

Differential expression of 11 β -hydroxysteroid dehydrogenase type 1 and 2 in mild and moderate/severe persistent allergic nasal mucosa and regulation of their expression by Th2 cytokines

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Clinical & Experimental Allergy

Summary

Background Glucocorticoids are used to treat allergic rhinitis, but the mechanisms by which they induce disease remission are unclear. 11 β -hydroxysteroid dehydrogenase (11 β -HSD) is a tissue-specific regulator of glucocorticoid responses, inducing the interconversion of inactive and active glucocorticoids.

Objective We analysed the expression and distribution patterns of 11 β -HSD1, 11 β -HSD2, and steroidogenic enzymes in normal and allergic nasal mucosa, and cytokine-driven regulation of their expression. The production levels of cortisol in normal, allergic nasal mucosa and in cultured epithelial cells stimulated with cytokines were also determined.

Methods The expression levels of 11 β -HSD1, 11 β -HSD2, steroidogenic enzymes (CYP11B1, CYP11A1), and cortisol in normal, mild, and moderate/severe persistent allergic nasal mucosa were assessed by real-time PCR, Western blot, immunohistochemistry, and ELISA. The expression levels of 11 β -HSD1, 11 β -HSD2, CYP11B1, CYP11A1, and cortisol were also determined in cultured nasal epithelial cell treated with IL-4, IL-5, IL-13, IL-17A, and IFN- γ . Conversion ratio of cortisone to cortisol was evaluated using siRNA technique, 11 β -HSD1 inhibitor, and the measurement of 11 β -HSD1 activity.

Results The expression levels of 11 β -HSD1, CYP11B1, and cortisol were up-regulated in mild and moderate/severe persistent allergic nasal mucosa. By contrast, 11 β -HSD2 expression was decreased in allergic nasal mucosa. In cultured epithelial cells treated with IL-4, IL-5, IL-13, and IL-17A, 11 β -HSD1 expression and activity increased in parallel with the expression levels of CYP11B1 and cortisol, but the production of 11 β -HSD2 decreased. CYP11A1 expression level was not changed in allergic nasal mucosa or in response to stimulation with cytokines. SiRNA technique or the measurement of 11 β -HSD1 activity showed that nasal epithelium activates cortisone to cortisol in a 11 β -HSD-dependent manner.

Conclusions and Clinical Relevance These results indicate that the localized anti-inflammatory effects of glucocorticoids are regulated by inflammatory cytokines, which can modulate the expression of 11 β -HSD1, 11 β -HSD2, and CYP11B1, and by the intracellular concentrations of bioactive glucocorticoids.

Keywords 11 β -HSD1, 11 β -HSD2, CYP11B1, CYP11A1, mild persistent allergic rhinitis, moderate/severe persistent allergic rhinitis

Submitted 21 February 2013; revised 15 August 2013; accepted 26 August 2013

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Cite this as: Y. J. Jun, S. J. Park, J. W. Hwang, T. H. Kim, K. J. Jung, J. Y. Jung, G. H. Hwang, S. H. Lee and S. H. Lee, *Clinical & Experimental Allergy*, 2014 (44) 197–211.

Introduction

Glucocorticoids regulate various metabolic and homeostatic processes in all tissues of the body, and their effects are controlled at the pre-receptor level by the activity of 11 β -hydroxysteroid dehydrogenase

(11 β -HSD) [1–3]. Two isoforms of 11 β -HSD have been identified, 11 β -HSD1 and 11 β -HSD2. 11 β -HSD1 acts as an oxidoreductase, generating active cortisol from cortisone, which potentiates the actions of glucocorticoids in tissues. The 11 β -HSD2 isoform inactivates cortisol to cortisone. Therefore, 11 β -HSD2 decreases the local

concentration of active glucocorticoids, while 11 β -HSD1 increases the concentration by inducing regeneration of active steroids. Thus, these enzymes play an important role in maintaining homeostasis by regulating local glucocorticoid concentrations [1–3]. Furthermore, changes in 11 β -HSD1 and 11 β -HSD2 expression and activity have been shown to be altered in various inflammatory diseases, suggesting that the reciprocal regulation of 11 β -HSD1 and 11 β -HSD2 expression contributes to the resolution of chronic inflammation by increasing intracellular glucocorticoid action in various tissues, including the lung, intestine, and skin [4–10]. For example, in chronic inflammatory diseases such as Crohn's disease, ulcerative colitis, and arthritis, expression of 11 β -HSD1 is significantly increased in inflamed tissue, whereas 11 β -HSD2 is decreased or absent in the inflamed samples [6, 8]. Several lines of evidences have demonstrated that various inflammatory mediators, including cytokines, modulate the expression of these enzymes in various tissues [4, 9].

Adrenal glands are the major source of endogenous glucocorticoids. However, extra-adrenal glucocorticoid synthesis was initially described in the thymic epithelium, and then, other tissues such as lung, skin, vascular endothelium, and intestinal epithelium have been described to express steroidogenic enzymes including cytochrome P450 11B1 (CYP11B1) and were found to be potential extra-adrenal sources of glucocorticoids [8, 10–18]. The extensive surface epithelial cells in the gastrointestinal tract, lung, and skin are in constant contact with the outside environment and are exposed to a plethora of pathogens, including bacteria and viruses. Thus, local glucocorticoid synthesis occurs in these immunologically important tissues that are sites of exposure to antigen and lymphocyte activation and contributes to the maintenance of immune homeostasis [10, 12, 19, 20]. The nasal mucosa lining the nasal cavity is also constantly exposed to ambient air, including bacteria and viruses, linking the innate and adaptive immune systems [21]. Therefore, it is feasible to hypothesize that nasal mucosa induces local reactivation of active corticosterone and acts as another extra-adrenal source of cortisol, resolving inflammatory processes and limiting tissue damage.

Allergic rhinitis, which is characterized by chronic inflammation, eosinophilic infiltration, nasal hyperresponsiveness to non-specific stimuli, hyperplasia of goblet cells, and subepithelial fibrosis, is an important, clinically prevalent disease world-wide [22]. Clinically, allergic rhinitis is characterized by bothersome symptoms, including rhinorrhea, nasal obstruction, sneezing, and nasal itch. Currently, allergic rhinitis is commonly classified as intermittent or persistent allergic rhinitis, based on the frequency and duration of symptoms irrespective of the nature of allergen exposure [23].

For moderate/severe intermittent or mild persistent as well as moderate/severe persistent allergic rhinitis, glucocorticoids are the first line of treatment. Thus, glucocorticoids are exogenously administered to treat a large number of diseases that require anti-inflammatory activity and are the most commonly used agents to treat allergic rhinitis, due to their ability to attenuate both the nasal inflammatory response and nasal hyper-reactivity [24]. In parallel with the well-established therapeutic effects of exogenously administered glucocorticoids in the treatment of allergic rhinitis, it has been suggested that endogenous glucocorticoids can reduce the severity of experimental allergic reactions in both humans and animals [25–28]. Nevertheless, there is very little information as to whether an endogenous steroidal system exists in human nasal mucosa or whether expression levels of factors involved in this pathway are altered in allergic rhinitis.

Therefore, the present study was undertaken to

- 1 Investigate the expression levels and distribution pattern of 11 β -HSD1 and 11 β -HSD2 in normal, mild, and moderate/severe persistent allergic nasal mucosa, to evaluate whether an endogenous steroidal system is present in allergic nasal mucosa.
- 2 Evaluate the synthesis of endogenous glucocorticoids and the expression of CYP11B1 and CYP11A1, which are enzymes essential for cortisol synthesis, in normal, mild, and moderate/severe persistent allergic nasal mucosa, to evaluate whether nasal mucosa is an extra-adrenal source of glucocorticoids.
- 3 Elucidate whether the expression levels of 11 β -HSD1, 11 β -HSD2, CYP11B1, CYP11A1, and cortisol are altered by stimulation of Th2 cytokines (IL-4, IL-5, and IL-13) and Th17 cytokine (IL-17A) relevant to allergic rhinitis, using an epithelial culture system.
- 4 Determine the functional role of 11 β -HSD1 and 11 β -HSD2 in conversion of cortisone to cortisol using 11 β -HSD1- and 11 β -HSD2-silencing siRNA or 11 β -HSD1 inhibitor.

