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RESEARCH PAPER

Macrolides increase the expression of 11β-hydroxysteroid dehydrogenase 1 in human sinonasal epithelium, contributing to glucocorticoid activation in sinonasal mucosa

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BACKGROUND AND PURPOSE

The anti-inflammatory and immunomodulatory effects of macrolides include the ability to decrease mucus secretion and inhibit inflammatory mediators in chronic rhinosinusitis. Nevertheless, their mechanisms of action remain to be determined. Here we have investigated the effects of macrolide antibiotics (clarithromycin, azithromycin and josamycin; representating the 14-, 15- and 16-membered macrolides) on endogenous steroids in human sinonasal epithelial cells and mouse nasal mucosa.

EXPERIMENTAL APPROACH

The effects of macrolides on the expression of steroid-converting enzymes [11β-hydroxysteroid dehydrogenase (11β-HSD1 and 11β-HSD2)], steroid-synthesizing enzymes (3β-HSD, CYP21, CYP11B1 and CYP11A1) and cortisol levels were assessed in cultured human epithelial cells. In control and adrenalectomized mice , these enzymes and corticosterone levels were evaluated in nasal mucosa and serum after administration of macrolides.

KEY RESULTS

The expression levels of 3β-HSD, CYP21, 11β-HSD1 and CYP11B1 increased in human epithelial cells treated with clarithromycin and azithromycin, whereas the expression levels of 11β-HSD2 and CYP11A1 were not affected. Josamycin had no effects on the expression of these enzymes. Cortisol levels increased in epithelial cells treated with clarithromycin or azithromycin. The expression of 3β-HSD, CYP11A1, CYP21, CYP11B1 and 11β-HSD1 was upregulated in nasal mucosa of mice treated with clarithromycin or azithromycin, but not in adrenalectomized mice.

CONCLUSIONS AND IMPLICATIONS

This study provides evidence that 14- and 15-membered macrolide antibiotics may affect the expression of steroid-synthesizing and steroid-converting enzymes in human sinonasal epithelial cells and mouse nasal mucosa, increasing the endogenous cortisol levels in sinonasal mucosa.

Abbreviations

11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase 2; 3β-HSD, 3β-hydroxysteroid dehydrogenase; CYP21A1, cytochrome P450, family 21, subfamily A, polypeptide 1; CYP11B1, cytochrome P450, family 11, subfamily B, polypeptide 1; CYP11A1, cytochrome P450, family 11, subfamily A, polypeptide 1; CRS, chronic rhinosinusitis; PBS-T, phosphate-buffered saline-Tween 20; PF, 915275, N-(6-amino-2-pyridinyl)-4'-cyano-[1,1'-biphenyl]-4-sulfonamide; siRNA, small interfering RNA

Tables of Links

TARGETS
Enzymes
11β-HSD-1, 11β-hydroxysteroid dehydrogenase 1
CYP11A1
CYP11B1

LIGANDS
Azithromycin
Cortisol
Cortisone

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Although considered an infectious process induced by bacteria, viruses and fungi, chronic rhinosinusitis (CRS) with and without nasal polyps is accepted as an example of persistent mucosal inflammation resulting from multiple causes (Benninger *et al.*, 2003; Adriaensen and Fokkens, 2013). Thus, the therapeutic potential of treatment that targets the common pathways of mucosal inflammation has resulted in significant interest in glucocorticoids and macrolides (Benninger *et al.*, 2003; Adriaensen and Fokkens, 2013). Glucocorticoids and macrolides share some similarities in their pharmacological action, including anti-inflammatory and immunomodulatory properties and both classes of compounds have been shown to improve the symptoms and signs of CRS and are recommended for the treatment of CRS by the European Position Paper on Rhinosinusitis and Nasal Polyposis (Fokkens *et al.*, 2012).

Macrolides are known to have many diverse biological activities, including the ability to modulate inflammation (Kanoh and Rubin, 2010). A recent study has indicated that macrolide antibiotics inhibit pro-inflammatory cytokine production *in vitro* and show similar potential to prednisolone (Wallwork *et al.*, 2002). A study comparing the clinical efficacy of mometasone furoate nasal spray and clarithromycin in CRS showed that there is no significant difference between these two treatments and that both result in significant improvements in subjective and objective outcome measures (Zeng *et al.*, 2011). Corticosteroids, used topically or systemically, are well established as the first option in the treatment of nasal polyposis (Gosepath and Mann, 2005). However, nasal polyps were also reduced in size in patients treated with clarithromycin or roxithromycin (Ichimura *et al.*, 1996; Yamada *et al.*, 2000). After endoscopic sinus surgery in CRS patients, corticosteroids in oral or topical forms are recommended for controlling the inflammation, and long-term therapy with oral macrolides is also recommended as an alternative therapy (Adriaensen and Fokkens, 2013; Maniakas and Desrosiers, 2014; Varvyanskaya and Lopatin, 2014). Patients who are treated with macrolides are less troubled by the side effects that are associated with the use of systemic steroids, especially mood swings, restlessness and weight gain (Zarogoulidis *et al.*, 2012). Consequently, macrolides have historically been used for their corticosteroid-sparing effect (Gosepath and Mann, 2005; Zeng *et al.*, 2011; Adriaensen and Fokkens, 2013). However, further investigations into the corticosteroid-sparing effects of macrolides are required to understand their anti-inflammatory and immunomodulatory effects in more detail.

The endogenous glucocorticoids in humans and rodents are hydrocortisone or cortisol, and corticosterone respectively, and they are synthesized in the adrenal cortex from cholesterol, secreted in response to adrenocorticotropic hormone (ACTH; Chrousos, 1995). However, the local concentration of glucocorticoids in peripheral tissues depends not only on the plasma concentration of cortisol or corticosterone but also on the tissue-specific metabolism of glucocorticoids metabolized by 11β-hydroxysteroid dehydrogenase (11β-HSD; Coutinho and Chapman, 2011). The two forms of 11β-HSD - 11β-HSD1 and 11β-HSD2 - modulate endogenous glucocorticoid activity within cells and tissues at the pre-receptor level (Draper and Stewart, 2005). The 11β-HSD1 converts cortisone into active cortisol, potentiating the effects of glucocorticoids in tissues, whereas 11β-HSD2 inactivates cortisol by conversion to cortisone. Therefore, 11β-HSD1 increases, and 11β-HSD2 decreases, the local concentration of active glucocorticoids (Draper and Stewart, 2005).

Glucocorticoids are synthesized in a range of extra-adrenal organs, such as thymus, brain, skin and vascular endothelium (Murphy, 1978; Vacchio *et al.*, 1994; Coste *et al.*, 2007; Noti *et al.*, 2009; Taves *et al.*, 2011; Hostettler *et al.*, 2012). Other tissues such as lung and intestinal epithelium express steroidogenic enzymes, including CYP11B1, and produce glucocorticoids (Murphy, 1978; Taves *et al*, 2011). The expression of steroidogenic enzymes such as CYP11B1 and CYP11A1 is also found in human nasal mucosa, which is also thought to act as a peripheral source of glucocorticoids (Jun *et al.*, 2014a).

To increase understanding of the effects of macrolide treatment, this present study was designed to elucidate the effect of clarithromycin (a 14-membered macrolide), azithromycin (a 15-membered macrolide) and josamycin (a 16-membered macrolide), respectively on the expression levels of 11β-HSD1, 11β-HSD2, 3β-HSD, CYP21, CYP11B1 and CYP11A1 in normal epithelium isolated from sinus mucosa. We also sought to investigate whether the conversion ratio of cortisone to cortisol in normal human epithelium and mouse nasal mucosa is affected by the administration of macrolides.

