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In vitro Metabolism of Ciclesonide in Human Nasal Epithelial Cells

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ABSTRACT: Ciclesonide, a corticosteroid in development for allergic rhinitis, is converted to the pharmacologically active metabolite, desisobutyryl-ciclesonide (des-CIC), and des-CIC is subsequently esterified with fatty acids. Various experiments were performed to investigate ciclesonide metabolism in human nasal epithelial cells (HNEC). Human nasal epithelial cells were incubated with (a) 0.1 μM ciclesonide for 1 h and medium without ciclesonide for up to 24 h, (b) esterase inhibitors for 0.5 h followed by 5 μM ciclesonide for 6 h, or (c) 1 μM des-CIC for 6 h followed by medium without des-CIC for up to 24 h. Ciclesonide, des-CIC and des-CIC fatty acid conjugate concentrations were determined by high-performance liquid chromatography with tandem mass spectrometry. The amount of ciclesonide in HNEC decreased approximately 93-fold from 0.5 to 24 h. In contrast, des-CIC was present at constant levels throughout the post-treatment period. Furthermore, fatty acid conjugates of des-CIC were retained in HNEC up to 24 h post-treatment. Carboxylesterase and cholinesterase inhibitors decreased ciclesonide metabolism $\geqslant$50%. The total amounts of des-CIC fatty acid conjugates decreased and the extracellular amounts of des-CIC increased with time. In conclusion, ciclesonide was rapidly converted to des-CIC by carboxylesterases and cholinesterases, and des-CIC underwent reversible fatty acid conjugation in HNEC. Copyright © 2006 John Wiley & Sons, Ltd.

Key words: allergic rhinitis; esterases; intranasal corticosteroids; nasal epithelial cells

Introduction

Allergic rhinitis (AR) is a chronic upper airway disease, characterized by sneezing, itching, nasal congestion and rhinorrhea [1]. Recent estimates suggest that AR affects 10–25% of the world's population [2]. Allergic rhinitis is associated with the development of sleep disturbances, decreased performance and productivity, and impaired quality of life [3,4]. Current pharmacologic treatments for AR include antihistamines, decongestants, leukotriene receptor antagonists and intranasal corticosteroids (INCS) [5]. Intranasal corticosteroids possess potent anti-inflammatory activity and effectively relieve nasal symptoms and nasal congestion. Furthermore, intranasal administration delivers the corticosteroid directly to the site of inflammation, the nasal epithelium [6]. Because of the high efficacy and favorable safety profile of INCS, current treatment guidelines recommend these agents as first-line therapy for moderate to severe persistent AR [7].

Ciclesonide is a novel corticosteroid that is administered as an inactive parent compound

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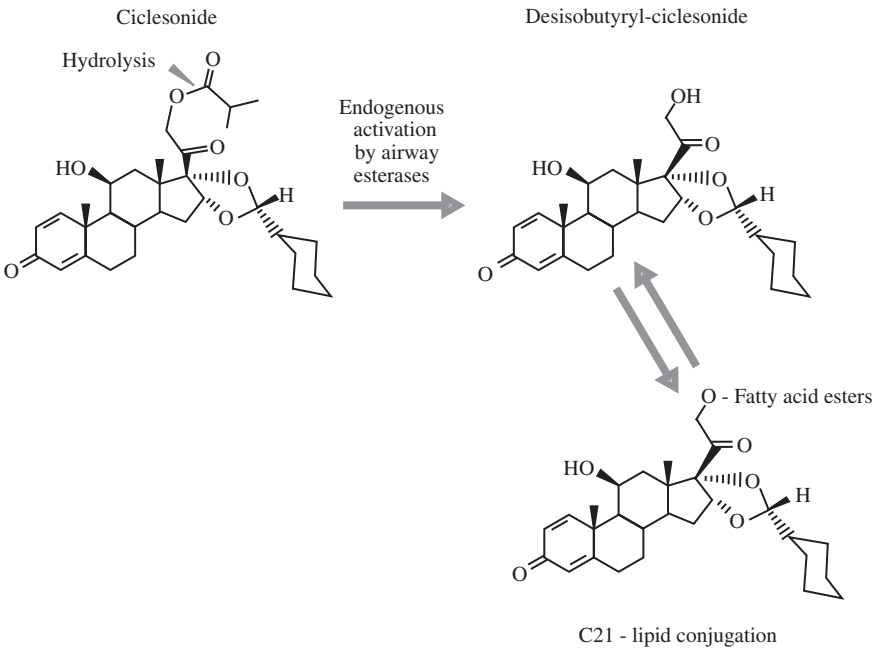