Methods

Subjects

Healthy nasal mucosa samples ($n = 45$) were obtained from the inferior turbinate of 45 patients who had normal nasal mucosa and were admitted for augmentation rhinoplasty. These patients had no history of nasal infection or allergy, smoking, or ongoing drug treatment. Allergic nasal mucosa samples were obtained from the inferior turbinate of patients with mild ($n = 45$) and moderate/severe persistent allergic rhinitis ($n = 45$). These patients had typical allergy symptoms, nasal eosinophilia, and a positive skin test results or positive RAST to house dust mites. Patients with allergic rhinitis

were classified as mild or moderate/severe persistent according to the allergic rhinitis and its impact on asthma criteria [23]. Exclusion criteria included current smoking, medical history of asthma and recurrent infections of the respiratory tract, and use of anti-allergic drugs during the 8 weeks that preceded the study. Before the tissue specimens were obtained, the Institutional Review Board at our institution approved the protocols, and all participants gave written informed consent.

All tissue samples were divided into three portions: the first and second pieces were frozen in liquid nitrogen and stored at -70°C for RNA and protein isolation, and the third sample was immersed in a fixative containing 4% paraformaldehyde and embedded in paraffin wax for immunohistochemistry. A part of the normal and allergic rhinitis mucosa samples was also used to isolate and culture epithelial cells.

In addition, to evaluate the effects of glucocorticoid on the expression levels of HSD1, HSD2, and Th2 cytokines (IL-4, IL-5, and IL-13) in allergic nasal mucosa, patients with allergic rhinitis were treated with glucocorticoid nasal spray for 4 weeks. Thereafter, nasal mucosa was obtained and was used for real-time PCR and Western blot.

Isolation and culture of epithelial cells

Normal nasal mucosa was treated with 0.5% dispase in a 1 : 1 mixture of DMEM and Ham's nutrient F12 supplemented with penicillin G sodium (50 IU/mL) and streptomycin sulphate (50 $\mu\text{g}/\text{mL}$) overnight at 4°C . Thereafter, nasal epithelial cells were freed from nasal mucosa and collected into a conical tube. The cells were cultured under air-liquid interface in serum-free bronchial epithelial growth medium (BEGM, Lonza Walkersville Inc., Walkersville, MD, USA) containing no bovine pituitary extract and thyroid hormone. Cultured cells were routinely 99–100% positive for cytokeratin staining at the time of harvest and were used for all *in vitro* studies at second passage only. When cells reached 80% confluence, they were stimulated with cytokines (10 and 30 ng/mL of IL-4, IL-5, IL-13, IL-17A, and IFN- γ ; Peprotech, NJ, USA) for 12 and 24 h in the presence or absence of cortisone (100 nM) to measure the effects of cytokines on the expression levels of HSD1 and HSD2 and on the conversion ratio of cortisone to cortisol. In addition, the cultured cells were used to evaluate whether the glucocorticoid and inflammatory cytokines may synergistically affect the expression levels of HSD1 and HSD2.

Sampling of blood and nasal secretion for analysis of cortisol

Blood secretion and nasal secretion were drawn between 08:00 and 09:00 in the morning when the

patients with allergic rhinitis or healthy subjects visited the outpatient clinic at baseline. Nasal lavage was performed by slowly instilling 10 mL sterile isotonic saline into each nostril using a syringe. The solution was retained for approximately 10 s in the nasal cavities and then was expelled by forward flexing the head, rinsing lavage liquid into a sterile plastic beaker. After centrifugation, serum and nasal lavage fluid were stored at -80°C until analysis.

11 β -HSD1 and 11 β -HSD2 small interfering RNA transfection experiments

Cultured epithelial cells were used as an *in vitro* model for functional assay of cortisol production. Small interfering RNA (siRNA) was synthesized as purified duplex (ST pharm Oligo center, SH, Kyung Ki Do, Korea). The sequences of the sense and antisense strands of the human 11 β -HSD1 and 11 β -HSD2 siRNA were as follows: 11 β -HSD1, CCA AAG CAC UGA UAC UUA U (sense), AUA AGU AUC AGU GCU UUG G (antisense), 11 β -HSD2, CCA AGA AAC UGG ACU CCA U (sense), and AUG GAG UCC AGU UUC UUG G (antisense). Cells were plated in 6-well plates at a density of 5×10^5 cells per well and transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Silencer negative control siRNA was introduced into cells by the same method. The efficiency of gene knock down was evaluated by RT-PCR. After transfection, cells were then treated as specified in the results section.

11 β -HSD1 activity assay

To measure 11 β -HSD1 activity in cytokine-treated epithelial cells, confluent epithelial cells were treated with IL-4, IL-5, and IL-13 and then lysed with assay buffer containing 1% Triton X-100 and 1 mM EDTA. The enzyme assays were performed using 50 μg of sample protein incubated for 1 h at 37°C in 600 μL of assay buffer containing 500 μM NADPH and 100 nM cortisone. Thereafter, the samples were placed in a boiling water for 5 min. 11 β -HSD1 activity was then assessed by measuring cortisol production using Cortisol ELISA kit from R&D Systems, and enzyme activity was expressed in as pmol of cortisol produced per milligram of protein.

Total RNA Isolation and real-time polymerase chain reaction (PCR)

Total RNA (1 μg) obtained from frozen nasal mucosa and cultured epithelial cells was reverse-transcribed in 20 μL of a reaction mixture containing 2.5 U of M-MLV reverse transcriptase (GIBCO BRL, Grand Island,

NY, USA). The primer sequences for each gene included in the study are listed in Table 1. Real-time PCR was carried out using an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR included 50 ng cDNA, 12.5 µL iTAQ SYBR supermix, and 200 nM of primers. PCR conditions for 40 cycles were 95°C denaturation for 30 s, 59°C annealing for 30 s, and 72°C extension for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control housekeeping gene. All PCRs were performed in triplicate. Target gene levels were expressed as the fold increase or decrease relative to the expression of GAPDH. The mean value of the replicates for each sample was calculated and expressed as the cycle threshold (Ct). The amount of gene expression was then calculated as the difference between the Ct value obtained for the target gene and that for GAPDH. Relative gene expression was determined by the $2^{-\Delta\Delta C_t}$ method.

Immunohistochemical and Western blot analysis

Immunohistochemical staining was performed using a peroxidase-labelled streptavidin–biotin technique. Briefly, paraffin sections (4 µm) were cut and placed on poly-L-lysine-coated glass slides. The sections were treated with 0.03% H₂O₂ in methanol for 15 min to quench endogenous peroxidase activity. After the sections were washed in phosphate-buffered saline (PBS), they were incubated overnight at 4°C with antibodies at the following dilutions: anti-11β-HSD1, 1 : 100; anti-11β-HSD2, 1 : 100; anti-CYP11B1, 1 : 500; and anti-CYP11A1,

1 : 500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The colour was developed using 3,3'-diaminobenzidine. As a negative control, the primary antibodies were replaced with non-immune goat IgG (Santa Cruz Biotechnology, Inc.).

For Western blot analysis, frozen tissue was crushed and vigorously vortexed in a buffer solution containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and a proteinase inhibitor cocktail. The extracted protein (50 µg) was resuspended in sodium dodecyl sulphate sample buffer and boiled for 5 min. Equal amounts of total protein were separated on 12% sodium dodecyl sulphate–polyacrylamide gels and transferred to Immobilon (Millipore, Bedford, MA, USA). The blots were blocked for 1 h at room temperature with PBS–Tween 20 containing 1% skim milk and then incubated with anti-11β-HSD1, anti-11β-HSD2, anti-CYP11B1, anti-CYP11A1, anti-IL-4, anti-IL-5, anti-IL-13, anti-IL-17A, or anti-IFN-γ antibodies in PBS-T overnight at room temperature. Antibody reactions were detected using the ECL detection kit (Amersham Bioscience, NJ, USA), followed by detection of chemiluminescence on X-ray film. As an internal control, β-actin expression was analysed in parallel blots using the β-actin antibody (Sigma, St. Louis, MO, USA). The intensity of detected bands was quantified using Scion Image Beta 4.0.2 (Scion Corporation, Frederick, MD, USA). Relative intensities of each of the protein signals were obtained by dividing their intensities by those of β-actin.

