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Differential expression of organic cation transporters in normal and polyps human nasal epithelium: Implications for *in vitro* drug delivery studies

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ABSTRACT

The aim of this study was to compare the expression of organic cation transporters (OCTs) in normal and polyps nasal epithelium. Primary cell cultures of human nasal epithelium (polyps and normal tissues) were compared by investigating the uptake of a fluorescent organic cation, [4-dimethylaminostyryl-N-methylpyridinium (4-Di-1-ASP)]. The effect of concentration, temperature, pH and competing inhibitors were investigated. Quantitative polymerase chain reaction (qPCR) was used to compare the OCTs gene expression levels in the cells. The K_m (μM) and V_{max} (μM/mg protein/15 min) for 4-Di-1-ASP uptake were higher in normal ($K_m = 3031 \pm 559.6$, $V_{max} = 70.8 \pm 8.8$) cells compared to polyps ($K_m = 952.4 \pm 207.8$, $V_{max} = 30.9 \pm 2.1$). qPCR results showed that OCT1-3 and organic cation/carnitine transporter 1-2 gene transcripts (OCTN1-2) were expressed in both normal and polyps cells at comparable levels, with OCT-3 having the highest expression level in both cultures. Kruskal–Wallis ANOVA showed that pH and specific inhibitors had similar effects on both normal and polyps cells ($p > 0.5$). Similarly, OCTs and OCTNs gene expression levels were similar. This study showed that polyps biopsies can be used for isolating cells to study organic cation transporters in human nasal epithelium as no major functional or molecular differences relative to normal cells could be found.

1. Introduction

Drugs used for treatment of various diseases, including those affecting the respiratory tract must attain significant concentrations at their site of action to be effective (Derendorf et al., 2006). This may be challenging, especially for drugs with poor absorption and permeation characteristics (Leonard et al., 2002). Drugs administered via the nasal or pulmonary route are absorbed by passive diffusion, through the paracellular pathway, or transported via drug transporters. The limited passive diffusion and paracellular pathways available for drug transport across the respiratory mucosa imply that endogenous solute carriers often act as drug carriers across the epithelial membranes (Sun et al., 2001; Thwaites and Anderson, 2007; Daniel and Kottra, 2004). These drug transporters are expressed in specific cell membranes of various tissues, where they have pivotal roles in determining the pharmacokinetic profiles of administered drugs including absorption, distribution, elimination and concentration at the target sites (Sai and Tsuji, 2004; Oswald et al., 2007; Koepsell et al., 2007; Wright et al., 2007)Organic cation transporters (OCTs) are recently identified class of proteins that play a major role in transporting positively charged molecules including endogenous substances (e.g. dopamine, choline) and drugs (Ciarimboli, 2008). Many currently available therapeutic drugs such as antihistamines, antibiotics and opiates are organic cations and thus rely on OCTs for distribution and elimination in the body (Mehrens et al., 2000). As suggested by their roles, these transporters are ubiquitously expressed in several organs in an array of isoforms (OCT1, OCT2, OCT3, OCTN1, OCTN2 and OCTN3, OCTN6). This family of solute carrier proteins are classified as uniporter in direction and either have electrogenic (OCT1-3) or electroneutral (OCTN1-3) properties and share common features such as a 12 trans-membrane alpha helices (Popp et al., 2005).

Epithelial remodeling due to chronic inflammation may also alter the characteristic of transporters responsible for the uptake and transport of some drugs used for treating inflammatory respiratory diseases (e.g. beta agonists and corticosteroids). Many drugs used for treating respiratory diseases are positively charged (e.g. salbutamol, salmeterol) compounds that are transported by