Methods

Isolation and culture of epithelial cells from normal sinus mucosa

Informed consent was obtained from all participants before the commencement of the study and the study protocol was approved by the Institutional Review Board of Korea University College of Medicine. Normal human sinus mucosa was removed from the healthy ethmoid sinus cavity of 23 patients (12 men and 11 women; age range, 25-35) during endoscopic endonasal reconstruction for blowout fracture, under general anesthesia. This procedure was usually performed in the morning. These patients were required to have been without infection of nasal or respiratory tract, allergy, asthma, smoking or ongoing drug treatment. The additional criterion for exclusion was recent use (previous 8 weeks) of antibiotics including macrolides.

Normal sinus mucosa was enzymically digested in 0.5% dispase for 24 h in DMEM/F-12 including penicillin and streptomycin at 4°C. Epithelial cells were collected and cultured, with an air–liquid interface, in bronchial epithelial growth medium without thyroid hormone and bovine pituitary extract. When they reached approximately 80–90% confluence, cultured cells were treated with clarithromycin, azithromycin or josamycin at concentrations of 10, 50 and 100 μM, respectively for 12, 24 and 36 h with or without cortisone (100 nM; this concentration is physiologically relevant). After treatment, the cultured cells were collected and the expression levels of 3β-HSD, CYP21, CYP11A1, CYP11B1, 11β-HSD1 and 11β-HSD2, were evaluated using real-time PCR or Western blotting. Furthermore, cultured epithelial cells were incubated with the 11β-HSD1 inhibitor PF915275 (1 μM) to evaluate the effect of 11β-HSD1 on cortisol production.

Animals

All animal care and experimental procedures were approved by the Committee of Care and Use of Laboratory Animals of Korea University, College of Medicine. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 288 animals were used in the experiments described here.

Adult male Balb/c mice (DBL, Chungcheong Buk-Do, Korea), between 5 and 6 weeks of age, were used in these experiments.Animals were divided into three treatment groups as follows: (1) the baseline control groups (n = 96); (2) the sham-operated control groups (n = 96); and (3) adrenalectomized groups (n = 96). Each group was divided into four subgroups; one subgroup did not receive macrolide antibiotics while other three subgroups were treated with an i.p. injection of clarithromycin (100 mg·kg^{-1}.), azithromycin (50 mg·kg^{-1}), or josamycin (50 mg·kg^{-1}) respectively. The doses used were based on published studies (Hori *et al.*, 1997; Yamamoto *et al.*, 2001; Kamemoto *et al.*, 2009).

Adrenalectomy was performed 2 days after obtaining the animals. The animals were anaesthetized with Ketapex (ketamine, 10 mg per 100 g) and Xylazil (xylazine, 1 mg per 100g). Dorsal midline and bilateral flank muscle incisions were then made, and the adrenal glands were identified and removed. The incisions were sutured and antiseptic treatment with povidone-iodine was applied to the wound daily to prevent infection and aid wound healing. A non-steroidal anti-inflammatory drug (meloxicam; s.c. at 1 mg·kg^{-1} q12 h for 3 days) was used to reduce the pain of the surgical wound postoperatively. The sham-operated mice underwent a similar procedure, except that their adrenal glands were left *in situ*.

Macrolides were injected intraperitoneally into mice once a day for 7 days in each group. A day after the final injection, mice were killed with an over-dose of anaesthetic, nasal mucosa and blood from cardiac puncture were collected, using EDTA as anticoagulant. Blood cells were centrifuged at 1500 x *g* for 10 min at 4°C to obtain plasma.

Plasma and nasal mucosa obtained from each group were stored at −80°C until used. Plasma was used to measure corticosterone, and nasal mucosa was used to analyse the expression levels of steroidogenic enzymes (3β-HSD, CYP21, CYP11B1 and CYP11A1) and steroid converting enzymes (11β-HSD1 and 11β-HSD2) in mouse nasal mucosa.

Nasal mucosa and adrenal glands were also collected for organ culture as previously reported (Cima *et al.*, 2004, Hostettler *et al.*, 2012). Nasal mucosa was rinsed in HBSS supplemented with 2% horse serum and was incubated for 10 min in media containing 1,4-dithiothreitol (1 mM) at 4°C to remove mucus. Thereafter, tissue pieces of nasal mucosa, equally distributed in a 24-well plate, were cultured in culture medium supplemented with 10% fetal calf serum at 37°C. Adrenal glands were cut in half and incubated in a 24-well plate in steroid-free medium. After 8 h, the cell-free supernatant was collected and the corticosterone concentration measured using a commercially available ELISA kit.

Small interfering RNA transfection experiments

Cultured epithelial cells were used to assess the synthesis of cortisol and were plated in 6-well culture dishes at a density of 5 × 10^5 cells per well. They were transfected with small interfering RNA (siRNA) and silencer negative control siRNA (ST Pharm Oligo Center, SH, Kyung Ki Do, Korea), using Lipofectamine 2000. The transfection efficiency of gene knockdown was determined using RT-PCR. Transfected cells were treated as described in the Results section.

Total RNA isolation, RT-PCR and real-time PCR

Total RNA (1 μg) obtained from epithelial cells was reverse-transcribed in a reaction mixture containing M-MLV reverse transcriptase. The primer sequences are listed in Table 1. RT-PCR was conducted to investigate the expression of each enzyme gene, and real-time PCR was performed with an iCycler (BioRad Laboratories, Hercules, CA, USA). Each PCR reaction was performed by the mixture including cDNA, iTAQ SYBR supermix and primers under the parameters (40 cycles × [95°C (15 s)], [60°C (20 s)], and [72°C (15 s)]. All PCR reactions were conducted in triplicate. The level of gene expression was calculated as the difference between the Ct value of the target gene and that of GAPDH used as control. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Sequences of PCR primers

Primer	Sequence
3β-HSD	S: 5′-GCCAATTTACACCTATCGACC-3′
	AS: 5′-TCCACCGTTTTCTGCTTG-3′
CYP17	S: 5′-ATCGGTGAGTTTGCTGTG-3′
	AS: 5′-GCTGGATTCAAGAAACGCTC-3′
CYP21	S: 5′-TCTTCCCGTTCCCCTTAAG-3′
	AS: 5′-TGGAGTTGATTTATTGGCTGG-3′
11β-HSD1	S: 5′-ACACACACACACACACACACAC-3′
	AS: 5′-ACCATGAGGTAGAAGCCACGTGTT-3′
11β-HSD2	S: 5′-ACATTAGCCGCGTGCTAGAGTTCA-3
	AS: 5′-CGCGCCAAAGAAATTCACCTCCAT-3′
CYP11B1	S: 5′-AAGGTGTCAGCAGGTTCTGTGTCT-3′
	AS: 5′-TCTCTGTGAGCTGTCTTGCCCTTT-3′
CYP11A1	S: 5′-ACATCAAGGCCAACGTCACAGAGA-3′
	AS: 5′-TTGCGTGCCATCTCATACAAGTGC-3′
GAPDH	S: 5′-ATCATCCCTGCCTCTACTGG-3′
	AS: 5′-GTCAGGTCCACCACTGACAC-3′
m3β-HSD	S: 5′-TCTCTTTAACCGCCACTTG-3′
	AS: 5′-TCCACTATTGTCCCTATCCAC-3′
mCYP17	S: 5′-AGGAGATTGACCAGTATGTAGG-3′
	AS: 5′-GGAGTCAATGTTAGCCTTGTG-3′
mCYP21	S: 5′-AGAGTCGGGACCATATTGTC-3′
	AS: 5′-CGTCTTTGCCATCCCTTTG-3′
m11β-HSD1	S: 5′-GGGATTGGAAGAGAAATGGC-3′
	AS: 5′-CAGCAATGTAGTGAGCAGAG-3′
m11β-HSD2	S: 5′-GGGTATCAAGGTCAGCATTATC-3
	AS: 5′-CGTGCTCAATGTAGTCTTCAC-3′
mCYP11B1	S: 5′-GGAGACACAAGAGAAAGAGG-3′
	AS: 5′-GCTATTGACATCTTGGGACAC-3′
mCYP11A1	S: 5′-TGGTGTCCTTTATAGCCTCC-3′
	AS: 5′-GCCATCTCATAAAGGTTCCAC-3′
mGAPDH	S: 5′-TTCAACGGCACAGTCAAG-3′
	AS: 5′-AGACTCCACGACATACTCAG-3′