Assessment of cortisol levels by ELISA

Cortisol levels in normal, mild, and moderate/severe persistent allergic nasal mucosa and cultured cells were measured using ELISA following the manufacturer's protocol (R&D systems, Abingdon, UK). Furthermore, cultured epithelial cells were incubated with cortisone (100 nM; Sigma), ACTH (10 nM; Sigma), 11β-HSD1 inhibitor (1 µM; Merck Millipore, MA, USA), and glucocorticoid (0.5 µM) according to the experimental protocol specified in the results section. Cultured cells were incubated with the above-mentioned compounds, together or in isolation, and then, cortisol levels were measured with cortisol parameter assay kits (R&D system). Data were expressed as ng cortisol/mg protein.

Statistical analyses

Statistical analyses were carried out using SPSS for Windows (version 16.0.0; SPSS, Chicago, IL, USA). Age differences among the 3 groups were calculated by one-way ANOVA, and the Bonferroni *post hoc* correction was applied. The difference in duration of rhinitis between the mild and moderate/severe persistent rhinitis groups was statistically evaluated using the

Table 1. Sequences of PCR primers

Primer	Size (bp)	Sequence
HSD1	171	S: 5'- ACACACACACACACACACACAC-3' AS: 5'- ACCATGAGGTAGAAGCCACGTGTT -3'
HSD2	158	S: 5'- ACATTAGCCGCTGCTAGAGTTCA-3' AS: 5'- CGCGCCAAAGAAATTCACCTCCAT-3'
CYP11B1	109	S: 5'- AAGGTGTCAGCAGGTCTGTGTCT-3' AS: 5'- TCTCTGTGAGCTGTCTTGCCTTT -3'
CYP11A1	91	S: 5'- ACATCAAGGCCAACGTACAGAGA-3' AS: 5'- TTGCGTGCCATCTCATACAAGTGC-3'
IL-4	169	S: 5'-TTGCTGCTCCAAGAACACAAGTGC-3' AS: 5'-TTCTGTCTGAGCCGTTTCAGGAAT-3'
IL-5	83	S: 5'-TAGCTCTTGAGAGCTGCCTACGTGTAT-3' AS: 5'-AAGCAGTGCCAAGGTCTCTTTCAC-3'
IL-13	120	S: 5'-TGGTCAACATCACCCAGAACCAGA-3' AS: 5'-AGCCTGACACGTTGATCAGGGATT-3'
IL-17A	144	S: TCACAATCCACGAAATCCAG AS: GTGAGGTGGATCGGTTGTAG
IFN-γ	182	S: 5'-TGCAGGTCATTAGATGTAGCGGA-3' AS:5'-TGTCTTCTTGATGGTCTCCACACTC-3'
GAPDH	122	S: 5'-ATCATCCCTGCCTCTACTGG-3' AS: 5'-GTCAGGTCCACCACTGACAC-3'

Mann–Whitney *U*-test. A one-way ANOVA, the Bonferroni *post hoc* correction ($\alpha = 0.0167$), and the Tukey test were performed to evaluate whether values obtained by real-time PCR, Western blot analysis, and ELISA significantly differ among the nasal mucosa of healthy controls and patients with mild and moderate/severe persistent allergic rhinitis. The level of significance was set at $P < 0.05$. Mann–Whitney's *post hoc* tests were performed to compare differences between the groups.

Results

Clinical characteristics of the subjects

The characteristics of patients with allergic rhinitis and healthy controls are shown in Table 2. Age was similarly distributed among the healthy controls and the patients with allergic rhinitis. The duration of disease did not significantly differ between the groups of patients with mild and moderate/severe persistent allergic rhinitis (Table 2). Serum total IgE levels were higher in patients with mild and moderate/severe persistent allergic rhinitis than in individuals with healthy nasal mucosa (Table 2).

Expression levels and distribution patterns of 11 β -HSD1, 11 β -HSD2, Th2 cytokine (IL-4, IL-5, IL-13), IL-17A, and IFN- γ in nasal mucosa

The expression levels of 11 β -HSD1, 11 β -HSD2, IL-4, IL-5, IL-13, IL-17A, and IFN- γ were determined in healthy, mild, and moderate/severe persistent allergic nasal mucosa using real-time PCR and Western blot (Fig. 1). The expression levels of 11 β -HSD1 were significantly increased in nasal mucosa of patients with mild and moderate/severe persistent allergic nasal mucosa compared with healthy nasal mucosa, whereas 11 β -HSD2 expression was decreased in patients with allergic rhinitis (Fig. 1). However, no significant difference was observed in the expression levels of either isoenzyme between mild and moderate/severe persistent allergic nasal mucosa (Fig. 1). The expression of

11 β -HSD1 and 11 β -HSD2 was also detected in cultured human nasal epithelial cells (Fig. 3).

The expression levels of Th2 cytokine (IL-4, IL-5, and IL-13) and Th17 cytokine (IL-17A) were significantly increased in mild and moderate/severe persistent allergic nasal mucosa, whereas IFN- γ expression levels were not different among three groups (Fig. 1).

Immunohistochemical staining showed a similar pattern in healthy nasal, mild, and moderate/severe persistent allergic nasal mucosa. In both healthy and allergic nasal mucosa, expression of 11 β -HSD1 and 11 β -HSD2 was exclusively localized to the superficial epithelium, submucosal glands, and vascular endothelial cells (Fig. 2a–f). These expressions were completely inhibited in the negative immunohistochemical control where primary antibodies were omitted and replaced with normal goat IgG (data not shown).

Effects of cytokine, glucocorticoid, and combined cytokine and glucocorticoid treatment on 11 β -HSD1 and 11 β -HSD2 expression in cultured epithelial cells

We examined the expression levels of 11 β -HSD1 and 11 β -HSD2 after stimulation with inflammatory cytokines in human nasal epithelial cell cultures using real-time PCR and Western blots (Fig. 3). Expression level of 11 β -HSD1 was increased 12 or 24 h after stimulation with IL-4, IL-5, IL-13, and IL-17A at concentrations of 10 or 30 ng/mL, compared with non-treated control (Fig. 3a,b). However, expression levels of 11 β -HSD2 decreased after treatment with IL-4, IL-5, IL-13, and IL-17A (Fig. 3c,d). However, IFN- γ has no effects on the expression levels of 11 β -HSD1 and 11 β -HSD2 (Fig. 3).

Dexamethasone, when used in isolation, increased the 11 β -HSD1 expression levels in cultured epithelial cells. However, the combination of dexamethasone and each cytokine induced dramatically increased expression of 11 β -HSD1 relative to either individual treatment (Fig. 4a,b).

In addition, 11 β -HSD1 activities were measured in cultured epithelial cells in the presence or absence of Th2 cytokines. As shown in Fig. 4c, IL-4, IL-5, and IL-13 increased the 11 β -HSD1 enzyme activities.

Expression of cortisol, CYP11B1, and CYP11A1, in normal, mild, and moderate/severe allergic rhinitis

To investigate the possible glucocorticoid synthesis in human nasal mucosa, we utilized multiple approaches to determine whether steroidogenic enzymes, CYP11B1 and CYP11A1, are expressed in human nasal mucosa and epithelial cells. We confirmed the expression of CYP11B1 and CYP11A1 transcripts in human nasal mucosa and cultured epithelial cells using real-time PCR (Figs 1a, 3e,g). To determine protein levels, we

Table 2. Clinical characteristics of patients

Characteristics	Healthy nasal mucosa (<i>n</i> = 45)	Mild allergic rhinitis (<i>n</i> = 45)	Moderate/severe allergic rhinitis (<i>n</i> = 45)
Female/Male	22/23	23/22	27/15
Age (years)	35.7 $\pm$ 3.7	38.9 $\pm$ 3.5	39.9 $\pm$ 9.8
Rhinitis duration (years)	NA	13.68 $\pm$ 1.3	17.55 $\pm$ 3.63
Serum total IgE (IU/mL)	20.99 $\pm$ 13.23	356.33 $\pm$ 162.75	377.64 $\pm$ 110.76