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cationic organic transporters (Ehrhardt et al., 2005; Horvath et al., 2007). Potential down-regulation of these transporters following chronic inflammatory diseases may affect drug disposition in the respiratory tract and the use of biopsies from such inflamed respiratory epithelium for *in vitro* drug delivery studies. Based on their availability, nasal polyps are often the primary source of biopsies for *in vitro* nasal drug delivery studies. Normal nasal biopsies are very difficult to obtain on a consistent basis for *in vitro* studies due to ethical considerations. It is therefore important to examine the fundamental differences and similarities between normal and polyps tissues and identify possible implications of using cells from polyps biopsies for *in vitro* studies (active transport studies). Basically, normal nasal mucosa consists of either stratified squamous epithelium or pseudostratified columnar ciliated epithelium with multiple seromucous glands in both superficial and deep layers, separated by large venous sinusoids. In contrast, histological appearance of nasal polyps varies from extremely edematous tissue with very few scattered glands (Lee et al., 2005). The aim of this study was to compare the expression levels and functional activity of organic cation transporters in normal and polyps human nasal tissue. Information from the studies may have clinical and drug delivery implications as polyps are often more readily available than normal tissues for *in vitro* human nasal drug delivery studies.

2. Materials and methods

2.1. Chemicals

4-(4-(Dimethylamino)styryl)-N-methylpyridinium iodide (4-Di-1-ASP), triton X-100, tetraethyl ammonium (TEA), choline, L-carnitine, verapamil, bovine serum albumin (BSA), pronase, isopropanol, Hanks' balanced salt (HBSS) and Penicillin/Streptomycin were supplied by Sigma (St. Louis, MO, USA). DMEM-F12 1/1, Oligo dT primer, M-MVL reverse transcriptase, cDNA buffer and dNTPs were purchased from Invitrogen (Burlington, ON, Canada). SYBR green mix was from Qiagen (Mississuaga, ON, Canada). Ultroser G was from Biosepra (St-Germain-en-Laye Cedex, France). Bicinchoninic acid (BCA) protein assay kit was from Millipore (Billerica, MA, USA).

2.2. Cell culture

The cell culture method used for the study has been described in detail elsewhere (Agu et al., 2001). Normal nasal epithelial cells were extracted from tissues obtained from patients that underwent endoscopic trans-nasal skull base surgery. Smokers and patients with chronic inflammatory respiratory diseases were excluded from the study. The use of human biopsies was approved by QEII Regional Hospital Research Ethics Board (REB #CDHA-RS/2006-352). The tissues were transported in DMEM-F12 1/1 culture medium supplemented with streptomycin 100 μg/ml and penicillin 100 IU/ml and used for cell culture. Biopsies were washed three times with physiological saline solution supplemented with antibiotics. The cells were dissociated enzymatically for a period of 16–24 h at 4 °C using 0.1% pronase. The pronase was deactivated with 10% NU-serum prior to cell washing with DMEM-F12 1/1. The washing solution was removed after centrifugation at 170 × *g* (1500 rpm) for 5 min on each occasion. The resulting suspension of cells was pre-plated on plastic for 1 h at 37 °C in a 95% O_2 and 5% CO_2 environment to reduce fibroblast contamination. Subsequently, the cells were counted and seeded on either 12- or 24-well plates (Fisher Scientific, ON, Canada) for uptake studies. The cells were incubated at 37 °C in a 95% O_2 and 5% CO_2 environment using DMEM F12 supplemented with Ultroser G 2%. The medium was changed every other day.

2.3. Drug uptake studies

Once these cells reached 90–100% confluence at about 21 days in culture, they were washed twice with warm transport medium (37 °C) supplemented with HEPES and glucose (HBSS, pH of 7.4). The cells were allowed to equilibrate in the buffer for 30 min in a tissue culture incubator at (37 °C, 5% CO_2/95%O_2). At the beginning of an uptake study 200 μl (24-well plates) or 400 μl (12-well plates) of appropriate concentrations of 4-Di-1-ASP solutions were added to properly labeled wells on either the 12- or 24-well plate. The plates were then incubated at the desired temperature for 15 min. The experiment was brought to an end by removing the test solution and immediately washing the cells three times with ice-cold transport medium. Subsequently, the cells were lysed with 500 μl (24-well plate) or 1000 μl (12-well plate) of 1% triton X-100 in 0.1N NaOH. Fluorescence detection of 4-Di-1-ASP was measured using a Modulus single tube multimode florescence reader (model 9200, Turner Bio systems CA, USA) with an excitation of 477 nm and emission of 557 nm. Protein content of the cells was measured with BCA protein assay kit according to the manufacturer's protocol.Quantitative and non-quantitative RT-PCR