Figure 1. Pathway of ciclesonide metabolism in human nasal epithelial cells. Adapted with permission from Dietzel *et al.* [8]

and is converted to the pharmacologically active metabolite, desisobutyryl-ciclesonide (des-CIC), by endogenous esterases in the upper and lower airways (Figure 1) [8]. Desisobutyryl-ciclesonide subsequently undergoes reversible fatty acid conjugation at the carbon-21 position [9]. Desisobutyryl-ciclesonide has a high affinity for the glucocorticoid receptor (relative glucocorticoid receptor binding affinities of des-CIC and ciclesonide are 1200 and 12, respectively; the dexamethasone reference being 100) and exhibits substantial *in vitro* and *in vivo* anti-inflammatory activity [10]. Low oral bioavailability (1%) [11], high protein binding (99%) [12] and high hepatic clearance (3961/h for des-CIC) [13] contribute to the low systemic bioavailability of ciclesonide and des-CIC. The clinical efficacy and improved safety profile of ciclesonide have been demonstrated in patients with asthma [14–16].

Ciclesonide is currently in clinical development for the treatment of asthma using a metered-dose inhaler. Ciclesonide is also in clinical development as an intranasal spray for the treatment of AR. The intranasal spray contains a hypotonic suspension of ciclesonide that increases local tissue uptake compared with a standard isotonic suspension [17].

Most of the currently available information on ciclesonide metabolism was ascertained from studies conducted in human alveolar epithelial cells and human lung precision-cut tissue slices [9,18]. The current study was undertaken to determine if a similar pattern of ciclesonide metabolism also occurred in human nasal epithelial cells (HNEC). The specific objectives of this study were to examine the *in vitro* activation of ciclesonide and esterification of des-CIC in HNEC, identify the specific esterases involved in the conversion of ciclesonide to des-CIC, and assess the reversibility of des-CIC fatty acid conjugation in HNEC.

Methods

This is a combined presentation of three related studies. The first study examined the *in vitro* metabolism of ciclesonide in HNEC. The second study identified the specific esterases involved in the conversion of ciclesonide to des-CIC. Finally,

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the reversibility of des-CIC fatty acid conjugation was assessed in HNEC.

Cultivation of human nasal epithelial cells and in vitro investigations

In vitro metabolism of ciclesonide in HNEC. Human nasal epithelial cells, obtained during specified surgical operations, were purchased from Oligene GmbH (Berlin, Germany). Immunohistochemical staining for cytokeration was positive. Human nasal epithelial cells were passaged $\leqslant 4$ times. No morphologic changes or differences in proliferation rates were observed in any dishes or wells. The cells were suspended in bronchial-tracheal epithelial cell growth medium (BTECGM; Toyobo, Japan), seeded ($\geqslant 6000$ cells/cm^2) in Biocoat Collagen I Cellware 100 mm dishes (BD Biosciences; Bedford, MA, United States), and incubated at 37 °C in 5% CO_2–95% humidified air for approximately 1 week until the cells became almost confluent. Each dish was washed 5 times with 10 ml of sterile Dulbecco's phosphate buffered saline (PBS). Then 5 ml of BTECGM minus bovine pituitary extract (BPE)–0.1% (w/v) bovine serum albumin (BSA) containing ciclesonide (0.1 μM) was added to each dish, and the cells were incubated at 37 °C for 1 h. After the incubation period was completed, the medium was aspirated, and each dish was washed 5 times with 10 ml of sterile PBS. Five ml of BTECGM minus BPE–0.1% (w/v) BSA was added to each dish, and the dishes were incubated for a further 0.5, 3, 6 and 24 h ($n = 5$ for each time point). At the indicated time points, the medium was removed by aspiration, 5 ml of 5 mM ethylenediamine tetraacetic acid (EDTA)-4Na-PBS was added to each dish, and the cells were incubated at 37 °C for 5 min. The cells were separated from the dishes and decanted into 15 ml tubes. Each dish was washed twice with 5 ml of ice-cold sterile PBS, and the wash solutions were combined with the previously collected cells. The cell suspension was centrifuged at 800 rpm ($134 \times g$) at 4 °C for 5 min. The cell pellet was immediately frozen in liquid nitrogen and stored at –80 °C until further use.