PCR, polymerase chain reaction; 11β-HSD, 11β-hydroxysteroid dehydrogenase; CYP11B1, cytochrome P450, family 11, subfamily B, polypeptide 1; CYP11A1, cytochrome P450, family 11, subfamily A, polypeptide 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CYP17, cytochrome P450c17; CYP21, cytochrome P450c21; 3β-HSD, hydroxy-delta-5-steroid dehydrogenase,; m, mouse.

Western blot analysis

For Western blot analysis, cultured cells were collected and vigorously vortexed in RIPA buffer. The extracted protein (50 μg) mixed in sodium dodecyl sulfate sample buffer was boiled for 5 min, run on 12% sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE) and transferred to Immobilon (Millipore, Bedford, MA, USA). The blots were blocked with PBS-Tween 20 containing 1% skim milk and then incubated with anti-11β-HSD1, anti-11β-HSD2, anti-CYP11B1, anti-3βHSD, anti-CYP21 or anti-CYP11A1 antibodies in PBS-T overnight at room temperature. Antibody reactions were detected using an ECL detection kit (Amersham Bioscience, NJ, USA), followed by detection of chemiluminescence on X-ray film. Relative intensities of each of the protein signals were determined by dividing their intensities by those of β-actin.

ELISA

Cortisol levels in supernatants of cultured cells were measured using ELISA according to the manufacturer's protocol (R&D Systems, Abingdon, UK). Data were expressed as pg cortisol mg^{-1} protein.

Data analysis

Results are shown a s means ± SEM. Statistical analyses were carried out using SPSS for Windows (version 16.0.0; SPSS, Chicago, IL, USA). One-way ANOVA, Bonferroni *post hoc* correction ($\alpha = 0.0167$), and Tukey test were conducted to evaluate the significant difference in data obtained by real-time PCR, Western blot analysis and ELISA among each groups. The Kolmogorov–Smimov test was conducted to determine whether the distribution of data was normal or not. Where distribution was not normal, Mann–Whitney *post hoc* tests were conducted to compare differences between the groups. The statistical significance was set to $P < 0.05$.

Materials

Dulbecco's modified Eagle's medium, Ham's F12 nutrient mixture and bronchial epithelial growth medium were obtained from Lonza Walkersville Incorporation (Walkersville, MD, USA). Penicillin G sodium, streptomycin sulfate, dispase, cortisone, metyrapone and josamycin were obtained from Sigma-Aldrich (Sigma, St. Louis, MO, USA). The 11β-HSD1 inhibitor (PF915275) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Clarithromycin and azithromycin were purchased from Abbott Korea and Pfizer Korea respectively. Povidone-iodine was purchased from Green Pharmacy (Seoul, Korea). Meloxicam was obtained from Korea Boehringer Ingelheim. M-MLV reverse transcriptase was obtained from GIBCO BRL (Grand Island, NY, USA). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). Corticosterone ELISA kit was purchased from Abcam (Cambridge, UK). Ketapex and Xylazil were obtained from Troy Laboratories, Australia.

Results

Effect of macrolide antibiotics on 11β-HSD1, 11β-HSD2, 3β-HSD, CYP21, CYP11A1 and CYP11B1 expression in cultured epithelial cells

To determine whether macrolide antibiotics could upregulate steroid-converting enzymes and steroid-synthesizing enzymes

in cultured epithelial cells, we evaluated the expression levels of 3β-HSD, CYP21, CYP11A1, CYP11B1, 11β-HSD1 and 11β-HSD2 after the addition of a macrolide to cultured epithelial cells. The expression of mRNA and protein of 11β-HSD1, 3β-HSD, CYP21 and CYP11B1 increased time- and dose-dependently after treatment with clarithromycin or azithromycin, whereas their expression levels were not affected in cells treated with josamycin (Figures 1, 2 and 3). The *P* value for each data are as follows; *P* value for dose-response of 11β-HSD1 and CYP11B1 mRNA and protein in cells treated with clarithromycin; 0.0007 and 0.028, 0.0008 and 0.04999 between c and 10 μM, 0.047 and 0.0489, 0.0456 and 0.0408 between 10 and 50 μM, 0.0005 and 0.0478, 0.0218 and 0.0208 between 50 and 100 μM, *P* value for time-response of 11β-HSD1 and CYP11B1 mRNA and protein in cells with clarithromycin; 0.00076 and 0.00032, 0.0019 and 0.0459 between 0 and 12 h, 0.00032 and 0.00121, 0.0299 and 0.0465 between 12 and 24 h, 0.03121 and 0.0389, 0.00198 and 0.00199 between 24 and 36 h, *P* value for dose-response of 11β-HSD1 and CYP11B1 mRNA and protein in cells treated with azithromycin; 0.0356 and 0.0463, 0.0469 and 0.0458 between 10 and 50 μM, 0.0256 and 0.0436, 0.0303 and 0.0411 between 50 and 100 μM, *P* value for time-response of 11β-HSD1 and CYP11B1 mRNA and protein in cells treated with azithromycin; 0.03431 and 0.0469, 0.047 and 0.0318 between 12 and 24 h, 0.04721 and 0.0421, 0.0479 and 0.0403 between 24 and 36 h, *P* value for dose-response of 3β-HSD and CYP21 mRNA in cells treated with clarithromycin; 0.0001 and 0.03 between c and 10 μM, 0.035 and 0.049 between 10 and 50 μM, 0.0174 and 0.047 between 50 and 100 μM, *P* value for dose-response of 3β-HSD and CYP21 mRNA in cells treated with azithromycin; 0.03 and 0.0001 between c and 10 μM, 0.00099 and 0.042 between 10 and 50 μM, 0.0469 and 0.0467 between 50 and 100 μM, *P* value for time-response of 3β-HSD and CYP21 protein in cells treated with clarithromycin; 0.0001 between 0 and 12 h, 0.0001 and 0.0489 between 12 and 24 h, 0.047 and 0.027 between 24 and 36 h, *P* value for time-response of 3β-HSD and CYP21 protein in cells treated with azithromycin, 0.0001 and 0.049 between 12 and 24 h, 0.00169 and 0.0001 between 24 and 36 h. The expression levels of 11β-HSD2 and CYP11A1 even after treatment with these macrolides were not affected in comparison to control (not shown). Cells exposed to vehicles (distilled water for clarithromycin and azithromycin and dimethyl sulfoxide for josamycin) did not show any effects on their expression levels (not shown).

