Regular Article

Azithromycin Inhibits Constitutive Airway Epithelial Sodium Channel Activation *in Vitro* and Modulates Downstream Pathogenesis *in Vivo*

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Epithelial sodium channel (ENaC) is an amiloride-sensitive sodium ion channel that is expressed in epithelial tissues. ENaC overexpression and/or hyperactivation in airway epithelial cells cause sodium over-absorption and dysregulated ciliary movement for mucus clearance; however, the agents that suppress constitutive airway ENaC activation are yet to be clinically available. Here, we focused on macrolides, which are widely used antibiotics that have many potential immunomodulatory effects. We examined whether macrolides could modulate constitutive ENaC activity and downstream events that typify cystic fibrosis (CF) and chronic obstructive pulmonary diseases (COPD) *in vitro* and *in vivo* models of ENaC overexpression. Treatment of ENaC-overexpressing human bronchial epithelial cells ($\beta\gamma$ ENaC-16HBE14o- cells) with three macrolides (erythromycin, clarithromycin, azithromycin) confirmed dose-dependent suppression of ENaC function. For *in vivo* studies, mice harboring airway specific β ENaC overexpression (C57BL/6J- β ENaC-transgenic mice) were treated orally with azithromycin, a well-established antimicrobial agent that has been widely prescribed. Azithromycin treatment modulated pulmonary mechanics, emphysematous phenotype and pulmonary dysfunction. Notably, a lower dose (3 mg kg^{-1}) of azithromycin significantly increased forced expiratory volume in 0.1s (FEV0.1), an inverse indicator of bronchoconstriction. Although not statistically significant, improvement of pulmonary obstructive parameters such as emphysema and lung dysfunction (FEV0.1%) was observed. Our results demonstrate that macrolides directly attenuate constitutive ENaC function *in vitro* and may be promising for the treatment of obstructive lung diseases with defective mucociliary clearance, possibly by targeting ENaC hyperactivation.

Key words epithelial sodium channel (ENaC); macrolide; emphysema; lung dysfunction; azithromycin

INTRODUCTION

Epithelial sodium channel (ENaC) is a sodium ion channel with heteromultimer (two α , one β , and one γ subunits) that is expressed in various epithelial tissues.^{1,2)} Especially, in the apical membrane of airway epithelial cells, ENaC works as an ion channel that controls airway surface liquid (ASL) and contributes to normal ciliary movement, leading to mucus clearance. On the other hand, its hyperactivation causes sodium over-absorption and dysfunction of ciliary movement.³⁾ Importantly, hyperactivation of ENaC was correlated with lung dysfunction in patients with cystic fibrosis (CF),⁴⁾ a common genetic disorder in Caucasians. Moreover, ENaC were also dysregulated in patients with chronic obstructive pulmonary disease (COPD),^{5,6)} a lung disease that exhibits inflammatory, emphysematous and mucus hypersecretory phenotypes. Consistently, airway-specific β ENaC-transgenic (Tg) mice show mucus obstructive lung phenotypes like CF and COPD,^{7,8)} implying that constitutive and hyperactive ENaC is being recog-

nized as therapeutic targets; however, the agents that suppress ENaC are yet to be clinically available.

The macrolide antibiotics are well-established antimicrobial agents that have had a critical role in the chemotherapy of respiratory infections.⁹⁾ Major members of the class include erythromycin, clarithromycin, and azithromycin.¹⁰⁾ Macrolide antibiotics inhibit protein synthesis by binding to the 50S ribosomal subunit of certain microorganisms. In addition to this antimicrobial mode of action, non-antibiotic immunomodulatory properties of macrolides are also considered to contribute in controlling pulmonary disease pathogenesis.⁹⁾ Due to their inhibitory effect on inflammatory mediators,¹¹⁾ regulatory role of mucus hypersecretion,¹²⁾ and modulatory action on host-defense mechanisms,¹³⁾ the potential application of macrolide antibiotics to a variety of lung diseases, including diffuse pan bronchiolitis (DPB),¹⁴⁾ bronchiectasis,¹⁵⁾ COPD¹⁶⁾ and CF,¹⁷⁾ were considered.