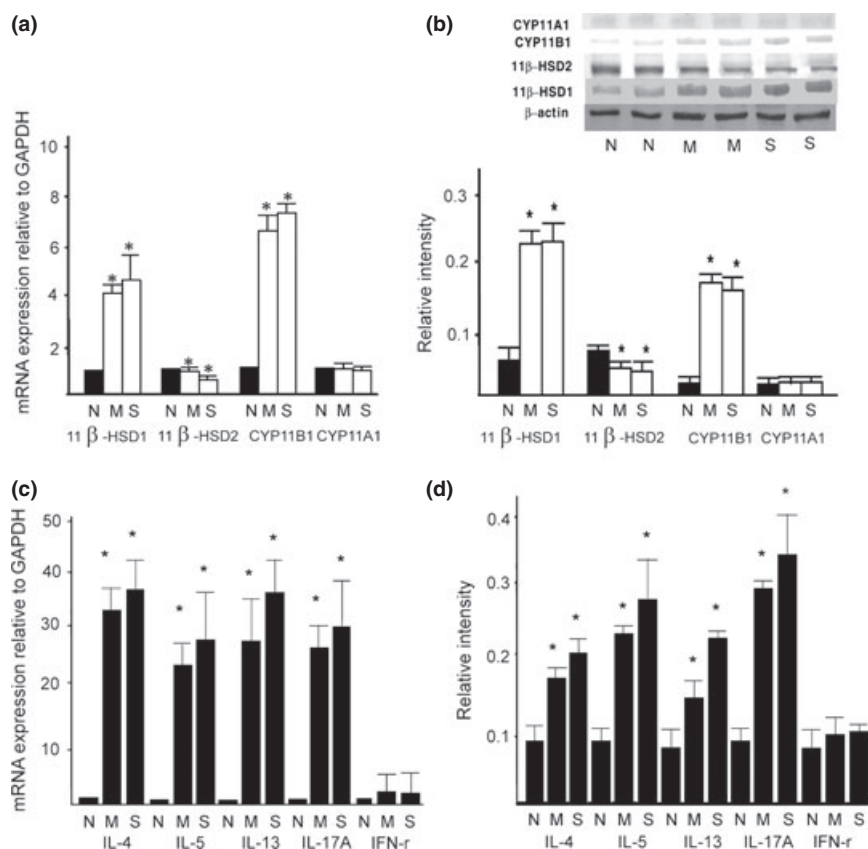


Fig. 1. Real-time PCR (a, c) and Western blot analysis (b, d) of 11β-HSD1, 11β-HSD2, CYP11B1, CYP11A1, IL-4, IL-5, IL-13, IL-17A, and IFN-γ expression in normal (N: $n = 30$), mild (M: $n = 30$), and moderate/severe persistent allergic nasal mucosa (S: $n = 30$). * indicates significant difference in the expression levels of 11β-HSD1, 11β-HSD2, CYP11B1, CYP11A1, IL-4, IL-5, IL-13, IL-17A, and IFN-γ among normal, mild, and moderate/severe persistent allergic nasal mucosa ($P < 0.05$). Results are expressed as mean $\pm$ SD. The panels of Western blot show representative data.

used both immunohistochemistry and Western blot. We found that CYP11B1 is present in normal human nasal mucosa and cultured epithelial cells, and its expression levels are altered in allergic nasal mucosa and increased after stimulation with Th2 cytokines and IL-17A (Figs 1b, 3f). CYP11A1 is also present in human nasal mucosa and cultured epithelial cells, but its expression levels are not changed in allergic nasal mucosa or even after treatment with cytokines (Figs 1b, 3h). The expression pattern of CYP11B1 mRNA and protein was similar to that of 11β-HSD1, with increased expression in mild and moderate/severe persistent allergic nasal mucosa compared with that in normal nasal mucosa, and there were no significant differences in its expression levels between mild and moderate/severe persistent allergic nasal mucosa (Fig. 1a,b). However, the expression levels of CYP11A1 mRNA and protein in allergic rhinitis were not changed in comparison with those in normal nasal mucosa (Fig. 1a,b). CYP11B1 and CYP11A1 were distributed in the superficial epithelium, submucosal glands, and vascular endothelium in normal, mild, and moderate/severe persistent allergic nasal mucosa

(Fig. 2g–l). The negative immunohistochemical controls did not exhibit any staining (data not shown). In cultured epithelial cells, the expression levels of CYP11B1 were increased after the stimulation with IL-4, IL-5, IL-13, and IL-17A, compared with non-stimulated epithelial cells (Fig. 3e,f). In contrast, CYP11A1 expression levels were not affected by stimulation with IL-4, IL-5, IL-13, or IL-17A (Fig. 3g,h).

To confirm that normal human nasal mucosa indeed produce glucocorticoids, we isolated proteins from cultured epithelial cells, normal, and allergic human nasal mucosa and measured cortisol production by ELISA. Moreover, cortisol levels were measured in nasal lavage fluid and serum obtained from control subjects, and patients with mild and moderate severe persistent allergic rhinitis. The cortisol levels were detected in normal nasal mucosa, and its levels were significantly increased in mild and moderate/severe persistent allergic nasal mucosa (Fig. 5a). However, cortisol levels did not differ between mild and moderate/severe persistent allergic nasal mucosa (Fig. 5a). Interestingly, cortisol levels in nasal lavage fluid increased significantly in mild and