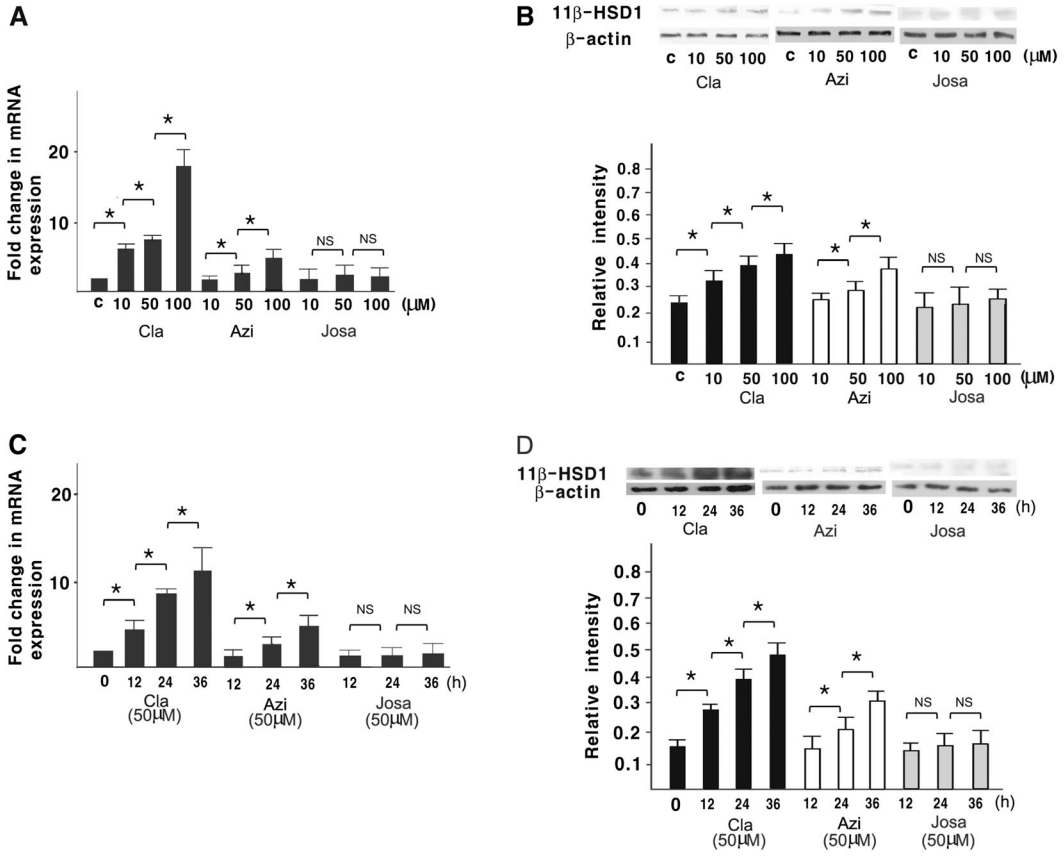

Figure 1

Concentration-dependent effects of macrolides on 11β-HSD1 mRNA and protein expression analysed by real-time PCR (A) and Western blot (B). Cells (n = 6 per treatment group) were treated with each macrolide at concentrations of 10, 50 and 100 μM for 24 h. Time-course effects of macrolide on 11β-HSD1 expression mRNA and protein analysed by real-time PCR (C) and Western blot (D). Cultured cells (n = 6 per group) were treated at concentrations of 50 μM for 0–36 h. Results are expressed as means ± SD. * $P<0.05$, significantly different as indicated; NS, not significant. Cla, clarithromycin; Azi, azithromycin; Josa, josamycin.