In pathophysiological states, ENaC hyperactivation is generally caused by two mechanisms; one involves direct activa-

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tion with cell surface ENaC cleavage exerted by intercellular proteases such as human neutrophil elastase (HNE),¹⁸⁾ and the other involves increased expression at transcriptional and post-translational levels by extracellular and intracellular signals.¹⁹⁾ Importantly, Tarran *et al.* showed that macrolides prevent HNE-induced ASL volume depletion through inhibition of HNE-stimulated ENaC activity.²⁰⁾ We postulate that treatment with macrolides may also inhibit overexpression-associated constitutive ENaC activation *in vitro* and modulate the pathogenesis of ENaC-hyperactive lung diseases. By utilizing ENaC-overexpressing human bronchial epithelial cells ($\beta\gamma$ ENaC-16HBE14o- cells),²¹⁾ we confirmed the suppressive effect of macrolides on constitutive ENaC function. Oral treatment with azithromycin in mice harboring airway specific β ENaC overexpression (C57BL/6J- β ENaC-Tg mice)⁷⁾ significantly increased the forced expiratory volume in 0.1 s (FEV_{0.1}), possibly leading to improvement of lung obstructive phenotypes such as FEV_{0.1}%, especially at a lower dose (3 mg kg⁻¹). Our results are the first to show that macrolides directly attenuate constitutive airway ENaC function, and may be beneficial for the treatment of obstructive lung diseases with defective mucociliary clearance.

MATERIALS AND METHODS

Reagents Azithromycin was purchased from TCI (Tokyo Kasei Kogyo, Tokyo, Japan). Erythromycin and clarithromycin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell Culture Human bronchial $\beta\gamma$ ENaC-stable 16HBE14o-cell line was established and grown as previously described.²¹⁾ Cells were maintained in MEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μ g/mL streptomycin. All cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C.

Transepithelial Measurements $\beta\gamma$ ENaC-stable 16HBE14o-cells were seeded onto Transwell® (12-mm) with polycarbonate membrane insert (0.4 μ m-pore) (Corning, Tewksbury, MA, U.S.A.). An epithelial volt ohmmeter (EVOM) with Ag:AgCl chopstick electrodes were utilized to measure transepithelial resistance (R_{te}) and potential difference (V_{te}) of monolayers as previously earlier (World Precision Instruments, Sarasota, FL, U.S.A.).²²⁾ Once the cells had grown confluent, 1 mM sodium butyrate and 100 μ M dexamethasone were added to the media.¹⁸⁾ After 24 h treatment, the equivalent current (I_{eq}) was calculated based on the data of R_{te} and V_{te}.²²⁾

C57BL/6J- β ENaC-Tg Mice and Treatments C57BL/6J- β ENaC-Tg mice were generated with the reproductive technique with the Center for Animal Resources and Development (CARD)²³⁾ and genotyped as previously described.⁷⁾ Age-matched wild-type (WT) C57BL/6J mice were used as a healthy control group. Adult male C57BL/6J- β ENaC-Tg mice (11 weeks old) were used for the experiment with pharmacological treatment. The mice were orally treated with methylcellulose (vehicle) or 3 and 10 mg kg⁻¹ azithromycin (AZ) once per day for 3 weeks. All mice were maintained in the animal house at Kumamoto University in accordance with the guidelines of the animal facility center of Kumamoto University and were fed with normal chow (Oriental Yeast, Tokyo, Japan) *ad libitum*. All experiments were performed according to the protocols approved by the Animal Welfare Committee of Kumamoto University and all methods were performed in

accordance with the relevant guidelines and regulations.

flexiVent Systems Pulmonary mechanics (resistance, compliance, and elastance) were measured by the forced oscillation technique with a computer-controlled small animal ventilator flexiVent (SCIREQ, Montreal, Canada) as previously described.⁷⁾ Briefly, mice were anesthetized and then mechanically ventilated by flexiVent system. Forced expiratory volume in 0.1 second (FEV_{0.1}), forced vital capacity (FVC) and pulmonary functional parameter FEV_{0.1}/FVC (FEV_{0.1}%) were also determined using the flexiVent software.