Total RNA was extracted from human nasal turbinates using TriZol (Invitrogen) according to the manufacturer's instructions. In brief, cells were lysed with 1 ml TriZol, followed by the addition of 200 μl of chloroform per 1 ml of TriZol and vortexed. The three resulting phases were separated by centrifuging for 15 min at 13,000 rpm at 4 °C. Only the colorless upper aqueous RNA phase was removed and vortexed with isopropanol to precipitate the RNA. The resulting sample was incubated at room temperature for 10 min before centrifugation for 10 min (13,000 rpm) at 4 °C. The resulting RNA pellet was washed twice with ice cold 75% ethanol and then re-suspended in ddH_2O. Concentration and purity of the RNA was assessed using spectrophotometry. All samples had A_{260}/A_{280} absorbance readings greater than 1.6 confirming high RNA purity. A 20 μl aliquot cDNA was synthesized from 0.5 μg

Table 1
Primers used for PCR studies.

Name	Gene symbol	Sequence	Position	Bp
Forward primer				
OCT1	SLC22A1	GATTTAAAGATGCTTTCCCTCG	1050–1077	141
OCT2	SLC22A2	GATGTACAACTGGTTCACGA	1222–1242	100
OCT3	SLC22A3	CTCTGATCATCTTTGGTATCCTG	1522–1545	103
OCTN1	SLC22A4	CTACATCGTCATGGGTAGTC	1631–1651	125
OCTN2	SLC22A5	TCTCCCTACTTCGTTTACCT	1649–1669	169
Reverse primer				
OCT1	SLC22A1	CCTGATAGAGCACAGAGTCC	1191–1171	141
OCT2	SLC22A2	GGGCAGAGTAGAAGAAATCC	1322–1302	100
OCT3	SLC22A3	TTCTACATCATCCACTGTCTC	1625–1604	103
OCTN1	SLC22A4	CCAGATCTGAACCATTTCAC	1756–1736	125
OCTN2	SLC22A5	CTGTGTTTCATTCCTTTGACTC	1818–1796	169

of total RNA using Invitrogen reverse transcription kit as recommended by the manufacturer.

PCR amplification was performed in a total volume of 20 μl containing 1 μl cDNA sample, 10 μl 2× SYBR green mix (Qiagen), 0.5 μM forward and reverse primers. Following 95 °C incubation for 15 min, forty cycles of PCR (94 °C/15 s; 58 °C/20 s; 72 °C/30 s) were then performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Threshold cycles (C_T) for duplicate reactions were determined using Sequence Detection System software (version 2.2.2) and relative transcript abundance calculated following normalization with a β-Actin PCR amplicon. Amplification of only a single species was verified by a dissociation curve for each reaction. The primers used for the study is summarized in Table 1. Reactions with RNA alone were used as negative control. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml).

2.5. Data and statistical and analyses

Unless stated otherwise, each experiment was carried out three times and the data expressed as mean ± SD. Michaelis–Menten kinetic constants (K_m, V_{max}) were calculated using non-linear regression curve-fitting program (Graphpad Prism 5.0, Graphpad Software Inc., CA, USA). Percentage inhibition, effect of pH and comparison of OCT gene expression levels between the normal and polyps nasal cells were performed using Kruskal–Wallis analysis of variance followed by Dunn's post-test. Significant p values were set at <0.05.

3. Results

3.1. Functional studies

Functional comparison of OCTs and OCTNs in the normal and polyps cells was undertaken by studying the effect of concentration, temperature, pH and presence of substrates/inhibitors on 4-Di-1-ASP uptake. Fig. 1 shows the effect of concentration on intracellular accumulation of 4-Di-ASP in normal and polyps human nasal

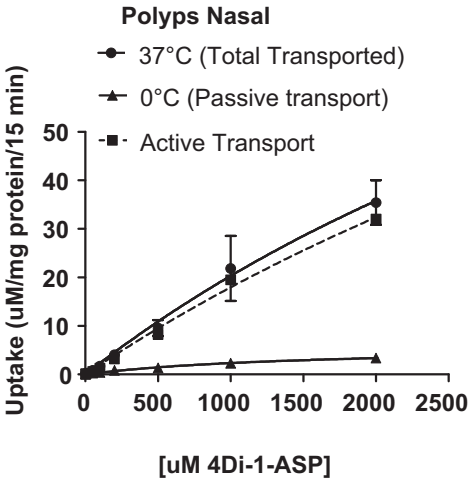

Fig. 2. Effect of temperature on the uptake of 4-Di-ASP in normal and polyps human nasal epithelium. Cells were incubated with the substrate on a shaker placed in refrigerated low-temperature incubator (0, 10 °C) and hybridization (37 °C) for 15 min. Data are expressed as mean ± SD (n = 3).

