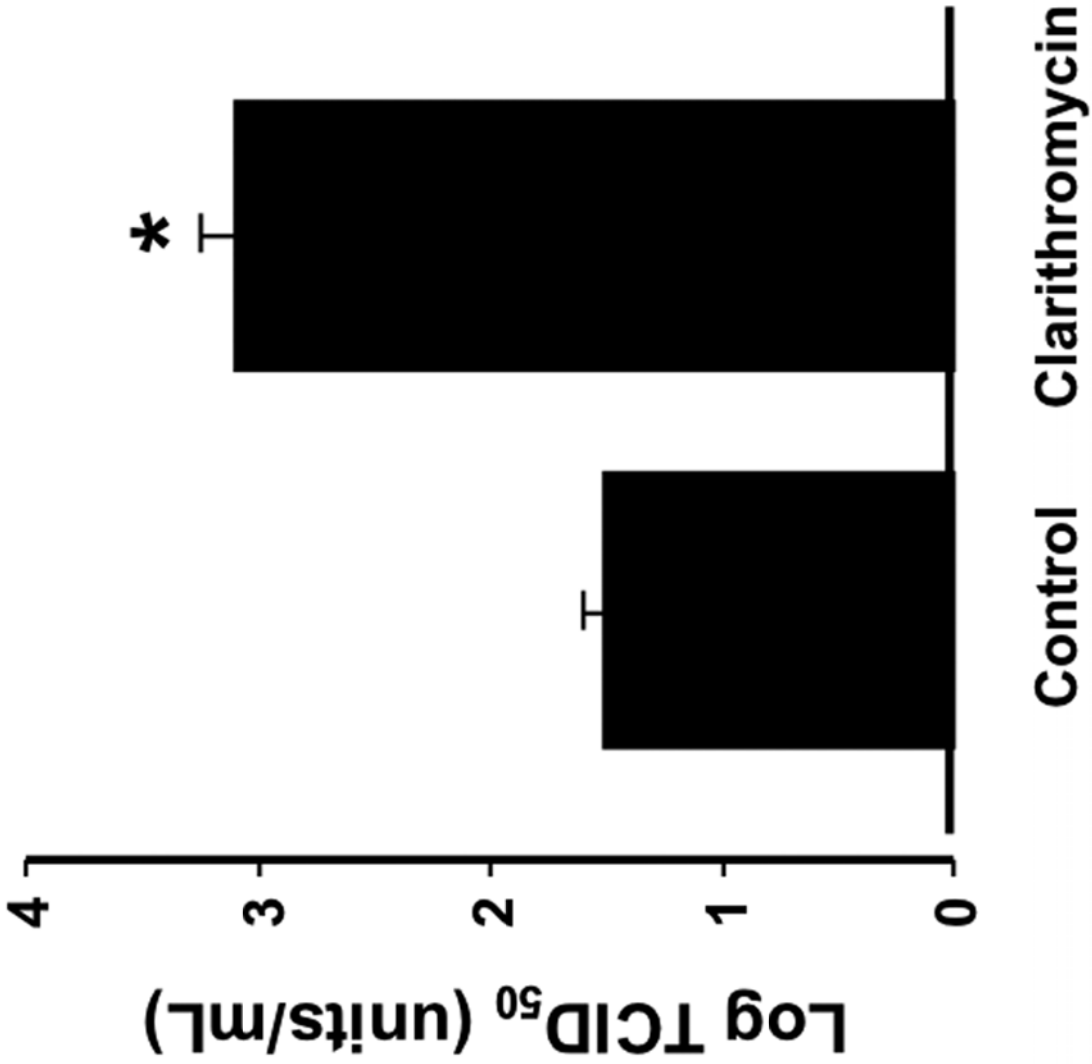

Fig 4

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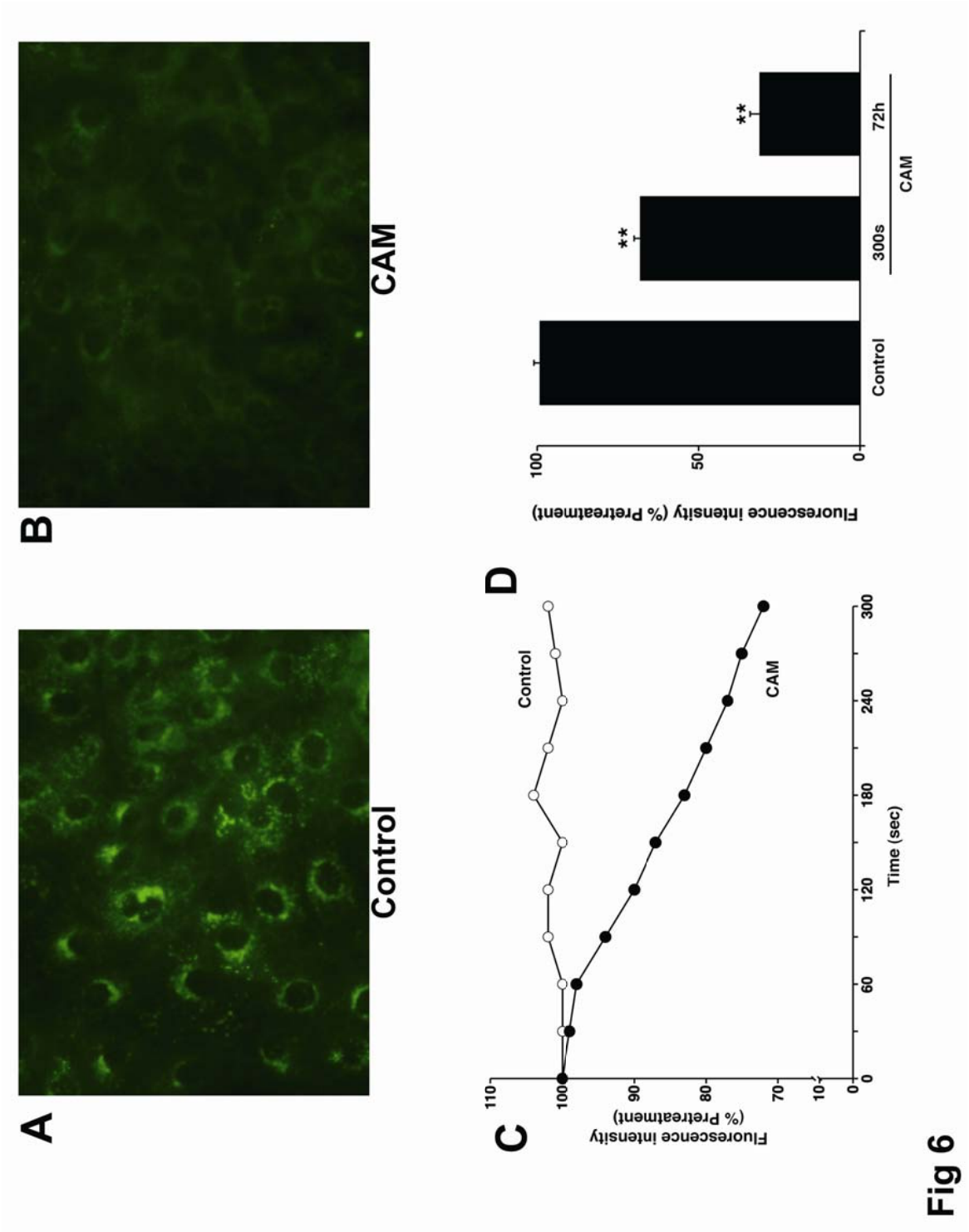