Histological Analyses and Measurement of Mean Linear Intercept (MLI) Measurement of MLI was performed as previously described.^{7,24)} Briefly, the left lobe of lung was fixed in 10% formalin and then condensed in paraffin using histoprocessor Histos-5 (Milestone, Italy). Histological sections (6- μ m thickness) were prepared with a rotary microtome Leica RM2125RT (Bensheim, Germany). The sections were subjected to periodic acid-Schiff (PAS) and alcian blue (pH 2.5) staining as well as to hematoxylin and eosin (H&E) staining. For MLI determination, ten lung sections were selected in an unbiased fashion and randomly drawn with 300- μ m width 6 lines, and the intersection points with the alveolar walls were counted. MLI was calculated by dividing the total length of lines drawn across the lung section by the number of intercepts encountered.

Statistical Analysis The result represents the mean $\pm$ standard error of the mean (S.E.M.) performed in indicated replicates and the data were analyzed by either Student's *t*-test or one-way ANOVA with Dunnett's or Tukey-Kramer tests (JMP software, SAS Institute) as indicated in each figure legend. The level of significance was set at $p < 0.05$.

RESULTS

Macrolide Antibiotics Suppress Basal ENaC Function in $\beta\gamma$ ENaC-Overexpressing 16HBE14o- Cells We first investigated the effect of macrolide antibiotics on $\beta\gamma$ ENaC-overexpressing 16HBE14o- cells,²¹⁾ a cellular model that mimics the molecular environment of lung tissue of C57BL/6J- β ENaC-Tg mice.⁷⁾ We treated $\beta\gamma$ ENaC-16HBE14o-cells with serial concentration of erythromycin, clarithromycin and azithromycin, which are the major members of macrolide antibiotics. All macrolide antibiotics significantly suppressed basal ENaC function in $\beta\gamma$ ENaC-overexpressing 16HBE14o-cells in a dose-dependent manner (Figs. 1A–C), implying that macrolides directly target ENaC to suppress its function.

Azithromycin Modulates Emphysematous and Lung Dysfunction Phenotypes in C57BL/6J- β ENaC-Tg Mice Because azithromycin is a well-established antimicrobial agent that has been widely prescribed in the world and has ENaC-inhibitory activity in $\beta\gamma$ ENaC-overexpressing 16HBE14o- cells (Fig. 1A), we determined if the treatment with azithromycin is beneficial to *in vivo* ENaC-overexpressing model, C57BL/6J- β ENaC-Tg mice. Two doses (3 and 10 mg kg⁻¹) of azithromycin were orally administered to C57BL/6J- β ENaC-Tg mice, and the effect on pulmonary mechanics, emphysematous phenotype and pulmonary dysfunction was determined. Azithromycin treatment induced a slight but not significant decrease in resistance (a level of constriction) (3 mg kg⁻¹; 0.613 ± 0.0403 vs. 0.524 ± 0.0469 , 10 mg kg⁻¹; 0.613 ± 0.0403 vs. 0.486 ± 0.0423) (Fig. 2A), but

Fig. 1. The Effect of Macrolides on the Activity of ENaC in β/γ ENaC-16HBE14o- Cells

The short-circuit current (Isc) measured by EVOM is shown as ENaC activity in β/γ ENaC-16HBE14o- cells treated with (A) azithromycin, (B) clarithromycin and (C) erythromycin (10 or 100 μ M) for 24 h. The data are shown as % of control. $n = 4$ /group. The experiment was repeated and confirmed that the results are reproducible. Compared with control; Dunnett test. $**p < 0.01$. Data are presented as means $\pm$ standard error of the mean (S.E.M.).

Fig. 2. The Effect of Azithromycin Treatment on Pulmonary Mechanics of C57BL/6J- β ENaC-Tg Mice

Pulmonary mechanics of resistance (A), compliance (B) and elastance (C) were assessed in WT or vehicle- or Azithromycin (3 or 10 mg/kg/d)-treated C57BL/6J- β ENaC-Tg mice. $n = 5-6$ mice/group. Compared with C57BL/6J- β ENaC-Tg control; Dunnett test. $*p < 0.05$, $***p < 0.001$. The data are expressed as mean $\pm$ S.E.M.