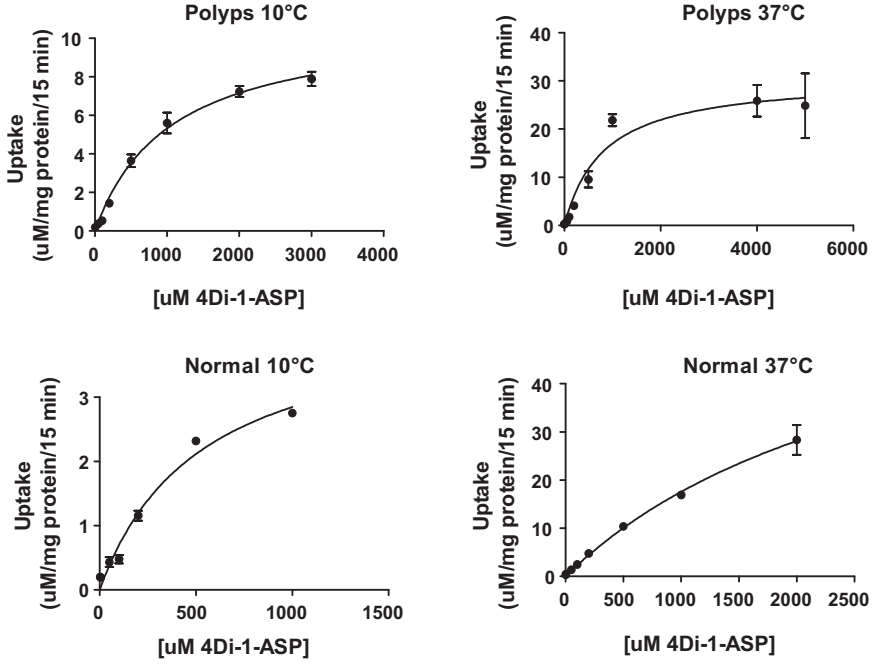

Fig. 1. Effect of concentration on the uptake of 4-Di-ASP in normal and polyps human nasal epithelium. Cells were incubated with the substrate at 37 °C on a shaker for 15 min. Data are expressed as mean ± SD (n = 3).

epithelium. The uptake of the compound in cells cultured from normal and polyps biopsies was rapid and follows Michaelis–Menten kinetics. The K_m (μM) and V_{max} (μM/mg protein/15 min) for 4-Di-1-ASP uptake in cells from normal tissue were 3031 ± 559.6 and 70.8 ± 8.8, respectively. These values were different from those calculated for polyps (K_m = 952.4 ± 207.8, V_{max} = 30.9 ± 2.1). The fact that the uptake of the compound at 0 °C was significantly lower than the values measured at 37 °C for both normal and polyps cells was an indication that the compound was actively taken up by the cells. The difference between the substrate uptake at 0 °C and uptake at 37 °C were taken into consideration during kinetic parameters calculation (Fig. 2). To further confirm the effect of temperature on active transport we investigated 4-Di-1-ASP uptake at a temperature where active transport is partially inhibited (10 °C). At this temperature, the rate of 4-Di-1-ASP uptake was between the values observed at 0 and 37 °C, respectively in both polyps and normal cells. For instance the rate of 4-Di-1-ASP (10 μM) uptake in polyps at 10 °C was 5.6 ± 0.5 μM/mg protein/15 min, while at 37 °C it was 21.9 ± 7.0 μM/mg protein/15 min. Also the compound was taken up by normal cells at 10 °C at a rate of 2.8 μM ± 0.1/mg protein/15 min compared to 16.9 ± 0.4 μM/mg protein/15 min at 37 °C (Fig. 1).