Esterases involved in the conversion of ciclesonide to des-CIC. Human nasal epithelial cells were cultivated as described above in Biocoat Collagen 1 Coat 6-well plates until they became confluent. After washing, 3 ml of BTECGM minus BPE–0.1% (w/v) BSA and 30 μl of 0.1, 1.0 or 10.0 μM of the following esterase inhibitors were added to each of 3 wells: paraoxon (carboxylesterase, cholinesterase and acetylcholinesterase inhibitor), bis (*p*-nitrophenyl) phosphate (BNPP; carboxylesterase inhibitor), tetraisopropyl pyrophosphoramide (iso-OMPA; cholinesterase inhibitor), eserine (acetylcholinesterase inhibitor), 4-hydroxymercuribenzoic acid sodium salt (PMB; arylesterase inhibitor), and EDTA (A-esterase inhibitor; Sigma-Aldrich Japan K.K., Tokyo, Japan). Cells were incubated for 0.5 h. Three μL of ciclesonide (5 μM) in dimethyl sulfoxide was added to each well, mixed, and incubated for 6 h. At the end of the incubation period, 1 ml of the incubation medium was collected, and the remaining medium was removed by aspiration. Cells that adhered to the 6-well plate were frozen in liquid nitrogen and stored at −80 °C.

Reversibility of des-CIC fatty acid conjugation. Human nasal epithelial cells were cultivated as described above in Biocoat Collagen 1 Coat 6-well plates until they became confluent. After washing, 1 ml of BTECGM minus BPE–0.1% (w/v) BSA containing des-CIC (1 μM) was added to each well, and the cells were incubated for 6 h. At the end of the incubation period, the medium was removed by aspiration, and the cells were washed with PBS. Cells that adhered to one of the 6-well plates were frozen in liquid nitrogen and stored at −80 °C. Three ml of BTECGM minus BPE–0.1% (w/v) BSA was added to each well in the remaining plates, and the cells were incubated for 3, 6 and 24 h ($n = 6$ for each time point). One ml of medium was collected from each well after 1, 3 and 6 h, and the remaining medium was replaced with 3 ml of fresh medium. After the incubation was completed, 1 ml of medium was collected from each well, and the remaining medium was removed by aspiration. Cells that adhered to the 6-well plate were frozen in liquid nitrogen and stored at −80 °C.

Preparation of cell extracts

In vitro metabolism of ciclesonide in HNEC. After adding 0.8 ml of high-performance liquid

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chromatography (HPLC)-grade ethanol (99.5%) to each tube containing a frozen cell pellet, the cells were pulverized with an ultrasonic homogenizer (Branson, Danbury, CT, United States). The mixture was centrifuged at 3000 rpm ($1882 \times g$) at 4 °C for 15 min, and the supernatant was stored at −20 °C until further use.