Fig. 3. The Effect of Azithromycin Treatment on Lung Function of C57BL/6J- β ENaC-Tg Mice

Lung function parameters are evaluated as indicated FVC (A), FEV0.1 (B), FEV0.1/FVC (FEV0.1%) (C) in WT or vehicle- or azithromycin (3 or 10 mg/kg/d)-treated C57BL/6J- β ENaC-Tg mice. $n = 5-6$ mice/group. Compared with C57BL/6J- β ENaC-Tg control; Dunnett test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. The data are expressed as mean $\pm$ S.E.M.

had no significant positive effects on elastance (the elastic rigidity), and compliance (a static elastic recoil pressure at a given lung volume) (Figs. 2B, C). In accordance with the data with resistance, azithromycin significantly increased forced expiratory volume in 0.1 second (FEV0.1), an inverse indicator of bronchoconstriction, especially at a lower dose (3 mg/kg⁻¹; 1.31 $\pm$ 0.0552 vs. 1.50 $\pm$ 0.0396, 10 mg/kg⁻¹; 1.31 $\pm$ 0.0552 vs. 1.41 $\pm$ 0.0689) (Fig. 3A). On the other hand, azithromycin

slightly increased the forced vital capacity (FVC) (an overall lung volume) (3 mg/kg⁻¹; 1.70 $\pm$ 0.0335 vs. 1.75 $\pm$ 0.0438, 10 mg/kg⁻¹; 1.70 $\pm$ 0.0335 vs. 1.78 $\pm$ 0.0558) (Fig. 3B), which resulted in a higher, but not statistically significant increase in FEV0.1/FVC (FEV0.1%) (an overall lung function), especially at a lower dose (3 mg/kg⁻¹; 77.4 $\pm$ 3.29 vs. 85.9 $\pm$ 2.78, 10 mg/kg⁻¹; 77.4 $\pm$ 3.29 vs. 79.1 $\pm$ 4.15) (Fig. 3C).

Consistent with the data on resistance, FVC and FEV0.1%,

Fig. 4. The Effect of Azithromycin Treatment on Pulmonary Emphysema Phenotype in the Lung of C57BL/6J-ENaC-Tg Mice

Emphysematous phenotypes in WT and C57BL/6J- β ENaC-Tg mice treated with vehicle or azithromycin (3 or 10 mg/kg/d). (A) Alcian blue-stained and PAS lung section of WT and C57BL/6J- β ENaC-Tg mice treated with vehicle or Azithromycin (3 or 10 mg/kg/d). Square diameter; 300 μ m. (B) Quantitative morphometric analysis of alveolar septa (the mean linear intercept; MLI). $n = 6$ mice/group. Compared with C57BL/6J- β ENaC-Tg control; Dunnett test. **** $p < 0.0001$. Data are means $\pm$ S.E.M. (Color figure can be accessed in the online version.)

low dose of azithromycin (3 mg kg⁻¹) improved the MLI, the most common morphometric method to assess emphysema in animal models (3 mg kg⁻¹; 56.1 $\pm$ 1.70 vs. 48.7 $\pm$ 2.87, 10 mg kg⁻¹; 56.1 $\pm$ 1.70 vs. 55.5 $\pm$ 2.54) (Figs. 4A, B). Overall, our data show that azithromycin modulates pulmonary mechanics, emphysematous phenotype and pulmonary dysfunction in C57BL/6J- β ENaC-Tg mice, and suggest that a lower dose (3 mg kg⁻¹) of azithromycin may improve airway obstructive parameters, possibly due to the inhibition of airway ENaC function *in vivo*.

DISCUSSION

ENaC overexpression/hyperactivation is an important hallmark in the pathogenesis of many types of lung diseases, such as COPD and CF,⁴⁻⁶ and causes impaired mucociliary clearance in the airway. Improvement of “mucostasis,” a complication in patients having defects in mucociliary clearance, by targeting ENaC is now widely accepted in the pulmonary research field.^{4,25} Because “mucostasis” results in the accumulation of mucus containing pathogenic microbes, targeting bacterial colonization by antibiotics is also an important aspect to achieve better therapeutic approaches.^{25,26} Importantly, macrolide antibiotics have the potential not only to kill bacteria to reduce bacteria-inflammation exacerbation cycles but also to exert a variety of anti-inflammatory and immunomodulatory activities without affecting bacterial burden.⁹ This bifunctional mode of macrolides is thought to be ideal for the treatment of respiratory diseases, but the risk for bacterial resistance is still considered as an important issue in the clinic.^{10,27} In this connection, recent studies focused on the pharmacological action of non-antibiotic macrolide derivatives.^{20,28-30} In the present study, we showed that macrolides directly attenuate constitutive airway ENaC function *in vitro* and demonstrated