Hydrogen ion concentration or pH plays an important role for optimal solute transport of some OCTs especially the electrogenic isoforms (OCTN1 and OCTN2). Therefore, it was important to investigate the effect of pH on the uptake of 4-Di-1-ASP in normal and polyps cells (Fig. 3). Based on the figures, pH-dependent increase in 4-Di-1-ASP was observed between pH 4 and 8.5 for both normal and polyps cells. Based on Kruskal–Wallis analysis, pH significantly affected 4-Di-ASP-uptake in both normal and polyps cells

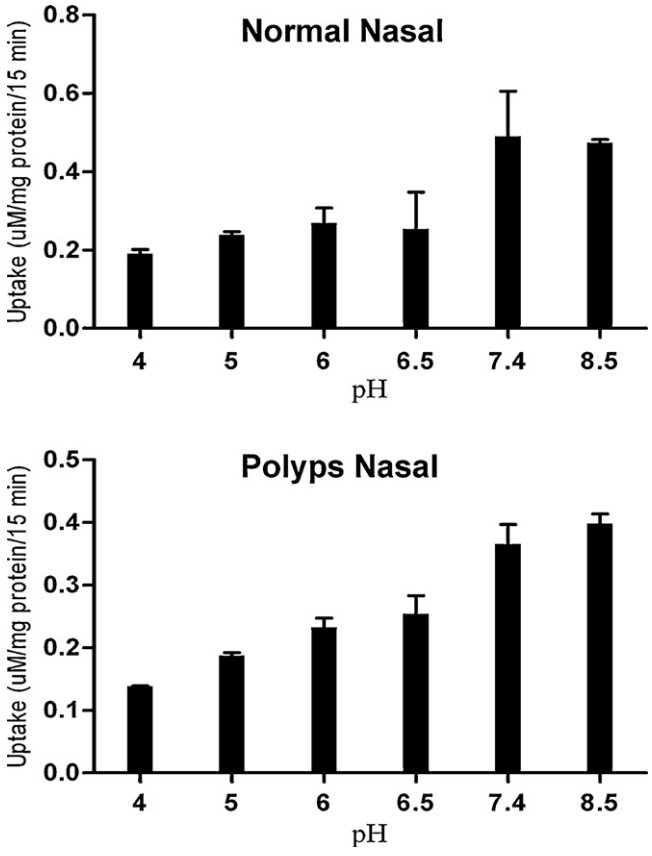

Fig. 3. Effect of pH on the uptake of 4-Di-ASP in normal and polyps human nasal epithelium. Cells were incubated with the substrate dissolved in HBSS adjusted to a specific pH using 0.1N hydrochloric acid or sodium hydroxide. Data are expressed as mean ± SD (n = 3).