Esterases involved in the conversion of ciclesonide to des-CIC and reversibility of des-CIC fatty acid conjugation. Human nasal epithelial cells, frozen in 6-well plates, were subjected to 2 freeze-thaw cycles; cells were gradually thawed at room temperature for approximately 1 h, frozen in liquid nitrogen, and stored at −80 °C for 1 h. One ml (reversibility study) or 2 ml (esterase study) of 0.1% (v/v) Tween-80–HPLC-grade ethanol (99.5%) was added to each well. The extracts were collected in 15 ml tubes. Each well was washed with 0.5 ml (reversibility study) or 1 ml (esterase study) of 0.1% (v/v) Tween-80–HPLC-grade ethanol (99.5%), and the wash solutions were combined in the 15 ml tubes. The mixture was centrifuged as described, and the supernatant was stored at −20 °C until further use.

Preparation of medium extracts

Esterases involved in the conversion of ciclesonide to des-CIC and reversibility of des-CIC fatty acid conjugation. One ml of HPLC-grade ethanol (99.5%) was added to 1 ml of collected medium, mixed thoroughly, and centrifuged at 7500 rpm ($10400 \times g$; esterase study) at 4 °C for 5 min or 4000 rpm ($3706 \times g$; reversibility study) at 4 °C for 15 min. Supernatants were stored at −20 °C until further use.

Liquid chromatography with tandem mass spectrometry (LC/MS/MS). Ciclesonide, des-CIC, des-CIC-oleate, des-CIC-palmitate and deuterated des-CIC (internal standard) were synthesized by ALTANA Pharma. Ethanol solutions containing these compounds were shown to be stable during the period of analysis. Ten μl of each standard solution was combined with 90 μl of extract from the medium and cells untreated with test drug, and 75 μl of this mixture and 25 μl of internal standard solution were combined and used as calibration-curve samples. Seventy-five μl of each medium and cell extract solution was combined with 25 μl of internal standard solution and used as measurement samples. All of the measured analyte data were within the acceptance criteria (the correlation coefficient of the calibration curve was >0.99; the accuracy of QC samples was $< \pm 15\%$ of the theoretical value for ciclesonide and des-CIC, and $< \pm 20\%$ of the theoretical value for des-CIC-oleate and des-CIC-palmitate).

Ciclesonide, des-CIC, des-CIC-oleate and des-CIC-palmitate were separated by HPLC (Agilent HP1100, Agilent Technologies, Tokyo, Japan), using an autosampler (Model SIL-HTC; Shimadzu Corporation, Kyoto, Japan) and a 5 μm Hypersil® Phenyl2 column (50×4.6 mm; Thermo Electron, K.K., Yokohama, Japan). The mobile phase consisted of 25% (v/v) acetonitrile-purified water–1 mM ammonium acetate and 95% (v/v) acetonitrile-purified water–1 mM ammonium acetate at a flow rate of 1.0 ml/min. Ciclesonide and its metabolites were further analysed by tandem mass spectrometry (MS/MS; API3000; Applied Biosystems, Tokyo, Japan), using a turbo ion spray and negative multiple reaction monitoring scan type.

Data analysis

Calibration curves of ciclesonide, des-CIC, des-CIC-oleate and des-CIC palmitate were prepared by linear regression analysis, using the peak area ratios of the standard solutions. The lower limits of quantitation were 0.10 ng/ml for ciclesonide, des-CIC-oleate and des-CIC-palmitate and 0.20 ng/ml for des-CIC. In the reversibility study, the lower limit of quantification of des-CIC-oleate in medium was 0.20 ng/ml. Data were expressed as arithmetic mean ± standard deviation. The effect of esterase inhibitors on ciclesonide metabolism was expressed as the percentage of ciclesonide converted to des-CIC, des-CIC-oleate and des-IC-palmitate. The reversibility of des-CIC fatty acid conjugation was assessed by comparing the total amounts of des-CIC with the total amounts of fatty acid conjugates of des-CIC at each time point.

