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Gene Expression for Carbonic Anhydrase Isoenzymes in Human Nasal Mucosa

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Abstract

Carbonic anhydrase (CA) is physiologically important in the reversible hydration reaction of CO_2; it is expressed in a number of isoforms (CA I–XIV) with varying degrees of enzymatic activity. In nasal chemesthesis, CA inhibition decreases the electrophysiologic response to CO_2, a common irritant test compound. CA enzymatic activity has been demonstrated in the human nasal mucosa using enzyme histochemical methods, but no systematic study of nasal mucosal CA isoenzyme gene expression has been published. We examined CA gene expression in superficial nasal mucosal scrapings from 15 subjects (6 females; 6 allergic rhinitics; age range, 21–56 years). Both non-quantitative and quantitative reverse transcription polymerase chain reaction (RT-PCR) were performed using primers for each gene coding for the 11 catalytically active CA isoenzymes and the housekeeping gene GADPH. Amplification products of GADPH and 10 of the 11 CA genes were detected in the specimens (CA VA was not detected). Relative expression of the CA genes was quantified using real-time PCR. Averaged across subjects, the relative abundance of the CA isoenzyme transcripts is as follows: CA XII > CA II > CA VB > CA IV > CA IX > CA III > CA XIV > CA I > CA VI > CA VII. Limited qualitative validation of gene expression was obtained by immunohistochemistry for CA I, CA II and CA IV. We also observed inter-individual variability in the expression of CA isoenzymes in human nasal mucosa, potentially contributing to differences in nasal chemosensitivity to CO_2 between individuals

Key words: carbon dioxide, carbonic anhydrase, gene expression, nasal epithelium, nasal irritation, quantitative PCR

Introduction

Carbon dioxide has been used extensively as a chemosensory testing agent because it is a 'pure' irritant which lacks a distinct odor (Cain, 1987). The mechanism of action of CO_2 excitation of sensory endings in both the oral and nasal cavities involves hydration and subsequent dissociation into hydrogen and bicarbonate ions (Bryant, 2000; Dessirier *et al.*, 2001). This reaction is facilitated by the enzyme carbonic anhydrase (CA) (EC 4.2.1.1), a zinc-containing metalloenzyme that catalyzes the reversible hydration of carbon dioxide. CA activity has been demonstrated in nasal mucosa using an enzyme-histochemical technique. (Brown *et al.*, 1984; Cavaliere *et al.*, 1996; Okamura *et al.*, 1996; Coates *et al.*, 1998). In animals, numerous CA isoenzymes and related proteins have been reported, eleven of which (CAI–VII, IX, and XII–XIV) are catalytically active (Chegwidden and Carter, 2000). These CA isoenzymes differ in kinetic properties, tissue distribution, and subcellular localization (Table 1).

CA plays different roles in various tissues. In the nasal mucosa its proposed role is in pH homeostasis in the nasal epithelium (tissue and/or mucus) and electrolyte transport across epithelial cells. This hypothesis is supported by the increase in pH of nasal secretion accompanied by changes in Na^+, K^+ and Cl^- concentrations observed after the inhibition of carbonic anhydrase activity by dichlorphenamide (Cavaliere *et al.*, 1996). Modulation of epithelial regulation of the pH of nasal secretion is suggested by the observation of pH increases in rhinitis of various etiologies (Cavaliere *et al.*, 1996).

CO_2 stimulation of nerve endings found in the cornea, nasal mucosa and oral cavity gives rise to pain and sensory irritation (Chen *et al.*, 1995; Simons *et al.*, 1999; Alimohammadi and Silver, 2001). Since these responses are mitigated by application of CA inhibitors, it is believed that CO_2 irritation responses are dependent on CA activity (reviewed in Bryant, 2000). The proposed perireceptor mechanism is that CA-catalyzed hydration of CO_2 results in a rapid elevation of intraepithelial H^+ (and a resulting decrease in the local pH) that is sensed by acid-sensitive receptors like ASIC (Waldman *et al.*, 1997) and VR1 (Caterina *et al.*, 2000), leading to pain mediated via the trigeminal nerve pathway.