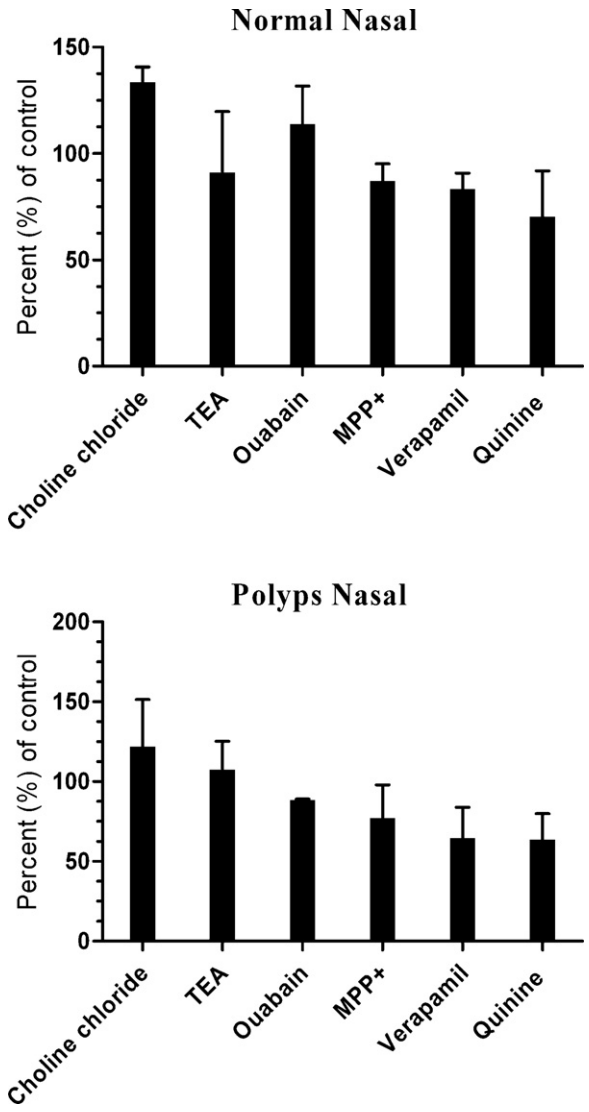

Fig. 4. Effect of inhibitors/OCTs substrates on the uptake of 4-Di-ASP in normal and polyps human nasal epithelium. Cells were pre-incubated with 1 mM inhibitors or substrates for 15 min, followed by uptake of the substrate in the presence of the competing or inhibiting compounds for an additional 15 min. Data are expressed as mean ± SD (n = 3).

(p = 0.0008, H = 31.8). However, Dunn's post-test analysis showed that pH had a similar effect on normal and polyps cells within the range of tested pH (p > 0.05). The comparable pH-dependent effect on 4-Di-1-ASP uptake in normal and polyps cells implies that the electrogenic OCTs (OCTN1 and OCTN2) were involved in the uptake of the compound in normal and polyps cells.

The specificity of 4-Di-1-ASP uptake in the cells from normal and polyps were compared using OCTs competitive and metabolic inhibitors (Fig. 4). The effect of the inhibitors was comparable in the two cell types. For instance whereas choline caused paradoxical increase in 4-Di-1-ASP uptake in both normal and polyps epithelial cells, there was no statistical difference in 4-Di-1-ASP uptake inhibition by 1 mM TEA, amiloride and oubain, respectively. Nevertheless, 1 mM verapamil and quinine resulted in significant reduction in 4-Di-1-ASP uptake in polyps and normal cells. Based on Kruskal–Wallis test (p = 0.0075, H = 27.1), and Dunn's post-test analysis for differences in inhibitors/substrates effect in normal and polyps cells (p > 0.05), it can be concluded that these compounds had comparable effects on normal and polyps epithelial cells (Fig. 5).