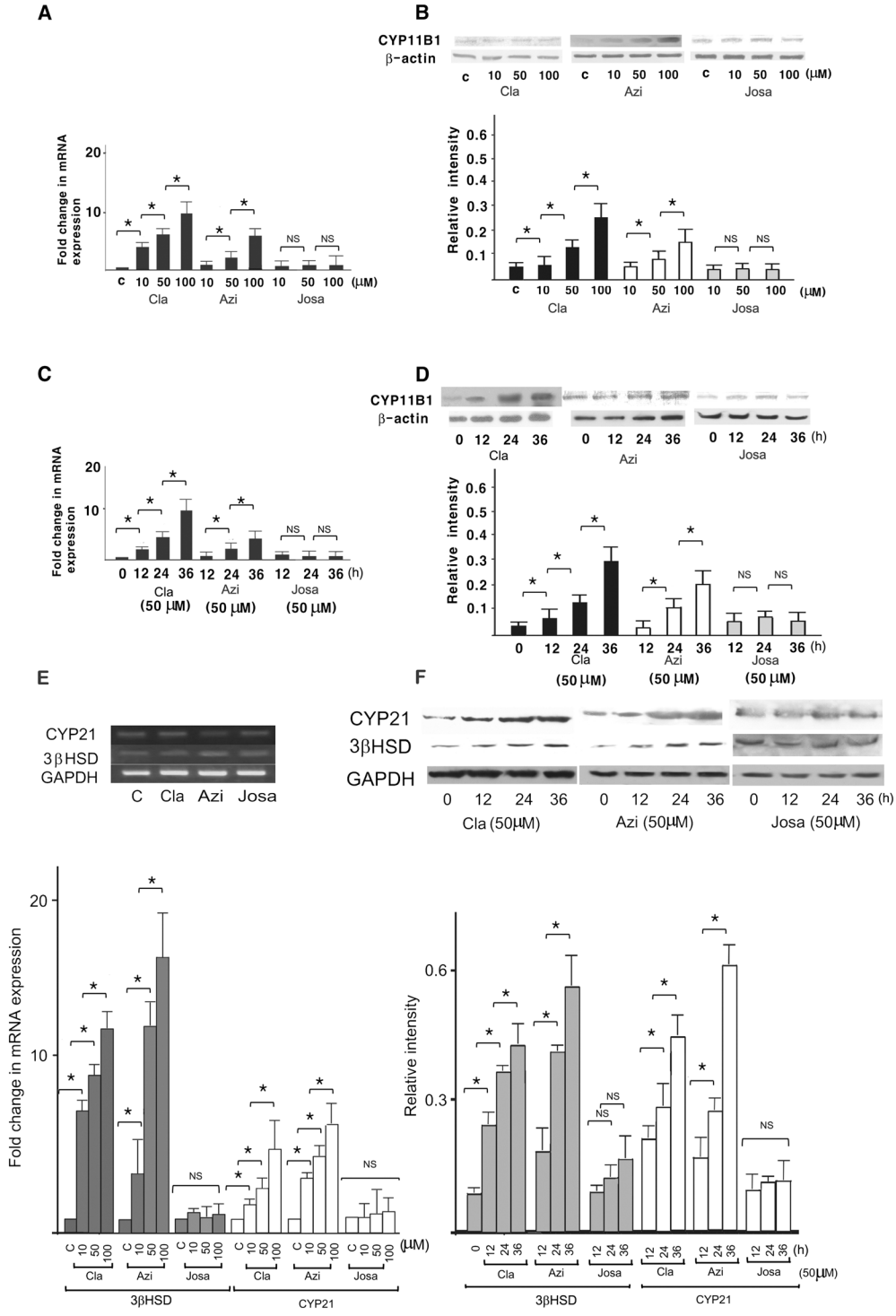

Figure 2

Concentration-dependent effects of each macrolide on CYP11B1 mRNA and protein expression analysed by real-time PCR (A) and Western blot (B). Cells (n = 6 per group) were treated with each macrolide at concentrations of 10, 50 and 100 μM for 24 h. Time-course effects of each macrolide on CYP11B1 mRNA and protein expression analysed by real-time PCR (C) and western blot (D). Cultured cells (n = 6 per treatment group) were treated with 50 μM macrolide, for 0–36 h. Concentration-dependent effects of each macrolide on 3β-HSD and CYP21 mRNA expression analysed by conventional RT-PCR (upper panel) and real-time PCR, and cells (n = 6 per treatment group) were treated with each macrolide at concentrations of 10, 50 and 100 μM for 24 h (E). Time-course effects of each macrolide on 3β-HSD and CYP21 protein by Western blot and cultured cells (n = 6 per group) were treated with 50 μM macrolide for 0–36 h (F). Results are expressed as means ± SD. * $P<0.05$, significantly different as indicated; NS, not significant. Cla, clarithromycin; Azi, azithromycin; Josa, josamycin.

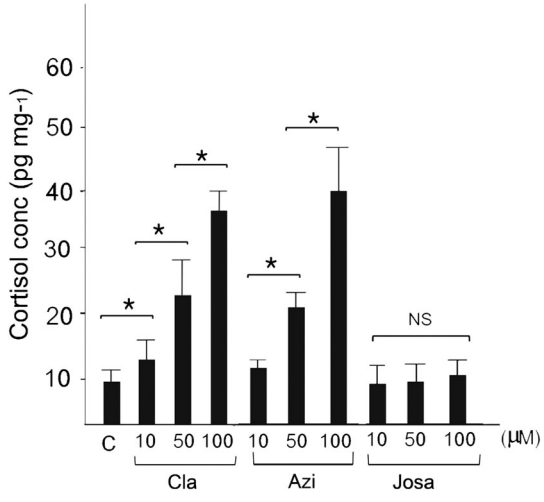

Figure 3

This graph demonstrates the cortisol levels after stimulation with clarithromycin, azithromycin and josamycin, respectively at concentrations of 10, 50 or 100 μM for 24 h in the absence of cortisone (n = 6 per treatment group). Results are expressed as means ± SD. Cla, clarithromycin; Azi, azithromycin; Josa, josamycin; NS, not significant.

Effect of macrolide antibiotics on the cortisol-synthesizing ability of cultured nasal epithelial cells

To evaluate the effect of macrolide antibiotics on the cortisol-synthesizing ability of epithelial cells, epithelial cells were incubated with clarithromycin, azithromycin or josamycin, with or without cortisone. Cortisol levels increased significantly in a dose- and time-dependent manner in cells treated with clarithromycin or azithromycin with or without cortisone, compared with cells without treatment (Figures 3 and 4). *P* value for each data are as follows; *P* value for cortisol levels in cells treated with clarithromycin in the absence of contisone; 0.0459 between control cells and 10 μM, 0.0437 between 10 and 50 μM, 0.0214 between 50 and 100 μM, *P* value for cortisol levels in cells treated with azithromycin in the absence of contisone; 0.00021 between 10 and 50 μM, 0.00063 between 50 and 100 μM, *P* value for cortisol levels in cells stimulated with clarithromycin (50 μM) for 0 and 36 hr with or without cortisone; 0.0016 and 0.0462 between control and 12 h, 0.0459 and 0.00289 between 12 and 24 h, 0.0389 and 0.00236 between 24 and 36 h, *P* value for cortisol levels in cells stimulated with azithromycin (50 μM) for 0 and 36 hr with or without cortisone; 0.00198 and 0.00049 between 12 and 24 h, 0.0335 and 0.0428 between 24 and 36 h. In cells treated with josamycin, cortisol levels were not increased compared with non-treated cells (Figures 3 and 4).

In order to confirm the role of macrolides in the activation of 11β-HSD1, in terms of the modulation of cortisol production, we used siRNA transfection to downregulate the expression of 11β-HSD1. Cells pretreated with 11β-HSD1-specific siRNA produced lower levels of cortisol even in the presence of clarithromycin or azithromycin, compared with cells pretreated with control siRNA in the presence of clarithromycin or azithromycin (Figure 5). When cells were incubated with the 11β-HSD1 inhibitor PF915275 in the presence of clarithromycin or azithromycin, cortisol levels decreased compared with cells without the inhibitor (Figure 5). *P*value for each data are as follows; 0.00001 between cells treated with clarithromycin (10 μM) and cells re-treated with control siRNA and clarithromycin (50 μM), 0.00129 between cells treated with both siRNA and clarithromycin (50 μM), and clarithromycin (100 μM), 0.00011 between cells treated with both siRNA and clarithromycin (50 μM), and cells pretreated with 11β-HSD1-specific siRNA, 0.00012 between cells treated with both

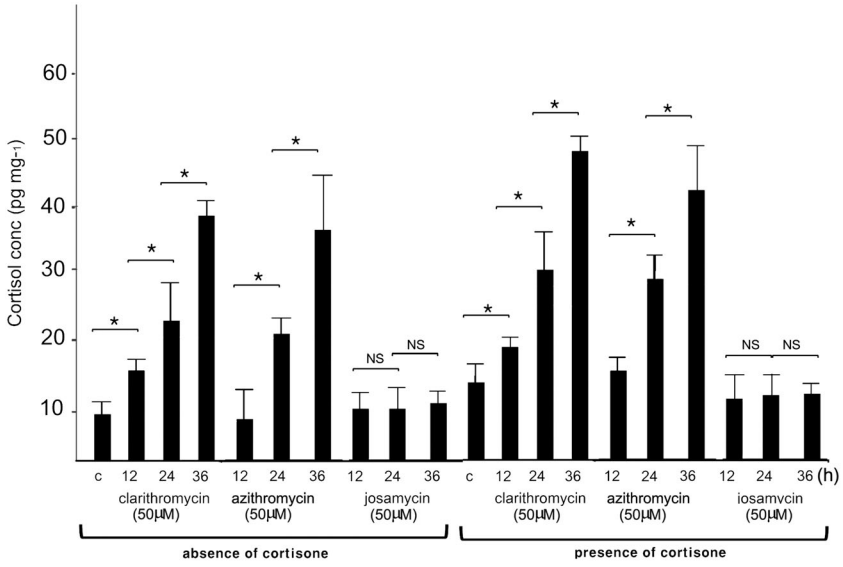

Figure 4

This graph shows the cortisol levels after stimulation for 0–36 h with each macrolide at a concentration of 50 μM in the presence or absence of cortisone (n = 6 per group). Results are expressed as means ± SD. NS, not significant.