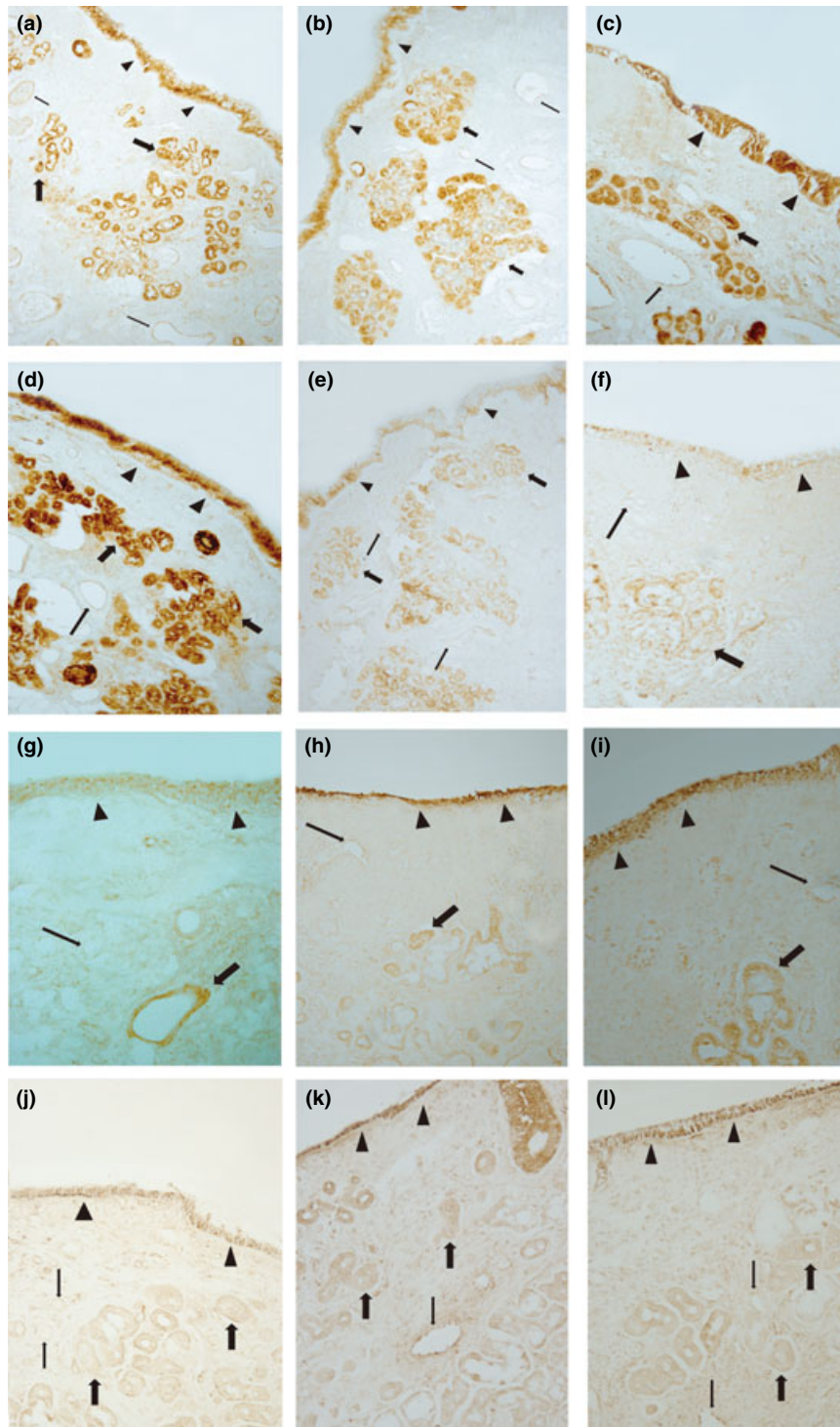


Fig. 2. Immunohistochemical localization of 11 β -HSD1 (a, b, c), 11 β -HSD2 (d, e, f), CYP11B1 (g, h, i), and CYP11A1 (j, k, l) in normal (a, d, g, j), mild (b, e, h, k), and moderate/severe persistent allergic nasal mucosa (c, f, i, l). In normal, mild, and moderate/severe persistent allergic nasal mucosa, 11 β -HSD1, 11 β -HSD2, CYP11B1, and CYP11A1 are detected in the surface epithelium (arrow head), submucosal glands (thick arrow), and vascular endothelium (thin arrow). Original magnification $\times 100$.

moderate/severe persistent allergic rhinitis, compared with control subjects, whereas cortisol levels in serum were not different among control subjects, mild, and moderate/severe persistent allergic rhinitis (Fig. 5b,c).

The role of the nasal epithelium in modulating cortisol bioavailability was examined by comparing the levels of cortisol accumulation in the incubation medium at 12 and 24 h following exposure to a physiologically

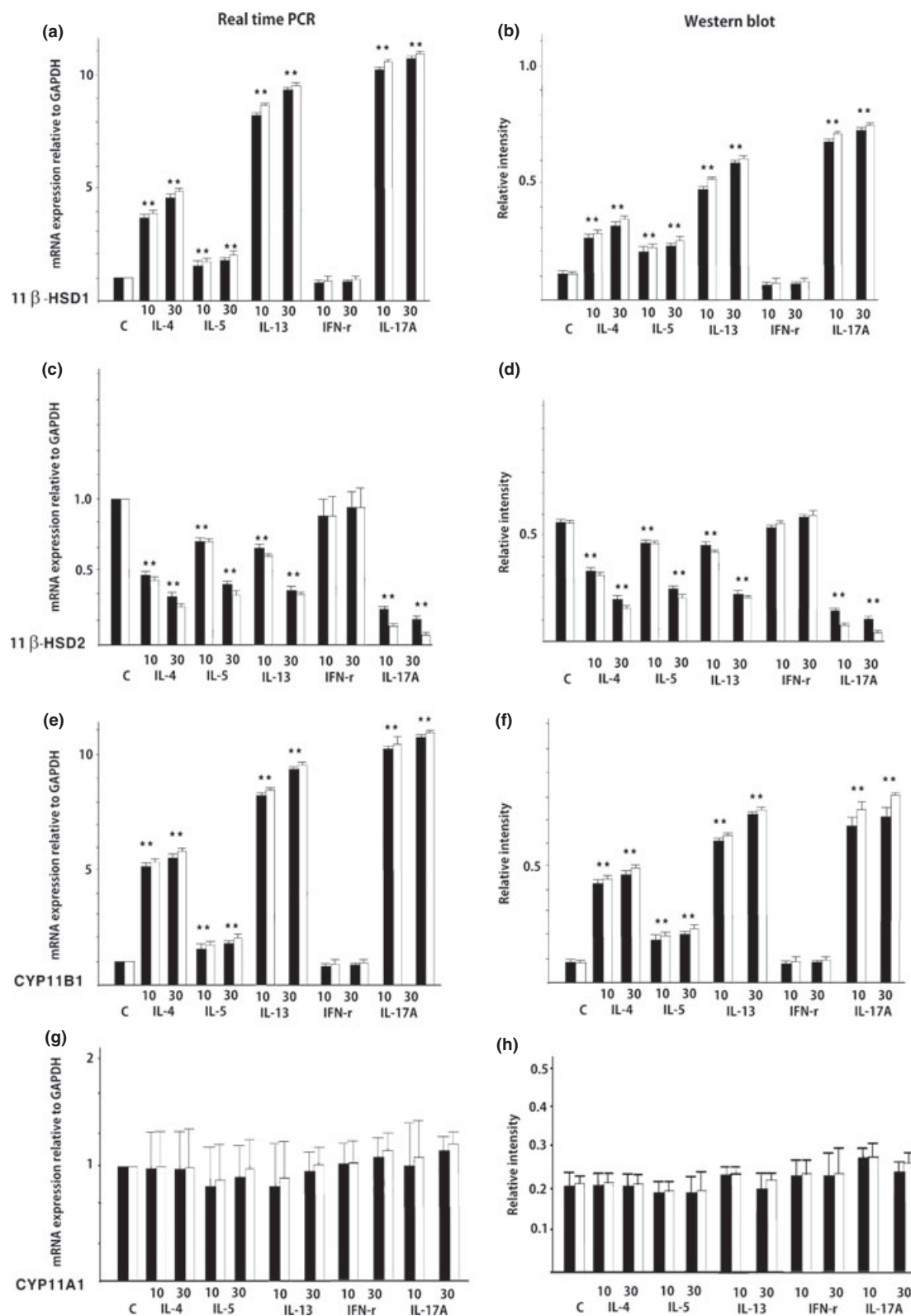


Fig. 3. Real-time PCR (a, c, e, g) and Western Blot analysis (b, d, f, h) of 11 β -HSD1 (a, b), 11 β -HSD2 (c, d), CYP11B1 (e, f), and CYP11A1 (g, h) expression levels in cultured epithelial cells after stimulation with IL-4, IL-5, IL-13, IFN- γ , and IL-17A (10 and 30 ng/mL), respectively, for 12 or 24 h. Black bar indicates stimulation for 12 h ($n = 6$), white bar indicates stimulation for 24 h ($n = 6$). Results are expressed as mean $\pm$ SD. * indicates statistical significant difference in 11 β -HSD1, 11 β -HSD2, and CYP11B1 expression levels between cytokine-stimulated cells and non-stimulated controls (C).