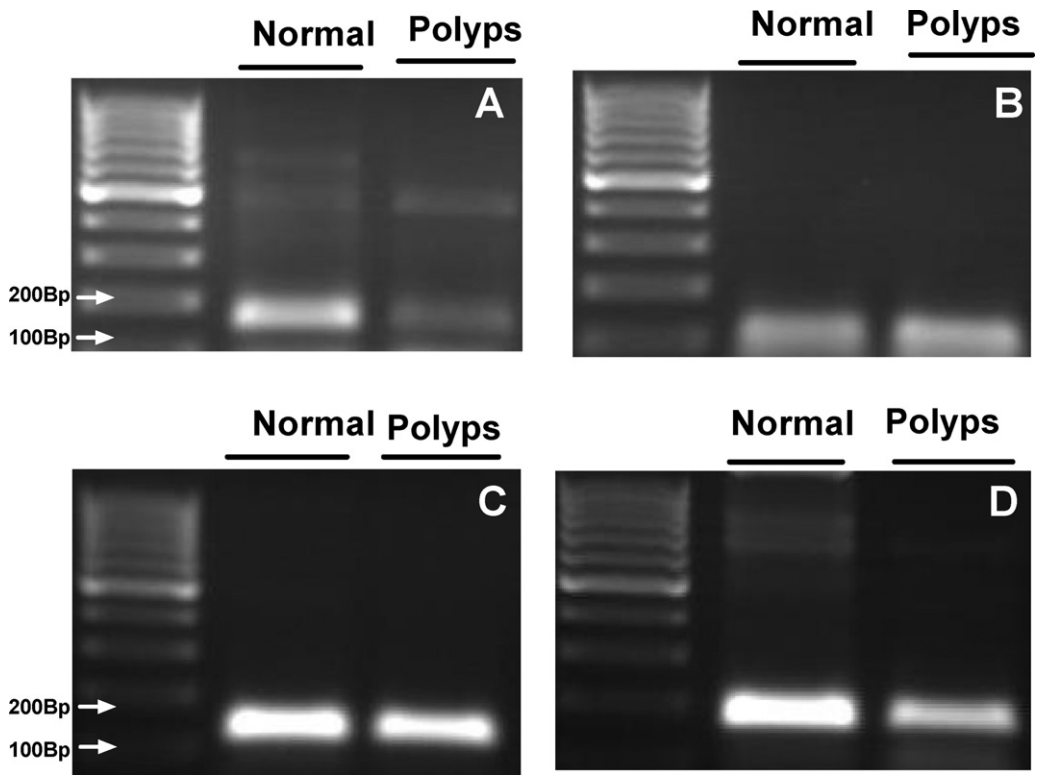

Fig. 5. Gene transcripts for OCT1 (A), OCT3 (B), OCTN1 (C) and OCTN2 (D) expression in normal and polyps nasal epithelium. Bands depict expected transcripts as follows: OCT1 (141 bp), OCT2 (100 bp), OCT3 (103 bp), OCTN1 (125 bp) and OCTN2 (169 bp). OCT2 was not detected in either normal or polyps tissue.

3.2. PCR-qPCR studies

Semi-quantitative and quantitative PCR methods were used to compare the differential expression of OCT1-3, OCTN1 and OCTN2 in cells cultured from normal and polyps biopsies. For quantification of the gene transcripts, β-actin was used as the house-keeping gene for relative gene expression determination. The result of the PCR studies is summarized in Fig. 5. As indicated in the figure, the expected genes for OCT1 (141 bp), OCT3 (103 bp), OCTN1 (125 bp) and OCTN2 (169 bp) were detected. Gene products for OCT2 were not detected in either normal or polyps cells (not shown). Quantitatively, the OCT isoforms were differentially expressed in the normal and polyps cells (Fig. 6). The order of OCTs expression in the normal cells was as follows: OCT3 ≫ OCTN2 > OCTN1 > OCT1 > OCT2. For polyps cells, a similar pattern was observed (OCT3 ≫ OCTN2 > OCTN1 > OCT1 > OCT2).

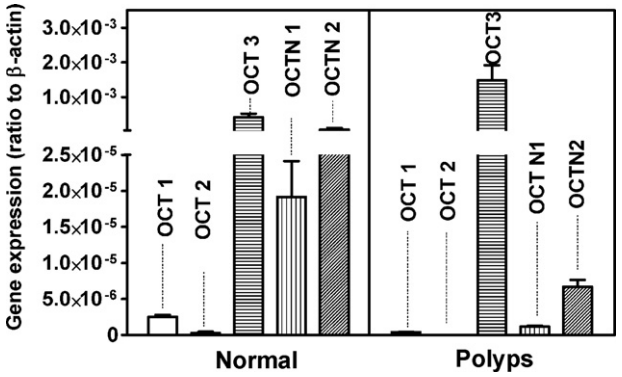

Fig. 6. Relative mRNA expression ($2^{-\Delta CT}$) of organic cation transporters in normal and polyps human nasal epithelium. Quantitative PCR data were based on 3 samples that were normalized with β-actin. Data are expressed as mean ± SD.

Based on quantitative and non-quantitative PCR studies, it may be inferred that OCT2 is not reproducibly expressed in both normal and polyps cells. Although the levels of OCT gene expression significantly varied in both normal and polyps cells ($p = 0.0049$, $H = 23.6$), Dunn's post-test analysis did not show a significant difference between the pattern and levels of expression of the various OCT isoforms in normal and polyps cells ($p > 0.5$).

4. Discussion

Cells from normal and polyps nasal tissues were studied to determine possible differences and similarities with respect to the expression and functional activity of organic cation transporters using 4-Di-1-ASP, a fluorescent low molecular weight organic cation transporter substrate with a pK_a of 3.6. Our first goal was to investigate whether chronic inflammation associated with polyps induces up- or down-regulation of the OCTs, and if such changes significantly affect solute uptake. If histological changes resulting from chronic inflammation affect drug uptake, this may have some implications for nasal absorption of organic cations. It may also be usefully in interpreting and extrapolating *in vitro* experimental drug uptake and transport studies generated using polyps biopsies to normal cells.