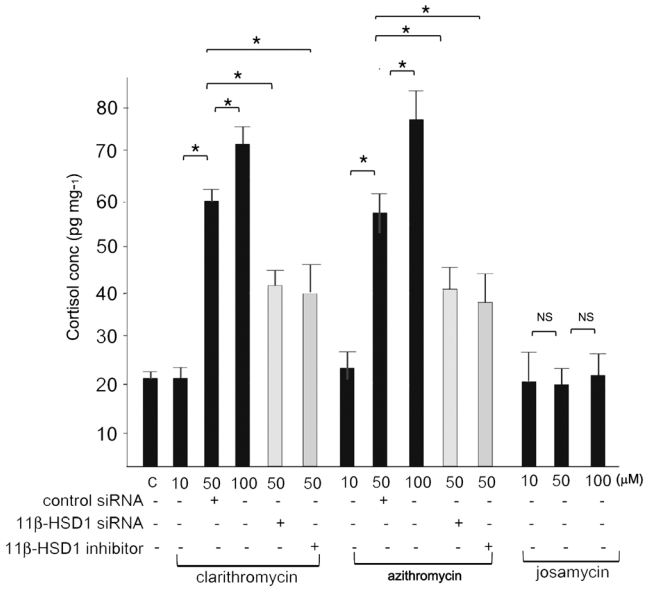

Figure 5

These data show the cortisol levels in cultured epithelial cells treated with clarithromycin (50 μM) or azithromycin (50 μM) for 24 h after inhibition of 11β-HSD1 activity using siRNA technology or the 11β-HSD1 inhibitor (PF915275; 1 μM) (n = 6 per group). The inhibition of 11β-HSD1 activity using 11β-HSD1 siRNA or PF915275 results in decreased cortisol levels. Results are expressed as means ± SD. NS, not significant.

siRNA and clarithromycin (50 μM), and cells treated with PF915275, 0.00001 between cells treated with azithromycin (10μM) and cells treated with both control siRNA and azithromycin (50 μM), 0.00121 between cells treated with both azithromycin (50 μM) and control siRNA and cells treated with azithromycin (100 μM), 0.00011 between cells treated with both azithromycin (50 μM) and control siRNA, and cells pretreated with 11β-HSD1-specific siRNA, 0.00012 between cells treated with both azithromycin (50 μM) and control siRNA, and cells treated with PF915275.

Effect of macrolides on the expression of steroidogenic enzymes and corticosterone-synthesizing ability of nasal mucosa of mice

Steroid-synthesizing enzymes (3β-HSD, CYP11A1, CYP21 and CYP11B1) and steroid-converting enzymes (11β-HSD1 and 11β-HSD2), enzymes required for the biosynthesis of corticosterone from cholesterol, were constitutively expressed within the nasal mucosa of control (Figure 6) and sham-operated mouse (not shown). However, the expression of 3β-HSD, CYP11A1, CYP21, CYP11B1 and 11β-HSD1 were strongly upregulated (3β-HSD (P =0.0001 and P =0.0001 between control and experimental groups treated with clarithromycin or azithromycin), CYP11A1 (P =0.0001 and P =0.0001 between control and experimental groups treated with clarithromycin or azithromycin), CYP21 (P =0.0001 and P =0.0001 between control and experimental groups treated with clarithromycin or azithromycin), CYP11B1 (P =0.0001 and P =0.0001 between control and experimental groups treated with clarithromycin or azithromycin), and 11β-HSD1 (P =0.0001 and P =0.0001 between control and experimental groups treated with clarithromycin or azithromycin) in nasal mucosa of mice treated with clarithromycin or azithromycin, in comparison to those of control and sham-operated mice, while the expression of 11β-HSD2 was not different among the nasal mucosa of control, sham-operated mouse, clarithromycin- and azithromycin-treated mice . These data were also confirmed using Western blot (not shown).

To investigate the role of adrenal glands on the effect of macrolides on the corticosterone production in nasal mucosa of mice, we analysed the expression levels of steroid-synthesizing enzymes and steroid-converting enzymes in nasal mucosa of adrenalectomized animals. However, the expression of 3β-HSD, CYP11A1, CYP21, CYP11B1 and 11β-HSD1 was not affected by clarithromycin or azithromycin in adrenalectomized mice (Figure 6).

We also measured the corticosterone levels in serum and cultured nasal mucosa of control, sham-operated mice and adrenalectomized mice. The results showed that the corticosterone levels were markedly increased in serum of control and sham-operated mice treated with clarithromycin or azithromycin, in comparison to the serum of mice without treatment (P =0.000023 and P =0.00001 between control and experimental groups treated with clarithromycin or azithromycin, P =0.000018 and P =0.00001 between sham-operated group without treatment and sham-operated group treated with clarithromycin or azithromycin). In adrenalectomized mice, the corticosterone levels in serum were strongly decreased, compared with those of control or sham-operated mice and were not increased in adrenalectomized mice treated with clarithromycin, azithromycin or josamycin (Figure 7).

In supernatants derived from cultures of nasal mucosa, the corticosterone levels were also increased in control and sham-operated mice treated with clarithromycin or azithromycin, in comparison to the nasal mucosa of mice without clarithromycin or azithromycin treatment and even in metyrapone-treated nasal mucosa (P=0.012 and P=0.021 between control cells without metyrapone treatment and clarithromycin-treated cells without metyrapone treatment or and azithromycin-treated cells without metyrapone treatment, P=0.0009 and P=0.0008 between control cells with metyrapone treatment and clarithromycin-treated cells with metyrapone treatment or and azithromycin-treated cells with metyrapone treatment, P=0.00201 and 0.00237 between sham-operated cells without metyrapone treatment and sham-operated cells treated with clarithromycin or azithromycin without metyrapone treatment, P=0.0111 and P=0.013 between sham-operated cells with metyrapone treatment and sham-operated cells stimulated with clarithromycin or azithromycin and metyrapone treatment). However, the corticosterone levels in nasal mucosa of adrenalectomized mice remained at low levels, and no significant induction was detected after treatment with clarithromycin or azithromycin (Figure 7).

Discussion and conclusions

In this work, the protein and mRNA expression levels of 3β-HSD, CYP21, 11β-HSD1 and CYP11B1 were increased in a