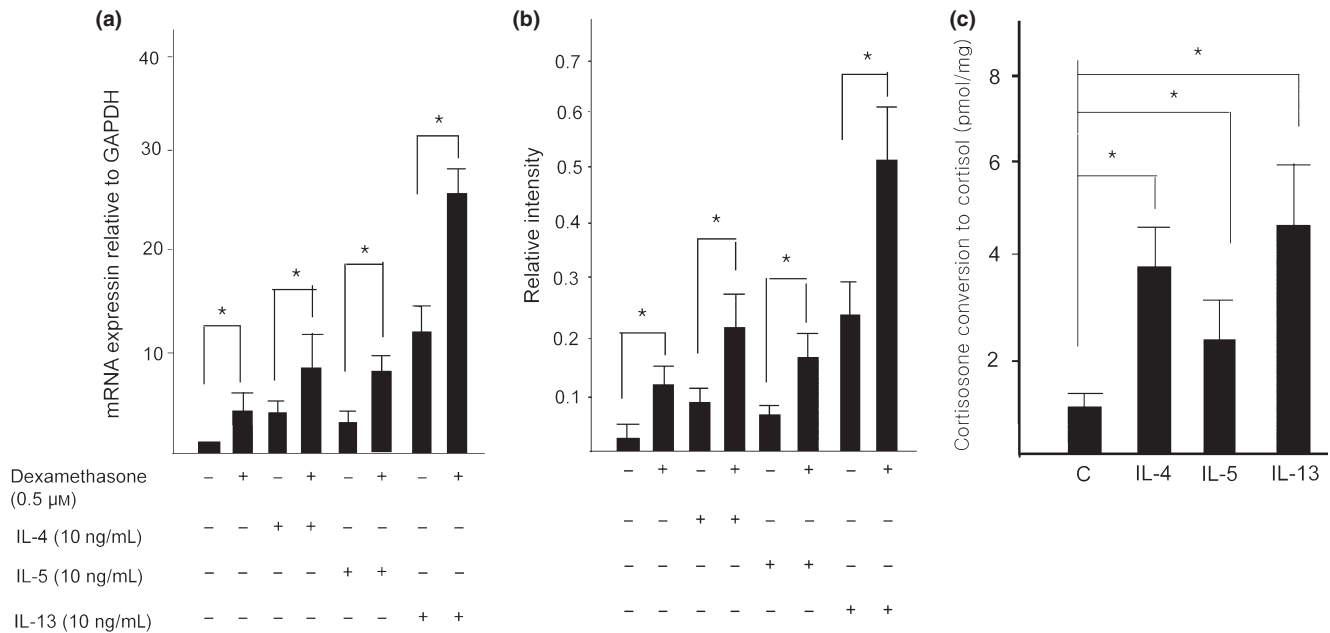


Fig. 4. Real-time PCR (a) and Western blot analysis (b) of 11 β -HSD1 expression levels in cultured epithelial cells after stimulation of Th2 cytokines (IL-4, IL-5, and IL-13) either alone or in combination with dexamethasone for 24 h. (c) 11 β -HSD1 activity measured after stimulation with IL-4 (10 ng/mL), IL-5 (10 ng/mL), and IL-13 (10 ng/mL), respectively, in the presence of cortisone (100 nM). * indicates statistically significance difference ($P < 0.05$).

relevant concentration of cortisone (100 nM), both in the absence and in the presence of pre-treatment of cytokines. The cortisol levels were also found in non-stimulated cultured epithelial cells and were increased 12 and 24 h after stimulation with IL-4, IL-5, IL-13, and IL-17A, at concentrations of 10 or 30 ng/mL in cultured epithelial cells in the presence or absence of cortisone (Fig. 5d).

Evaluation of cortisol-synthesizing ability in human nasal epithelial cells and regulation of cortisol levels through HSD1 activity

The present study evaluated whether human nasal epithelial cells were capable of producing cortisol. Cells were cultured with ACTH, 11 β -HSD1 inhibitor, or both. Cortisol levels were significantly increased in cells treated with ACTH, compared with non-stimulating cells. But when cells were treated with 11 β -HSD1 inhibitor in the presence or absence of ACTH, cortisol levels were decreased compared with cells treated with ACTH alone (Fig. 6a). These data show that nasal epithelial cells are not only able to activate cortisone to cortisol but also respond to stimulation with ACTH by initiating de novo cortisol production.

To evaluate whether human nasal epithelial cells metabolize cortisol, human nasal epithelial cells were incubated with cortisone alone or in the presence of 11 β -HSD1 inhibitor. In cells treated with

cortisone alone, the level of cortisol increased, but when pre-treated with 11 β -HSD1 inhibitor, the cortisol levels were decreased in comparison with cells incubated with cortisone alone. These findings indicate that human nasal epithelial cells can convert cortisone to cortisol through 11 β -HSD1 enzyme activity (Fig. 6b).

To confirm the role of 11 β -HSD1 and 11 β -HSD2 in cortisol activation and deactivation, we employed siRNA technology in the presence or absence of cortisone. Cells silenced with 11 β -HSD1-specific siRNA produced lower levels of cortisol when compared to controls, whereas in 11 β -HSD2-silenced cells, cortisol levels were increased in comparison with controls. These data indicate that both 11 β -HSD enzymes can regulate the local concentration of cortisol in human nasal epithelial cells (Fig. 6c).

Effects of glucocorticoid treatment on the expression levels of 11 β -HSD1, 11 β -HSD2, and Th2 cytokines (IL-4, IL-5, and IL-13) in patients with allergic rhinitis

The expression levels of 11 β -HSD1, 11 β -HSD2, and Th2 cytokines (IL-4, IL-5, and IL-13) were measured in nasal mucosa of patients with allergic rhinitis after treatment with intranasal steroid spray for 1 month. Their expression levels were not significantly different among normal controls, mild, and moderate/severe persistent allergic rhinitis (Fig. 7a–d).

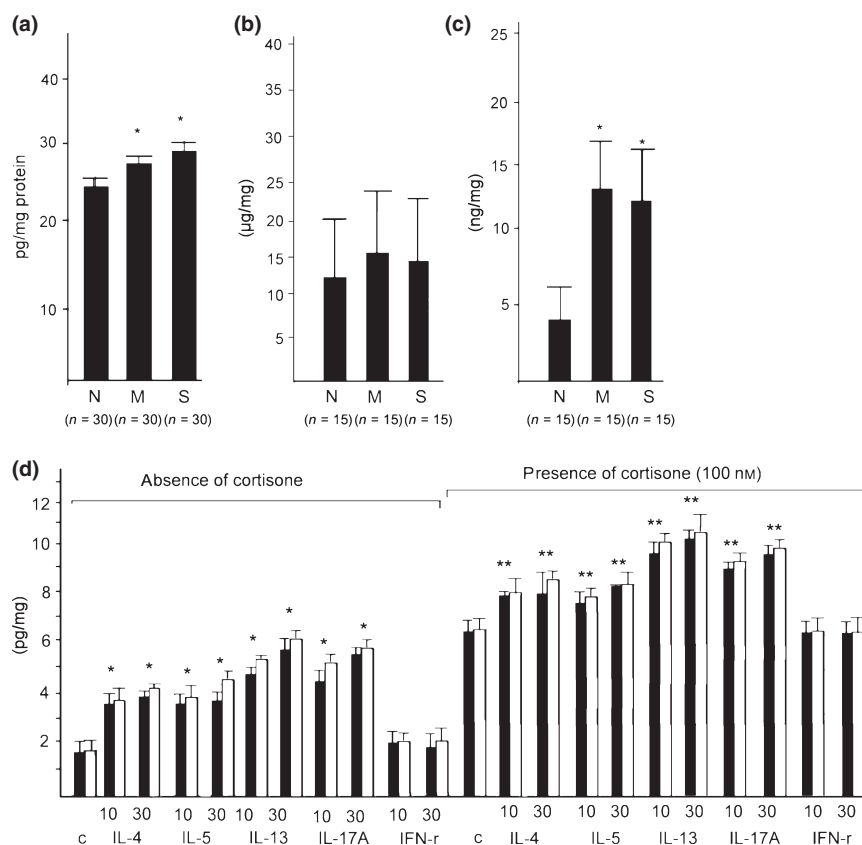


Fig. 5. Analysis of cortisol levels in nasal mucosa (a), serum (b), and nasal lavage fluid (c) of normal subjects (N) and patients with mild (M) and moderate/severe persistent (S) allergic nasal mucosa. * indicates statistical significant difference in cortisol levels among normal, mild, and moderate/severe persistent allergic nasal mucosa. (b) Analysis of cortisol levels in cultured epithelial cells after stimulation with IL-4, IL-5, IL-13, IL-17A, and IFN- γ (10 and 30 ng/mL), respectively, for 12 or 24 h in the presence of cortisone. Black bar indicates stimulation for 12 h ($n = 6$), and white bar indicates stimulation for 24 h ($n = 6$). Results are expressed as mean $\pm$ SD. * indicates statistically significant difference in cortisol levels between cytokine-stimulated and non-stimulated control groups (c). ** indicates statistically significant difference in cortisol levels between cytokine-stimulated and non-stimulated control groups (c) in the presence of cortisone.

Discussion

In this study, precise cellular localization and expression patterns of 11 β -HSD isoforms were identified in human nasal mucosa, indicating that glucocorticoid regulation by 11 β -HSD isoforms does occur in human nasal mucosa. Furthermore, our data demonstrated that up-regulation of cortisol-activating 11 β -HSD1 is accompanied by down-regulation of cortisol-inactivating 11 β -HSD2 in allergic rhinitis. Interestingly, CYP11B1, a steroidogenic enzyme required for cortisol production, is constitutively expressed in normal human nasal mucosa and is up-regulated in mild and moderate/severe persistent allergic rhinitis. In parallel with these findings, we observed that cortisol is present at levels detectable by ELISA in normal nasal mucosa and is increased in mild and moderate/severe allergic nasal mucosa. Furthermore, our results indicate that these enzymes, which regulate glucocorticoid concentration in human nasal mucosa, are differentially enhanced by cytokines relevant to allergic

rhinitis. Collectively, our results indicate that allergic inflammation is associated with stimulation of 11 β -HSD1 and inhibition of 11 β -HSD2, resulting in increased generation of active glucocorticoids in nasal mucosa.