The major parameters used to compare the functional and molecular expression of the OCTs in normal and polyps cells were effect of concentration, temperature, pH and inhibitors/substrates on 4-Di-1-ASP uptake. 4-Di-1-ASP was selected as an organic cation substrate based on its fluorescent characteristics and validation as substrate for OCTs and OCTNs (Pietruck and Ullrich, 1995). Also semi-quantitative and quantitative expression of organic cation genes was also used. As indicated in Figs. 1 and 2, 4-Di-1-ASP was actively absorbed in normal and polyps cells in concentration-, temperature-dependent and saturable manner. The higher V_{max} and K_m values at 37 °C for normal and polyps cells compared

to at 10 °C (partial active transport) indicates that cells isolated from both biopsies behaved the same way when exposed to different temperatures. Quantitatively, the observed temperature-dependency as exemplified with the uptake of 1000 μM 4-Di-1-ASP in polyps cells at 37 °C compared to 10 °C (4-fold decrease) was comparable to 6-fold reduced uptake observed in normal cells.

The effect of pH and inhibitors on 4-Di-1-ASP uptake in normal nasal and polyps cells also yielded comparable results. For instance, concentration-dependent increase in uptake was observed in normal and polyps cells between pH 4.5 and 8.5. Similarly, there was no statistical difference on the effect of the inhibitors on 4-Di-1-ASP uptake in normal and polyps biopsies. The slightly higher, but not statistically significant effect of the inhibitors/substrates in polyps cells relative to normal cells again correlates with the expression level of OCTs in the polyps tissue. TEA, which has a high affinity for OCT1 and OCT2 transporters did not significantly inhibit 4-Di-1-ASP uptake in both normal and polyps, an indication that the uptake of the compound in both normal and polyps cells was most likely mediated by OCT3, OCTN1 and OCTN2.

One of the critical differences between normal nasal mucosa and polyp is the number and cytoarchitecture of the seromucous glands and goblet cells (Bernstein, 2001). Despite the various possible changes in polyps histology, the hallmark of its histopathology is marked tissue eosinophilia, edema, and alteration of the cytoarchitecture of the surface epithelium (Bernstein, 2001). These changes often lead to altered ion and water transport (Lee et al., 2005), but not to changes in organic cation transporters. This was highlighted by lack of significant differences on the effect of temp, inhibitors and gene expression levels of OCT1-3, OCTN1 and OCTN2 in polyps and normal cells. Nevertheless, it is important to take into account the fact that histochemically polyps may be broadly classified into four groups that include (Pawankar, 2003): (1) esinophilic edematous polyps (edematous stroma with a large number of eosinophils); (2) chronic inflammatory or fibrotic polyps (large number of inflammatory cells mainly lymphocytes and neutrophils with fewer eosinophils); (3) seromucinous gland polyps; and (4) atypical stromal polyps. The polyps used in our studies were mainly of a chronic inflammatory type. Although we did not find significant differences between cells from these biopsies and normal cells with respect to organic cation transport, it may still be possible that the expression of OCTs is significantly different in other types of polyps. Either up- or down-regulation of OCTs in a specific type of polyps may alter nasal drug absorption. It may also affect pulmonary drug absorption because polypopsis is a chronic inflammatory multifactorial disease that is often associated with asthma and other respiratory diseases like cystic fibrosis, primary ciliary dyskinesia, and aspirin sensitivity (Lee et al., 2005).

5. Conclusions

The results of this study showed that chronic inflammation resulting in polyps did not significantly alter the expression levels of OCTs in human nasal epithelium. Also, functional attributes of the transporters were not significantly altered. This implies that there may not be any significant inter-individual variability in drug absorption and therapeutic response for drugs that are substrates for the transporters in patients with polyps compared to those without the disease. The study also showed that polyps biopsies may be used for *in vitro* studies involving OCTs without compromising the results of the experiments.

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