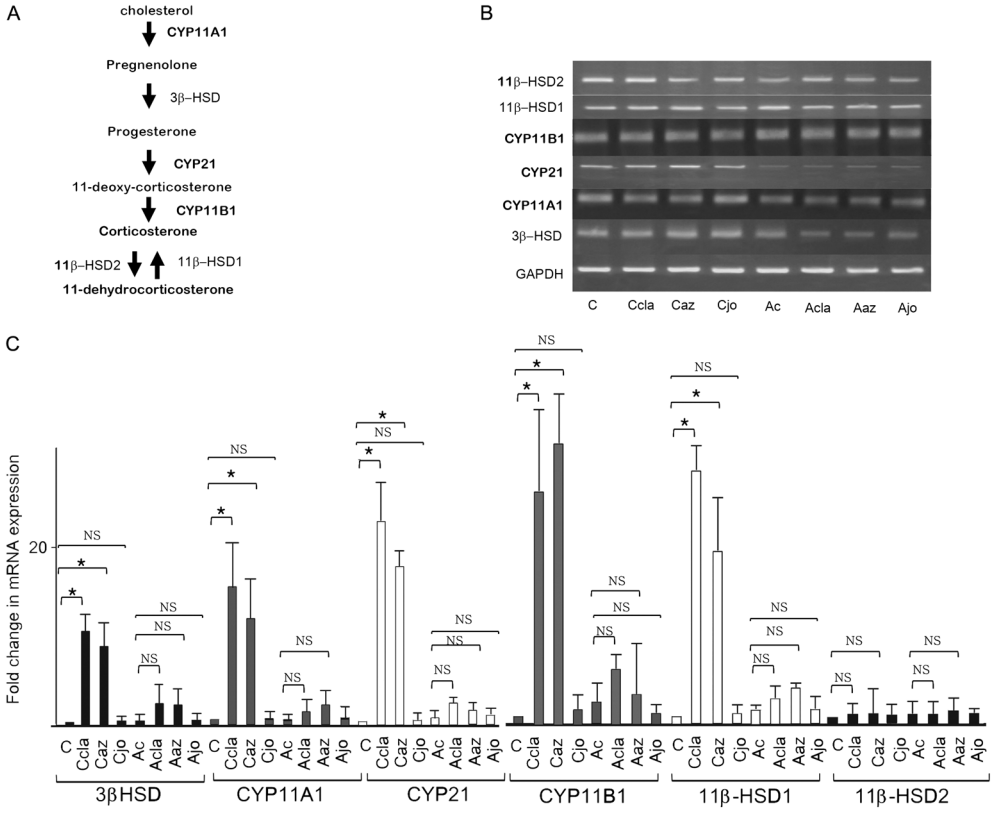

Figure 6

(A) Metabolic pathway demonstrating the production of corticosterone from cholesterol. (B) These panels show the expression of steroidogenic enzymes (CYP11A1, 3β-HSD, CYP21 and CYP11B1) and steroid-converting enzymes (11β-HSD1 and 11β-HSD2) in mouse nasal mucosa evaluated by RT-PCR. (C) This graph demonstrates levels of steroidogenic enzymes (3β-HSD, CYP11A1, CYP21 and CYP11B1) and steroid-converting enzymes (11β-HSD1 and 11β-HSD2) in mouse nasal mucosa evaluated by real-time PCR ($n = 6$ per treatment group). Results are expressed as means ± SD. c, baseline control groups; Ccla, control groups treated with clarithromycin; Caz, control groups treated with azithromycin; Cjo, control groups treated with josamycin; Ac, adrenalectomized groups; Acla, adrenalectomized groups with treated with clarithromycin; Aaz, adrenalectomized groups treated with azithromycin; Ajo, adrenalectomized groups treated with josamycin; NS, not significant.

concentration- and time-dependent manner in cultured epithelial cells treated with clarithromycin (a 14-membered macrolide) or azithromycin (a 15-membered macrolide), as compared with non-treated epithelial cells. Additionally, treatment of cells with these macrolides resulted in increased production of cortisol and these cortisol levels were decreased by the addition of the 11β-HSD1 inhibitor, PF915275. Furthermore, cells in which 11β-HSD1 expression was knocked down showed decreased levels of cortisol even after treatment with these macrolides. However, protein and mRNA expression levels of 3β-HSD, CYP21, 11β-HSD1 and CYP11B1 in cells treated with josamycin (a 16-membered macrolide) were not increased compared with non-treated cells. Collectively, our data indicate that the administration of clarithromycin or azithromycin, the representative agents of 14- and 15-membered macrolides, may be associated with increased expression of 3β-HSD, CYP21, 11β-HSD1 and CYP11B1, contributing to increased production of active cortisol in the epithelial cells of the sinonasal mucosa. In addition, the effect of macrolides on the local glucocorticoid synthesis was evaluated in mouse nasal mucosa. The results showed that the expression levels of steroid-converting and steroid-synthesizing enzymes in nasal mucosa were increased in normal controls and sham-operated groups treated with clarithromycin or azithromycin, while these enzymes were decreased in adrenalectomized groups even after treatment with these macrolides. In parallel with these results, corticosterone production in cultured nasal mucosa was increased in normal control and sham-operated groups treated with clarithromycin or azithromycin but was decreased in adrenalectomized groups treated with these macrolides. Interestingly, our results suggest that adrenalectomy may affect the expression levels of these enzymes in nasal mucosa, implying that local synthesis of these enzymes in nasal mucosa was regulated by the adrenal glands.

Cortisol in humans is synthesized from cholesterol in the adrenal cortex and then secreted in response to ACTH (Chrousos, 1995). There is also evidence suggesting that systemic administration of macrolides can regulate endogenous cortisol levels in plasma (Hori *et al.*, 1996, 1997; Yamamoto *et al.*, 2001). Administration of erythromycin or clarithromycin resulted in increased endogenous glucocorticoid levels in mice by activating the hypothalamo-pituitary-adrenocortical axis (HPA). These results suggested that this

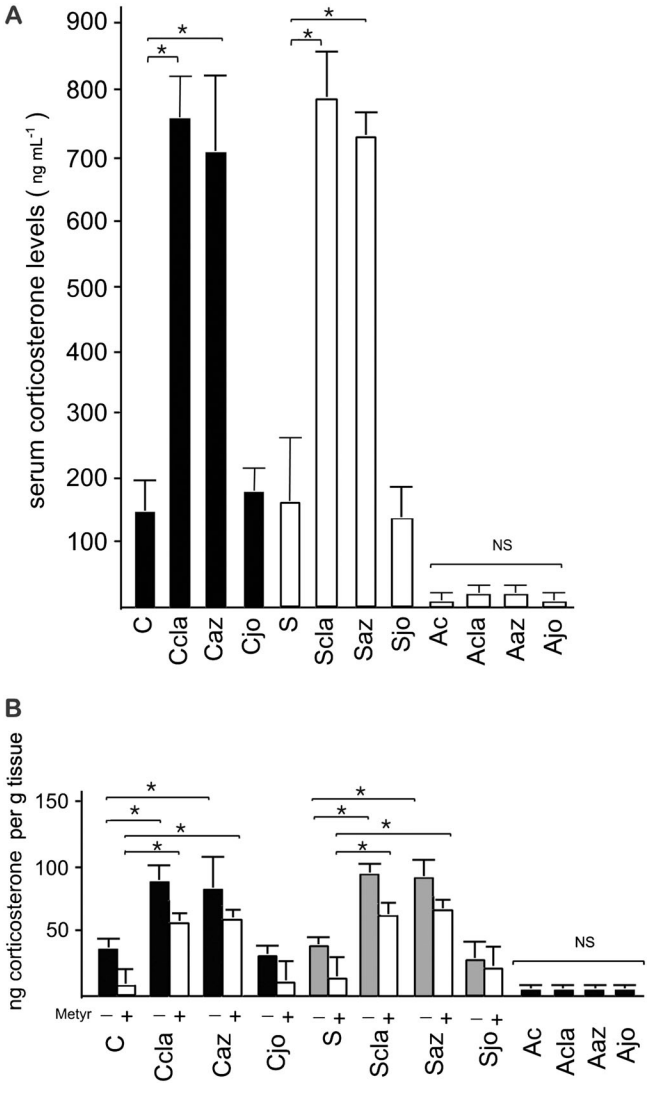

Figure 7

(A) Corticosterone levels in serum of mice (n = 6 per treatment group). (B) Corticosterone synthesis by the nasal mucosa in the presence or absence of metyrapone (250 μg mL^{-1}; n = 6 per group). Results are expressed as means ± SD. c, baseline control groups; Ccla, control groups treated with clarithromycin; Caz, control groups treated with azithromycin; Cjo, control groups treated with josamycin; S, sham-operated control groups; Scla, sham-operated groups treated with clarithromycin; Saz, sham-operated groups treated with azithromycin; Sjo, sham-operated groups treated with josamycin; Ac, adrenalectomized groups; Acla, adrenalectomized groups treated with clarithromycin; Aaz, adrenalectomized groups treated with azithromycin; Ajo, adrenalectomized groups treated with josamycin).

increase in glucocorticoid levels is another action of the drug that could be relevant to the treatment of chronic inflammatory diseases of the respiratory tract (Hori *et al.*, 1996, 1997). Another study also demonstrated that roxithromycin (a 14-membered macrolide) markedly increased endogenous plasma corticosterone levels, which directly affects the HPA (Yamamoto *et al.*, 2001). However, josamycin, one of the 16-membered macrolides, did not affect plasma corticosterone levels, suggesting that the ability of the macrolides to cause corticosterone hyperproduction may be one of the characteristic features of 14-membered macrolides (Yamamoto *et al.*, 2001). Taken together, these results suggest that the anti-inflammatory effect of 14-membered macrolides such as erythromycin, clarithromycin and roxithromycin may be due to their ability to induce corticosteroid production. This suggestion is supported by previous studies showing that macrolides, including erythromycin, have been used as glucocorticoid-sparing agents in the treatment of severe steroid-dependent asthma (Spahn *et al.*, 2001; Reiter *et al.*, 2013).