Fig 6

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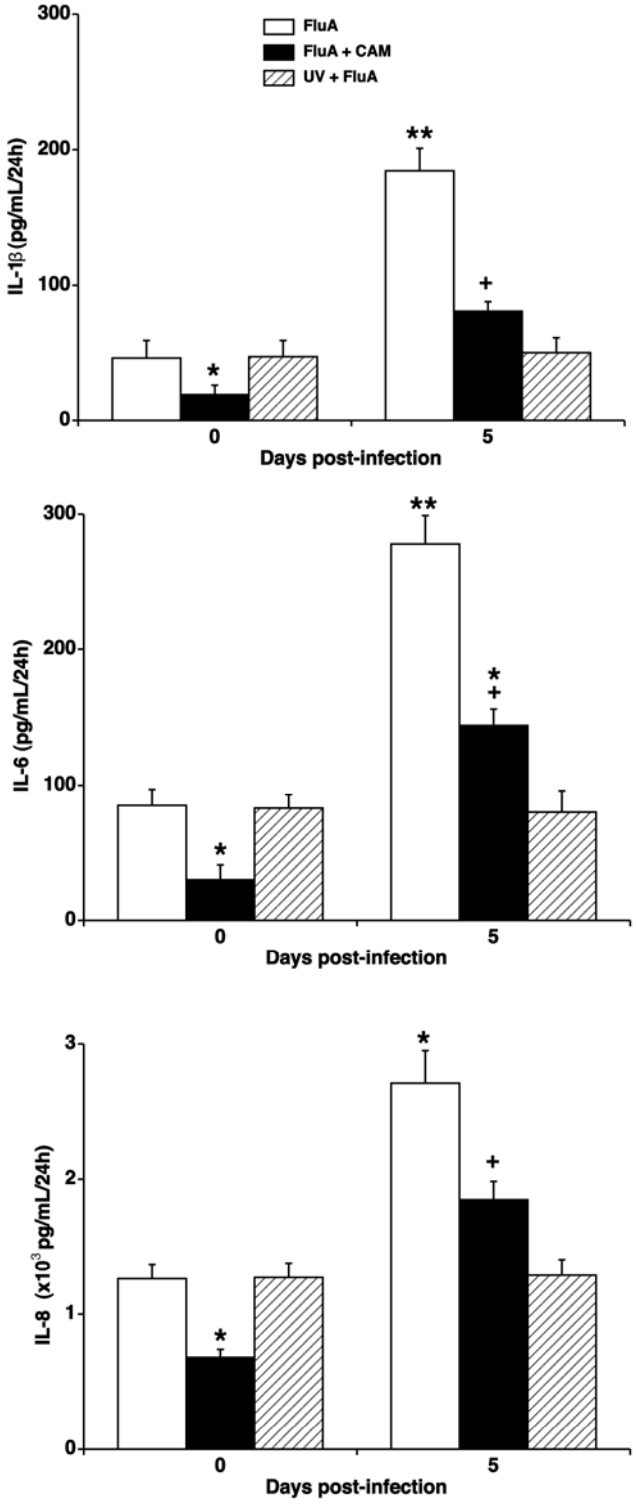