the modulatory roles of azithromycin in C57BL/6J- β ENaC-Tg mice, possibly through the inhibition of constitutive ENaC activity. However, there are two concerns in the present *in vivo* study. First, despite beneficial pulmo-modulatory effects of azithromycin (3 mg kg⁻¹) in C57BL/6J- β ENaC-Tg mice, statistically significant improvement was limited to FEV0.1 parameter. Because other parameters, including resistance, FEV0.1% and MLI, tended to improve with a low dose of azithromycin treatment, further verification study using more replicates may be important to conclude its efficacy. Second, our study could not exclude the possibility that antibiotic function of macrolides contributes to its beneficial effect. To clarify this point, non-antibiotic macrolide derivatives, *e.g.* EM703,²⁸ EM900,²⁹ CSY0073³⁰ and GS-459755,²⁰ should be tested in future study.

Beneficial effects by ENaC inhibition in β ENaC-Tg mice have been well-described in previous several papers. For example, Zhou *et al.* described the importance of early and continuous but not late and temporary inhibitions of ENaC activity by amiloride in the suppressive effect on all phenotypes of β ENaC-Tg mice.³¹ Moreover, Scot *et al.* demonstrated that SPX-101, a peptide mimetic of SPLUNC1 (short palate, lung, and nasal epithelial clone 1) that induces ENaC internalization and suppresses its function, increases mucus transport in β -ENaC-Tg mice.³² Thus, ENaC-targeting has beneficial effects on the pulmonary symptom in mice models. Notably, this “ENaC-targeting concept” is now applied to human treatment. Because several clinical trials are actually ongoing to target ENaC inhibition,^{33,34} our study showing azithromycin-dependent ENaC inhibitory role *in vitro* and modulatory role on ENaC-associated pulmonary symptoms *in vivo* may provide important information.

Despite lines of evidence for the muco-regulatory function of macrolide, the mechanism underlying ENaC inhibi-

tion by macrolides is obscure. Tarran *et al.* showed that the non-antibiotic macrolide GS-459755 inhibits the ASL volume depletion induced by human neutrophil elastase, possibly by inhibition of proteolytic activation of ENaC,²⁰⁾ implying that preventing ENaC cleavage may also be the mode of action of macrolide. Importantly, we previously showed that treatment of C57BL/6J- β ENaC-Tg mice with ONO-3403, a serine protease inhibitor that also suppresses neutrophil elastase, significantly improves pulmonary emphysema and dysfunction, suggesting that not only constitutive overexpression of ENaC but also proteolytic cleavage of ENaC is crucial for the pathophysiology of airway epithelial tissue in C57BL/6J- β ENaC-Tg mice.⁷⁾ Thus, the pulmo-modulatory effect of macrolides may be partly due to its inhibitory action on ENaC cleavage.

Finally, one key issue that needs to be discussed in the clinical aspect is the fact that only lower dose of azithromycin was effective *in vivo*. Azithromycin has a better pharmacokinetic profile compared to others,³⁵⁾ so that relatively higher dose (10 mg kg⁻¹) of azithromycin may have more detrimental rather than ameliorative effects on the lung. Consistently, the clinical dose used for long-term treatment with azithromycin having a pharmacological benefit in human is 500 mg 3 times per week, which corresponds to 3.57 mg kg⁻¹ in mice once a day (calculated by considering patient weight as 60 kg).³⁶⁾ If we consider long-term macrolides treatment, clinical regimen should be carefully set for azithromycin. Further *in vivo* experiments with erythromycin or clarithromycin may reveal better options for therapeutics. Overall, our results provide insight on macrolides as airway ENaC inhibitor *in vitro* and possibly *in vivo*. Future studies on the macrolide derivatives may open up new treatment options to patients with obstructive lung diseases with defective mucociliary clearance.

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Conflict of Interest The authors declare no conflict of interest.

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