It is now clear that the effect of endogenous glucocorticoids can be regulated at the level of tissues as well as the HPA axis pathway (Murphy, 1978; Vacchio *et al.*, 1994; Draper and Stewart, 2005; Coste *et al.*, 2007; Noti *et al.*, 2009; Taves *et al.*, 2011; Hostettler *et al.*, 2012; Jun *et al.*, 2014a,b). This is because of the activation of glucocorticoid-modifying enzymes in a range of cell types (Murphy, 1978; Vacchio *et al.*, 1994; Draper and Stewart, 2005; Coste *et al.*, 2007; Noti *et al.*, 2009; Taves *et al.*, 2011; Hostettler *et al.*, 2012; Jun *et al.*, 2014a). In our previous studies, we found that 11β-HSD1 and 11β-HSD2 were expressed in normal sinonasal mucosa, where they are predominantly distributed in superficial epithelium and submucosal glands (Jun *et al.*, 2014a,b). In cultured, normal sinonasal epithelial cells, silencing of 11β-HSD1 using siRNA or PF915275 reduced the level of active cortisol (Jun *et al.*, 2014a,b). 11β-HSD1 levels were increased by exogenous glucocorticoids and inflammatory cytokines, which suggested that an increase in 11β-HSD1 may downregulate inflammatory response through an autocrine feedback-mediated increase of endogenous cortisol levels (Sun and Myatt, 2003; Kaur *et al.*, 2010; Jun *et al.*, 2014a,b). Similar feedback regulation has been identified in several cell types associated with immune responses, including dendritic cells and lymphocytes, where each cell type appears to use 11β-HSD1 in this fashion to attenuate distinct immune responses (Thieringer *et al.*, 2001; Freeman *et al.*, 2005; Zhang *et al.*, 2005). In this work, the expression of 11β-HSD1 is increased in epithelial cells after treatment with clarithromycin or azithromycin and that this increase was in turn associated with increased production of cortisol. Cortisol levels were decreased by silencing 11β-HSD1 with siRNA or by inhibiting it with PF915275 even in the presence of clarithromycin or azithromycin. However, josamycin had no effect on the expression of 11β-HSD1 and endogenous cortisol levels in epithelial cells. Furthermore, the effects of clarithromycin and azithromycin on the expression levels of steroid-synthesizing and steroid-converting enzymes and cortisol production were not significantly different. Therefore, the experiments in our study offer new evidence that 14- and 15-membered macrolides may regulate endogenous cortisol levels in tissue by modulating the expression levels of 11β-HSD1. Taken together, these results suggest that 14- and 15-membered macrolides may function as anti-inflammatory agents by increasing endogenous cortisol levels, locally, in peripheral tissues . However, a recent report revealed that a novel macrolide fluoroketolide restored corticosteroid sensitivity by inhibition of P13K signalling under oxidative stress (Kobayashi *et al*, 2013). Further studies are needed to clarify this possible new mechanism of action of macrolides.

Many studies have investigated the presence of these steroidogenic enzymes in various extra-adrenal organs to evaluate local glucocorticoid synthesis. Their results demonstrate that corticosteroid synthesis occurs in various extra-adrenal organs. Most of the accumulated evidence is from PCR studies of steroid-synthesizing and steroid-converting enzyme mRNA and corticosteroid synthesis *in vitro*. However, the expression of each enzyme vary between the different organs and not consistently regulated by various factors (Cima *et al*., 2004; Hannen *et al*., 2011; Taves *et al*., 2011). Previously, our studies indicated that CYP11B1 and CYP11A1, enzymes relevant to cortisol synthesis, were expressed in normal sinonasal mucosa (Jun *et al*., 2014a,b). The first step in the synthesis of glucocorticoids is the synthesis of pregnenolone from cholesterol, which is catalysed by CYP11A1. The final step of cortisol biosynthesis is performed by CYP11B1, converting 11-deoxycortisol to cortisol (Sewer and Waterman, 2003). Therefore, evidence showing CYP11A1 and CYP11B1 expression supports the formation of glucocorticoids in peripheral tissues. Furthermore, we found that cultured sinus mucosal epithelial cells could synthesize cortisol after stimulation with ACTH, thereby acting as a glucocorticoid-producing organ (Jun *et al*., 2014b). Interestingly, in this work, clarithromycin and azithromycin administration led to an increased expression of 3β-HSD, CYP21 and CYP11B1 in cultured epithelial cells of sinonasal mucosa. Additionally, cortisol levels were increased after treatment with these macrolides in the absence of cortisone. Therefore, we surmise that macrolides may increase cortisol synthesis in nasal epithelium by inducing 3β-HSD, CYP21, 11β-HSD1 and CYP11B1 expression. These results were observed in an animal study that shows that steroid-synthesizing and steroid-converting enzymes are expressed in mouse nasal mucosa, and their levels are increased after administration of clarithromycin or azithromycin. However, in adrenalectomized mice treated with clarithromycin or azithromycin, the expression levels of these enzymes were not upregulated in nasal mucosa. These results are in agreement with other earlier studies demonstrating decreased expression levels of 11β-HSD1 in various organs of adrenalectomized animals (Low *et al*., 1994; Jamieson *et al*., 2000; Nwe *et al*., 2000a,b). Furthermore, endogenous cortisol levels in nasal mucosa were not upregulated in adrenalectomized mice treated with clarithromycin or azithromycin. These results are in accordance with the results of Hostettler *et al*. (2012), demonstrating that adrenalectomy not only eliminated serum glucocorticoids but also prevented anti-CD3-induced local lung glucocorticoid synthesis. Further studies are required to investigate these mechanisms.

Collectively, our present results provide the possibility that macrolide therapy may improve the inflammatory reaction in CRS by increasing the production of endogenous cortisol through regulating the steroid-converting enzymes and steroid-synthesizing enzymes in sinonasal mucosa. Further studies are needed to evaluate the effects of macrolides on the endogenous cortisol levels in patients with CRC.

In conclusion, these results suggest the possibility that the anti-inflammatory and immunomodulatory effects of 14- and 15-membered macrolides result from increased endogenous cortisol levels in superficial epithelium of the healthy sinus mucosa.

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Author contributions

Conception and design were carried out by S J P and S H L.

Development of methodology was carried out by S J P, J H K and S H L.

Acquisition of data was carried out by S J P, H K K, S H K, S H L, H J K, K W K and T H K.

Analysis and interpretation of data were carried out by S J P, H K K, S H K, S H L, H J K, K W K and T H K.

Writing study supervision was carried out by S J P and S H L.

Conflict of interest

There is no conflict of interest.

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