Fig 7

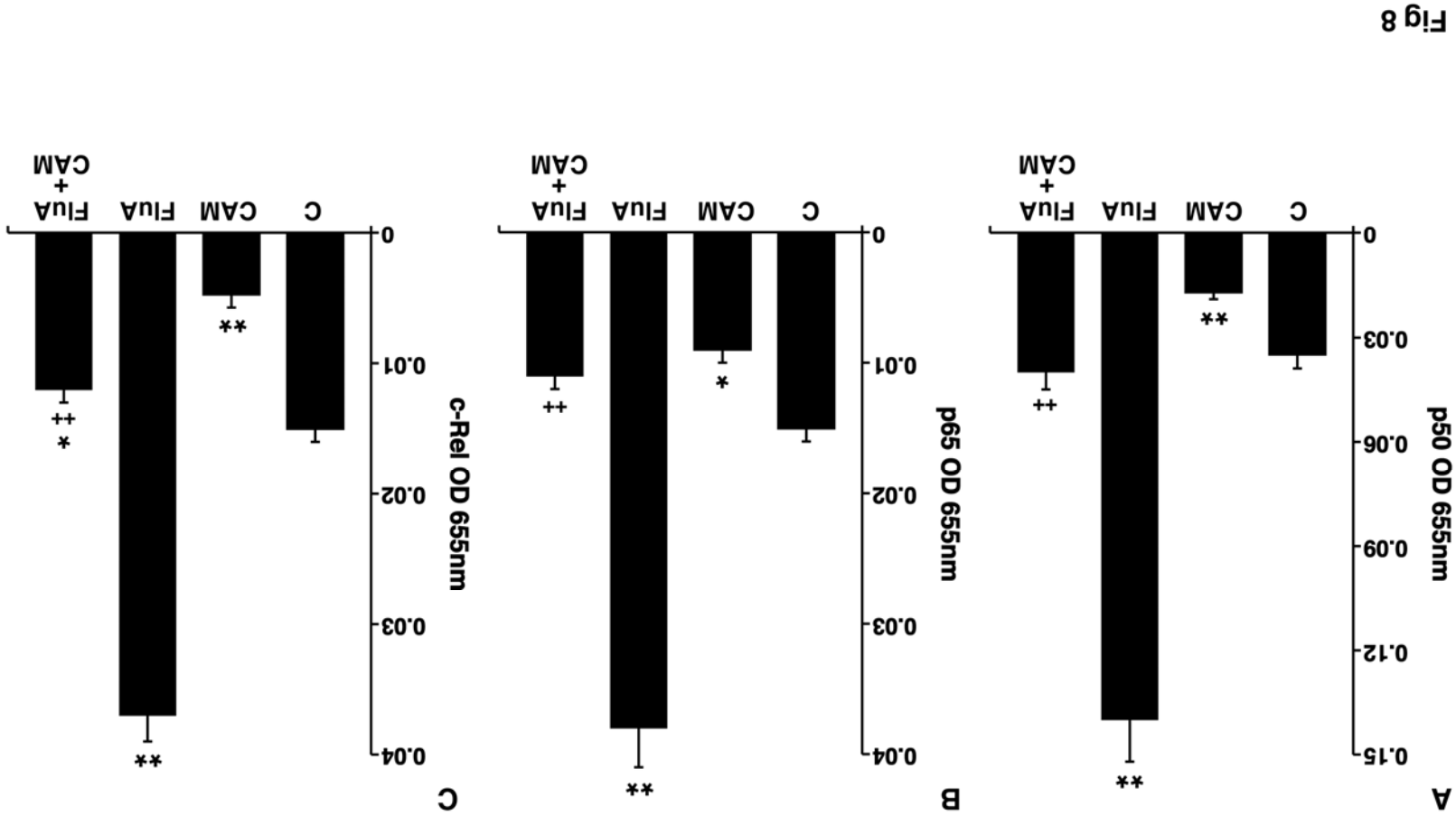

Fig 8