11 β -HSD1 is present in many tissues including the liver, lung, kidney, testis, brain, fat, and the immune system [29]. 11 β -HSD2 acts as a protector of the non-selective mineralocorticoid receptors by preventing them from being occupied by glucocorticoids and has also been found in kidney, placenta, skin, brain, gastrointestinal tract, thymus, spleen, and lung [30–33]. To our knowledge, this study is the first to demonstrate the expression of both glucocorticoid-metabolizing enzymes (11 β -HSD1 and 11 β -HSD2) in the human nasal mucosa and cultured human nasal epithelial cells. Considering the evidence that 11 β -HSD1 activates cortisone to cortisol and 11 β -HSD2 performs the opposite reaction [29], 11 β -HSD possibly regulates the concentration of glucocorticoids in human nasal mucosa, providing a physiological balance between active cortisol

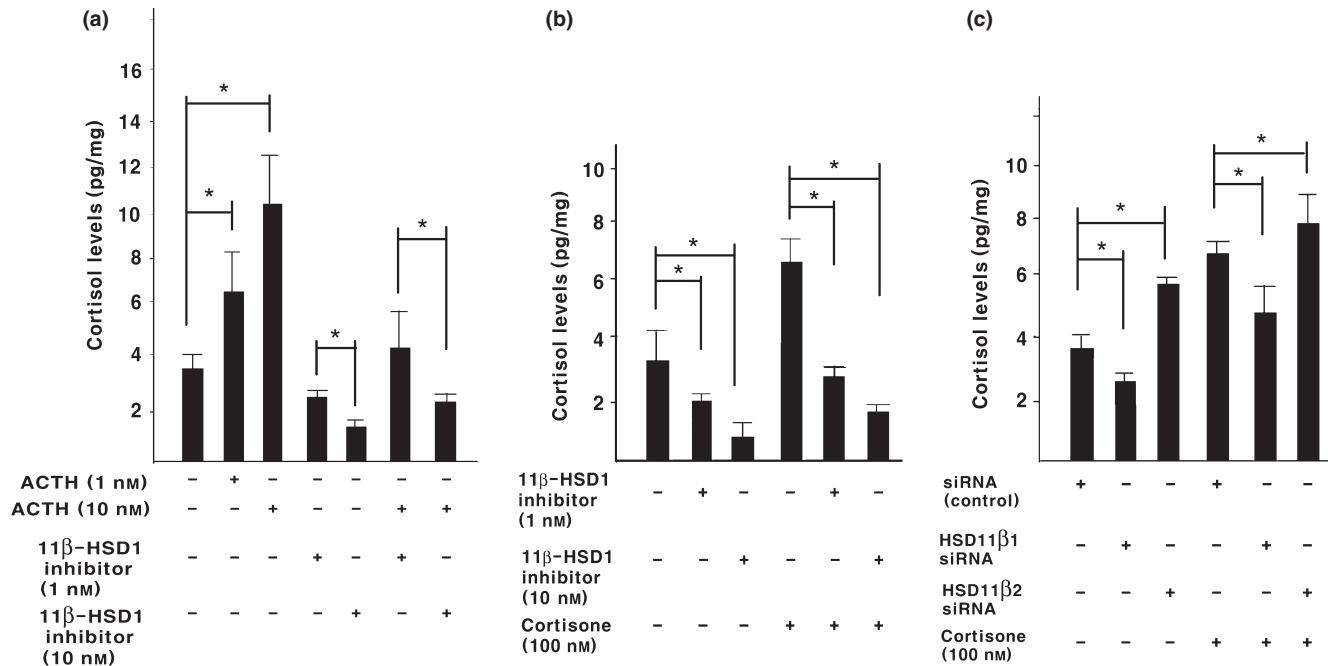


Fig. 6. (a) ACTH induces cortisol synthesis in nasal epithelial cells. Nasal epithelial cells were incubated for 24 h with ACTH (1, 10 nM) in the presence or absence of 11 β -HSD1 inhibitor (1, 10 nM). (b) Nasal epithelial cells were treated for 24 h with cortisone and/or 11 β -HSD1 inhibitor. (c) Nasal epithelial cells were transfected with 11 β -HSD1-specific siRNAs, 11 β -HSD2-specific siRNAs, or scrambled siRNA as control and then incubated with medium in the presence or absence of cortisone. The cortisol levels were measured with ELISA. The results are shown as a mean of six independent experiments. * indicates $P < 0.05$.

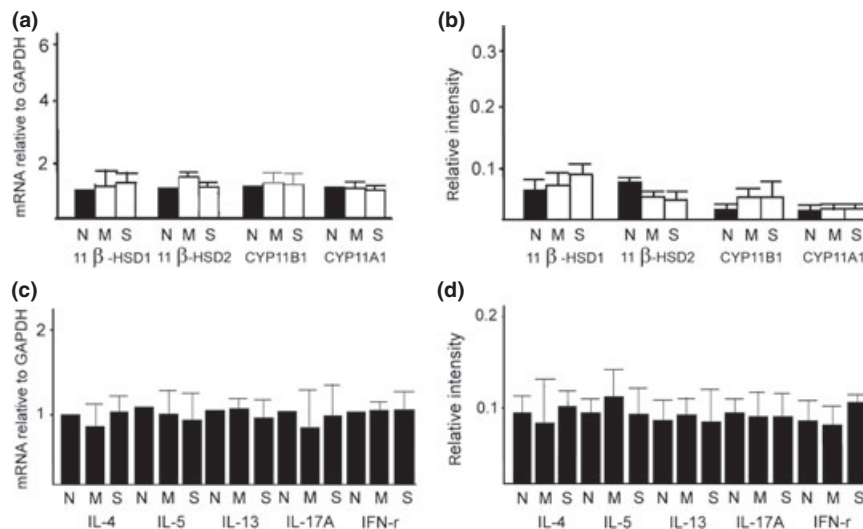


Fig. 7. Real-time PCR (a) and Western blot analysis (b) for expression levels of 11 β -HSD1 and 11 β -HSD2 in control subjects (N: $n = 15$) and patients with mild (M: $n = 15$) and moderate/severe persistent allergic rhinitis (S: $n = 15$) treated with intranasal steroid spray for 1 month. Real-time PCR (c) and Western blot analysis (d) of IL-4, IL-5, IL-13, IL-17A, and IFN- γ in control subjects and patients with mild and moderate/severe persistent allergic rhinitis treated with intranasal steroid spray for 1 month.

and inactive cortisone as a critical factor in the determination of tissue sensitivity to cortisol. This suggestion is supported by the present results that silencing of either 11 β -HSD1 or 11 β -HSD2 by siRNA or selective 11 β -HSD1 inhibitor resulted in significant changes in

the level of active cortisol in cultured nasal epithelial cells, and cortisol levels in nasal lavage fluid are increased in patients with allergic rhinitis, compared with normal subjects. Similar to our data, 11 β -HSD1 has been shown to be up-regulated in human tissue

and in an animal model of inflammatory bowel disease, whereas 11 β -HSD2 has shown to be down-regulated [7, 8, 34]. The up-regulation of 11 β -HSD1 is found in other *in vivo* inflammatory responses such as human and rodent arthritis [6]. The hypothesis arising from these observations is that inflammation is associated with stimulation of 11 β -HSD1 leading to increased production of active glucocorticoids, namely, cortisol, and that changes in glucocorticoid metabolism can contribute to negative feedback suppressing further inflammatory responses. This hypothesis is supported by the observation that 11 β -HSD1 is stimulated by IL-1 β and TNF- α in renal mesangial cells and this may, in turn, modulate intrarenal inflammatory responses [35]. Another study also identified a Th2-cytokine-driven homeostatic feedback mechanism in airway smooth muscle that enhances its responsiveness to endogenous glucocorticoids by up-regulating 11 β -HSD1 activity, inhibiting the adverse effects of the cytokine on airway function [9]. In study using skin wound model, inhibition of cortisol synthesis increased IL-1 β production, suggesting that cortisol synthesis in epidermis may serve as a local negative feedback to proinflammatory cytokine [13].