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Results

In vitro metabolism of ciclesonide in human nasal epithelial cells

Ciclesonide, des-CIC, and fatty acid conjugates of des-CIC were detected in HNEC up to 24 h post-treatment (Table 1). The amount of ciclesonide decreased approximately 93-fold from 28.82 ± 2.01 pmol/dish at 0.5 h to 0.31 ± 0.07 pmol/dish at 24 h. The largest decrease in ciclesonide amounts occurred during the first 3 h post-treatment. In contrast, des-CIC remained at constant levels throughout the 24 h post-treatment period (range, 6.08 ± 0.78 pmol/dish at 0.5 h to 5.39 ± 0.99 pmol/dish at 24 h). The amount of des-CIC fatty acid conjugates, especially des-CIC-oleate, increased with time in HNEC. Desisobutyryl-ciclesonide-oleate levels increased from 17.13 ± 1.55 pmol/dish at 0.5 h to 48.43 ± 7.46 pmol/dish at 24 h. Low levels of des-CIC-palmitate were also detected in HNEC throughout the post-treatment period.

Esterases involved in the conversion of ciclesonide to desisobutyryl-ciclesonide in human nasal epithelial cells

In the absence of exogenous esterase inhibitors, the majority of ciclesonide (approximately 90%) was converted to des-CIC and des-CIC fatty acid conjugates in HNEC (Table 2). Paraoxon, a nonspecific B esterase inhibitor, reduced the formation of ciclesonide metabolites approximately 75% at each of the tested inhibitor concentrations (0.1–10 μM). Increasing concentrations of BNPP and iso-OMPA, a carboxylesterase- and cholinesterase-specific inhibitor, respectively, progressively decreased ciclesonide metabolism in HNEC. Approximately 50% and >70% of metabolite formation were inhibited by 1 and 10 μM, respectively, of BNPP or iso-OMPA. Eserine, an acetylcholinesterase inhibitor, only achieved substantial inhibition (57%) of ciclesonide metabolism at a concentration of 10 μM. The arylesterase and A-esterase specific inhibitors, PMB and EDTA, did not affect ciclesonide metabolism in HNEC.

Table 2. Percentage of ciclesonide metabolites[a] in human nasal epithelial cells and medium after incubation with ciclesonide and various inhibitors

Target esterase	Inhibitor	Concentration of inhibitor (μM)	Mean percentage of metabolites ± SD
—	No inhibitor	—	90.2 ± 0.8
Carboxylesterase, cholinesterase, acetylcholinesterase	Paraoxon	0.1	23.4 ± 0.6
		1.0	21.7 ± 1.6
		10.0	22.5 ± 0.5
Carboxylesterase	BNPP	0.1	81.7 ± 1.6
		1.0	44.1 ± 3.0
		10.0	22.4 ± 0.8
Cholinesterase	iso-OMPA	0.1	88.8 ± 0.9
		1.0	51.4 ± 0.7
		10.0	26.4 ± 0.9
Acetylcholinesterase	Eserine	0.1	91.1 ± 3.3
		1.0	75.8 ± 2.1
		10.0	43.4 ± 0.4
Arylesterase	PMB	0.1	93.5 ± 0.6
		1.0	92.5 ± 0.6
		10.0	93.5 ± 1.3
A-esterase	EDTA	0.1	95.5 ± 0.9
		1.0	94.2 ± 2.3
		10.0	95.3 ± 0.4

SD, standard deviation; BNPP, bis (p-nitrophenyl) phosphate; iso-OMPA, tetraisopropyl pyrophosphoramide; PMB, 4-hydroxymercuribenzoic acid sodium salt; EDTA, ethylenediamine tetraacetic acid.[a] Desisobutyryl-ciclesonide (des-CIC) and des-CIC fatty acid conjugates.

Table 1. Mean amount of ciclesonide and its metabolites in HNEC

Cell collection time (h)	Mean amount (pmol/dish ± SD)			
	Ciclesonide	des-CIC	des-CIC-oleate	des-CIC-palmitate
0.5	28.82 ± 2.01	6.08 ± 0.78	17.13 ± 1.55	0.48 ± 0.05
3	5.12 ± 1.94	5.76 ± 1.90	21.59 ± 4.64	0.71 ± 0.18
6	2.50 ± 0.51	7.34 ± 1.55	32.58 ± 5.04	1.30 ± 0.21
24	0.31 ± 0.07	5.39 ± 0.99	48.43 ± 7.46	1.87 ± 0.34

HNEC, human nasal epithelial cells; SD, standard deviation; des-CIC, desisobutyryl-ciclesonide.