The mechanism of reciprocal regulation of the two isoforms of 11 β -HSD has not previously been observed in allergic rhinitis. In a human bronchial epithelial cell line, endotoxin inhibited 11 β -HSD2 enzyme activity in a dose-dependent manner. Likewise, TNF- α inhibited both activity and mRNA expression of 11 β -HSD2 [36]. In vascular smooth muscle cells, up-regulation of 11 β -HSD1 was induced after exposure to IL-1 β and TNF- α , suggesting that the induction of 11 β -HSD1 plays a role in regulating inflammatory responses in arterial vessels [37]. Induced expression of 11 β -HSD1 by IL-1 β and TNF- α was also found in human airway smooth muscle cells [38], and another study showed that the expression of 11 β -HSD1 in airway smooth muscle cells is up-regulated in the presence of IL-13, whereas 11 β -HSD2 expression is inhibited [9]. 11 β -HSD1 expression in human monocytes was found to be induced by the Th2 cytokines, IL-4, and IL-13, and this effect was inhibited by the Th1 cytokine, IFN- γ [39]. Therefore, it is conceivable that the reciprocal changes in 11 β -HSD1 and 11 β -HSD2 expression in allergic rhinitis may be induced by inflammatory mediators including cytokines relevant to allergic rhinitis. Indeed, our data demonstrate that the expression of 11 β -HSD1 was up-regulated after stimulation with IL-4, IL-5, IL-13, and IL-17A, compared with non-treated controls. In contrast, expression levels of 11 β -HSD2 were decreased after treatment with IL-4, IL-6, IL-13, and IL-17A. Furthermore, increased cortisol bioavailabilities associated with increased 11 β -HSD enzyme activities were found in cultured epithelial cells after stimulation of cytokines. Therefore, we conclude that the reciprocal

regulation of 11 β -HSD isoforms by cytokines may contribute to the regulation of intracellular corticosteroid metabolism in allergic nasal mucosa, serving to enhance pre-receptor glucocorticoid bioavailability and contributing to feedback control of allergic inflammation. Our findings support the notions that inflammatory cytokines can induce the formation of biologically active glucocorticoids through the interactions between enzymes relating to glucocorticoid metabolism and cytokines that can persist in the target tissue through autocrine or paracrine mechanism [37, 40–43].

The adrenal glands are the predominant source of endogenous glucocorticoid in the body [44]. Recently, extra-adrenal sources of glucocorticoids have been described. Initially, Vacchio et al. [12] found that the thymic epithelium expresses the entire enzyme cascade required for glucocorticoid synthesis and can synthesize glucocorticoids in a paracrine manner. More recently, skin has also been proposed to synthesize glucocorticoids [13]. The initial step in synthesis and secretion of glucocorticoids from cholesterol is the conversion of cholesterol to pregnenolone, induced by the CYP11A1 enzyme. The final step of cortisol biosynthesis converting 11-deoxycortisol to cortisol is performed by the 11 β -hydroxylase, CYP11B1 [45]. Therefore, evidence of CYP11A1 and CYP11B1 expression supports the formation of glucocorticoids [45]. The presence of these critical enzymes that participate in steroidogenesis has also been identified in the heart and intestine, suggesting the possibility of adrenal-independent glucocorticoid synthesis *in situ* [14, 15]. Recently, cortisol production by fibroblasts, melanocytes, and isolated hair follicles was demonstrated *in vitro* [16–18]. There is no previously reported evidence that an endogenous steroidal system exists in nasal mucosa. In this study, we investigated the possibility of local glucocorticoid production in human nasal mucosa. RT-PCR showed the transcription of CYP11A1 and CYP11B1 in human nasal mucosa, and Western blot and immunohistochemistry identified anti-CYP11B1 and CYP11A1 protein band in human nasal mucosa, where they are distributed in the epithelial layer, submucosal gland, and vascular endothelium. As mentioned above, this study showed that the two isoforms of 11 β -HSD, regulating local metabolism and action of glucocorticoids at the tissue level, are present in human nasal mucosa. Taken together, these data suggest the possibility that human nasal mucosa is another extra-adrenal site synthesizing cortisol. To provide direct evidence that cortisol is synthesized in human nasal mucosa, the production of glucocorticoids in cultured epithelial cells and in protein isolated from human nasal mucosa was detected by ELISA. Furthermore, the present study showed that cultured epithelial cells can synthesize cortisol *de novo* after stimulation with ACTH. Collectively, our data demonstrate that the

human nasal mucosa is capable of synthesizing and releasing considerable amounts of glucocorticoids, suggesting that nasal mucosa is another extra-adrenal cortisol-synthesizing site. These results are in accordance with those of studies showing that cortisol produced in cultured keratinocyte or skin is easily detectable by ELISA [13, 46]. Further studies are required to evaluate the full complement of enzymes and co-factors necessary for *de novo* production of glucocorticoids from cholesterol in human nasal mucosa.

Interestingly, CYP11B1, a critical steroidogenic enzyme, constitutively expressed in the human nasal mucosa, is increased in mild and moderate/severe persistent allergic nasal mucosa. Furthermore, we found that the increased expression levels of CYP11B1 and cortisol are induced upon stimulation with Th2 cytokines in cultured nasal epithelial cells. Consistent with these findings, the induction of experimental colitis in wild-type mice led to substantial up-regulation of mRNA transcripts of steroidogenic enzymes as well as to a clear induction of local synthesis of intestinal glucocorticoid, suggesting intestinal glucocorticoid synthesis as part of a negative feedback response [8, 15]. In patients with inflammatory arthritis, local generation of active glucocorticoids was demonstrated on the basis of synovial fluid corticosteroid measurements, and this activity correlated with measures of inflammation [47]. These data also suggest the possibility that increased local generation of steroids reduces inflammation and hasten resolution. Taken together, our data also suggest that, in parallel with the induction of 11 β -HSD1, Th2 cytokines contribute to the increased production of CYP11B1 in allergic nasal mucosa, resulting in the local generation of cortisol and inducing a negative feedback response. Nevertheless, the expression levels of CYP11A1, another steroidogenic enzyme, were not changed in allergic nasal mucosa, compared with normal nasal mucosa and in cultured epithelial cells even after stimulation with cytokines. The reasons for the discrepancy among these data are not clear at present to interpret with accuracy. Future studies involving the tissue-specific deletion of steroidogenic enzymes may help to better define the role of extra-adrenal glucocorticoid synthesis in human nasal mucosa and determine its role in allergic nasal mucosa.

The therapeutic effects of glucocorticoids are attributed to various properties including the prevention of the release of various inflammatory cytokines from nasal mucosa. The actions of glucocorticoids and

inflammatory cytokines usually oppose each other at sites of inflammation [23, 24]. Interestingly, in the present study, when cultured nasal epithelial cells were stimulated with glucocorticoids or cytokines each other, the expression levels of 11 β -HSD1 were increased. The combination of cytokines and glucocorticoids substantially increased 11 β -HSD1 levels relative to either individual treatment, which is in obvious contrast to the opposing effects of glucocorticoids and inflammatory cytokines at sites of inflammation. Our data were in accordance with results showing that combined treatment of inflammatory cytokines and glucocorticoids synergistically induces 11 β -HSD1 expression and activity in synovial fibroblasts and osteoblasts [48]. In the amnion fibroblasts, glucocorticoids can positively induce 11 β -HSD1 expression, which was further strengthened by inflammatory cytokines [49]. However, in patients with allergic rhinitis treated with intranasal steroid spray for 1 month, the expression levels of 11 β -HSD1 and Th2 cytokines in nasal mucosa were not different from those of control subjects. The detailed mechanisms of this paradoxical interaction of glucocorticoids and inflammatory cytokines in human nasal mucosa need further investigation. Understanding these paradoxical interactions of glucocorticoids and cytokines in allergic rhinitis could provide important clues in revealing the pathogenesis of allergic rhinitis.

In conclusion, allergic rhinitis is associated with an increase in 11 β -HSD1 expression and a reciprocal decline in 11 β -HSD2, suggesting that these changes may facilitate the anti-inflammatory function of glucocorticoids by regulating the production of cortisol at human nasal mucosa.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Communication Disorders Center, Korea University, Korea, and The Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2010-0004094). This research was also supported by a Korea University grant, Korea.

Conflict of interest

The authors declare no conflict of interest.

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