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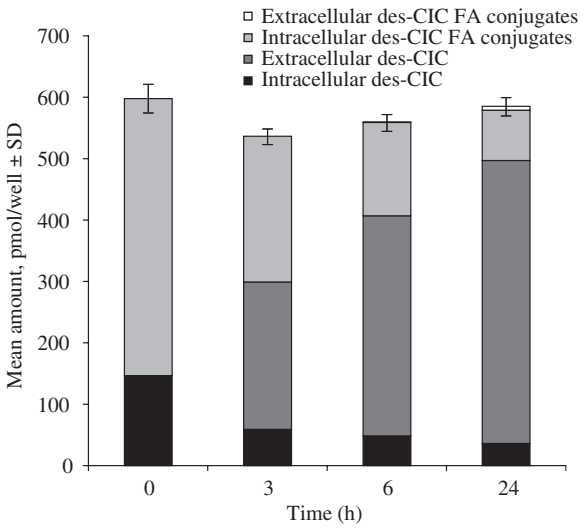

Figure 2. Amount of desisobutyryl-ciclesonide (des-CIC) and des-CIC fatty acid conjugates in human nasal epithelial cells and medium. Data are expressed as mean ± standard deviation

Reversibility of desisobutyryl-ciclesonide fatty acid conjugation in human nasal epithelial cells

The total amount of analytes at 3 h, 6 h and 24 h after treatment were 89.75%, 93.61% and 97.89%, respectively, of the total amount of analytes at 0 h. The total amount of des-CIC increased during the post-treatment period and accounted for approximately 85% of total analytes (497.00 ± 4.44 pmol/well) 24 h after treatment (Figure 2). However, the total amount of des-CIC fatty acid conjugates declined over time and represented only 15% of total analytes 24 h post-treatment. Intracellular amounts of des-CIC decreased from 146.6 ± 11.5 pmol/well at 0 h to 36.1 ± 1.7 pmol/well at 24 h. Similarly, intracellular amounts of des-CIC fatty acid conjugates decreased from 451.2 ± 15.0 pmol/well at 0 h to 82.0 ± 12.5 pmol/well at 24 h. However, des-CIC progressively accumulated in the medium. In contrast, extracellular concentrations of des-CIC-oleate and des-CIC-palmitate were almost undetectable at all time points post-treatment.

Discussion

This study extended previous studies of ciclesonide metabolism conducted in human alveolar epithelial cells and human lung precision-cut tissue slices [9,18]. Similar to the human lung, ciclesonide was initially converted to des-CIC, and des-CIC was subsequently esterified with fatty acids in HNEC. The initial conversion of ciclesonide to des-CIC occurred rapidly; however, hydrolysis decreased as the pool of ciclesonide was depleted and fatty acid conjugates of des–CIC were formed over time. Desisobutyryl-ciclesonide remained at stable amounts (range, 6.08 pmol/dish at 0.5 h to 5.39 pmol/dish at 24 h) throughout the 24 h post-treatment period, suggesting that steady-state conditions may have been reached whereby the amount of des-CIC formed from ciclesonide hydrolysis was in equilibrium with the amount of des-CIC used for fatty acid conjugation. Fatty acid conjugates of des-CIC were detected in HNEC throughout the 24 h post-treatment period. Desisobutyryl-ciclesonide-oleate was the major fatty acid conjugate formed in HNEC, possibly because of higher local concentrations of oleate versus other fatty acids (e.g. palmitate). Previous studies have described a similar pattern of ciclesonide metabolism in human alveolar epithelial cells and human lung precision-cut tissue slices [9,18]. Furthermore, fatty acid conjugates of des-CIC were also detected in the rat lung after daily inhalation of ciclesonide for 4 weeks [19].

The conversion of ciclesonide to des-CIC in HNEC was mediated by esterases, especially carboxylesterases and cholinesterases. Acetylcholinesterases may also have lesser involvement in ciclesonide hydrolysis in HNEC. Esterase-mediated ciclesonide activation has previously been described in human bronchial epithelial cells, human lung tissue and human liver tissue [20]. Furthermore, the recent demonstration of the conversion of ciclesonide to des–CIC in rabbits suggests that esterases are also present in nasal mucosal tissue [17]. Carboxylesterases and cholinesterases belong to the serine hydrolyase superfamily of enzymes, members of which are highly conserved in a triad of amino acids (serine, glutamic acid and histidine) located in the active site [21].

Desisobutyryl-ciclesonide and des-CIC fatty acid conjugates were retained for >24 h in HNEC. In a previous study, administration of a hypotonic suspension of ciclesonide to rabbits also

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resulted in the prolonged retention of des-CIC and des-CIC-oleate in nasal mucosa [17]. Retention on the nasal epithelium allows ciclesonide to provide sustained anti-inflammatory activity and, thereby, supports once-daily dosing with a 24 h effect. Intranasal ciclesonide 200 μg was superior to placebo in relieving nasal symptoms in adult and adolescent patients with seasonal AR when administered as a once-daily dose [22]. Although ciclesonide was administered once daily in the morning, the signs and symptoms of AR were adequately controlled throughout the intervening 24 h period.

Nave *et al*. [9,19] also suggested that formation of reversible fatty acid conjugates in the lung enabled once-daily ciclesonide to provide prolonged anti-inflammatory activity for the effective treatment of asthma. However, it was not known if des-CIC could form reversible fatty acid conjugates within the nasal epithelium. In the present study, reversible formation of des-CIC fatty acid conjugates was investigated by the removal of the medium, which mimics the *in vivo* process whereby des-CIC is excreted from the nasal mucosa into the systemic circulation. The levels of intracellular des-CIC fatty acid conjugates, formed during a 6 h incubation period with des-CIC, decreased throughout the 24 h post-treatment period (reversibility study), indicating that conversion of des-CIC to des-CIC fatty acid conjugates was reversible and that stored fatty acid conjugates may revert to the pharmacologically active moiety. Budesonide, another INCS used in the treatment of AR, also undergoes reversible fatty acid conjugation [23]. Formation of budesonide esters has been demonstrated in nasal mucosa. Budesonide is also retained in nasal mucosa to a greater extent compared with fluticasone propionate [24]. Moreover, des-CIC-oleate is 5-fold more lipophilic than budesonide-oleate [25]. Consequently, des-CIC-oleate may display longer retention than budesonide-oleate in the nasal mucosa. In contrast to ciclesonide and budesonide, fluticasone propionate does not form fatty acid conjugates.

Although des-CIC was detected extracellularly in the current *in vitro* studies, results from previous studies that used the inhaled formulation of ciclesonide suggest that high protein binding and high hepatic clearance [12,13] may minimize the potential for systemic side effects in patients receiving ciclesonide treatment. Furthermore, the systemic bioavailability of ciclesonide was demonstrated to be extremely low in a recent phase I study. After intranasal administration of supratherapeutic daily doses of ciclesonide 800 μg for 14 days, mean maximal serum concentrations of des-CIC were below the lower limits of quantification (10 pg/ml), despite the use of a highly sensitive assay [26].

In conclusion, this study demonstrates that ciclesonide is converted to the active metabolite, des-CIC, by carboxylesterases and cholinesterases and that des-CIC subsequently undergoes reversible fatty acid conjugation in HNEC. These findings are consistent with the previous report of ciclesonide activation and retention in rabbit nasal mucosal tissue [17]. Activation and retention of ciclesonide on the nasal mucosa may provide sustained anti-inflammatory activity and thus lead to symptom relief in patients with AR.

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