Despite the increasing evidence on the importance of CA activity in the nasal mucosa, there has been a dearth of

Table 1 Cellular localization and activity of CA isoenzymes (Chegwidden and Carter, 2000)

Isoenzyme	Localization	Activity	Tissue expression
CA I	Intracellular, cytoplasmic	Moderate	Red blood cell, intestine
CA II	Intracellular, cytoplasmic	High	Ubiquitous
CA III	Intracellular, cytoplasmic	Low	Red muscle, adipose tissue
CA IV	Extracellular, membrane-bound	High	Kidney, lung, gut, brain, eye, capillary endothelium
CA VA	Intracellular, mitochondrial	High	Liver, skeletal muscle, kidney
CA VB	Intracellular, mitochondrial	High	Widespread (except liver)
CA VI	Extracellular, secreted	Moderate	Salivary glands
CA VII	Intracellular, cytoplasmic	High	Brain, salivary gland, lung
CA IX	Extracellular, transmembrane	High/moderate	Gastric mucosa, various tumors
CA XII	Extracellular, transmembrane	High/moderate	Widespread
CA XIV	Extracellular, transmembrane	Low	Widespread

studies on the activity of individual carbonic anhydrase isoenzymes in this tissue. Our interest has been to catalogue the CA isoenzyme genes that are expressed in the nasal mucosa. Apart from a single study claiming that CAXII is expressed in the nasal mucosa of allergic rhinitic (but not rhinitic) subjects, the relative distribution of CA isoenzymes in the nasal mucosa is incompletely characterized (Berkes, 2002).

Psychophysically, we have been examining the distribution of CO_2 detection thresholds as a function of allergy status, age and gender (Shusterman *et al.*, 2001). Inter-individual variability in chemesthetic sensitivity to irritants, including CO_2, has been explored in other laboratories as well (reviewed in Shusterman, 2002). Despite evidence of a neuromodulatory effect of allergy on airway nerve function (Undem *et al.*, 2000), the biological basis of these observed differences remains largely unexplained. Thus our ultimate objective for this research is to characterize CA activity, as indexed by CA mRNA levels in nasal scrapings, as a potential explanatory variable in chemosensory studies involving CO_2. Our immediate goal in this preliminary study is to analyze for the relative quantitative expression of known active CA isoenzymes using RNA derived from nasal scrapings from individuals with known allergy status, age and gender. We are interested in learning if there is a significant difference in gene expression for the various catalytically active CA isoenzyme across pooled subjects and if there are associations between individual subject characteristics (i.e. age, allergy status and gender) and CA isoenzyme gene expression.

Materials and methods

Subjects

Fifteen subjects were recruited through posters and newspaper advertisements. Inclusion criteria included ages 18–69 year and 'general good health'; exclusion criteria were: (i) asthma, (ii) active smoking (within 6 months) and (iii) pregnancy or lactation. Questionnaires were administered and confirmatory allergy skin tests involving 13 common aeroallergens were performed on all prospective subjects to classify them as either AR (allergic rhinitic) or NR (non-rhinitic). Allergic rhinitics consisted of 6 subjects (4 males and 2 females, 21–55 years of age; mean age 35.0) while non-rhinitics consisted of 9 subjects (5 males and 4 females, 28–56 years of age; mean age 40.2). All subjects read and signed an informed consent document approved by the Committee on Human Research of the University of California, San Francisco prior to the experimental study.

Tissue sampling from nasal epithelium

Nasal tissue scrapings of the medial surface of the inferior turbinates were taken bilaterally under direct visual inspection using a Rhinoprobe (Arlington Scientific, Arlington, TX) disposable plastic curette (1.5 mm diameter, 0.5 mm depth). Scrapings were immediately dispersed in 600 µl of RLT Lysis buffer (Rneasy RNA mini kit; Qiagen, Santa Clarita, CA) by vortexing for 30 s and immediately stored at –80°C prior to RNA extraction. To verify the cell populations obtained by this method (i.e. respiratory vs. transitional vs. squamous epithelium), an additional nasal scraping was taken, smeared on a slide and stained with Wright–Giemsa Stain.

Preparation, quantitation of total RNA and RT-PCR

Total RNA from nasal scrapings was isolated using commercial kits (Qiashredder and Rneasy kit; Qiagen), following the manufacturer's instructions. After extraction, the RNA was treated with DNaseI (DNA-free, Ambion, Austin, TX) as follows: to 50 µl of total RNA in water, we added 5 µl of DNA-free buffer, 2 µl of Superase RNAse inhibitor (Ambion) and 1 µl of DNaseI and incubated the mixture at 37°C for 20 min. To clean up the RNA, the mixture was loaded onto an Rneasy column and total RNA was eluted with water in a volume of 40 µl. The quantity and quality of the RNA samples were determined using the RNA 6000 LabChip kit (Agilent Technologies, Inc, Wilmington, DE). To detect the expression of CA isoenzyme genes we carried out two-step reverse transcription polymerase chain reaction (RT-PCR) analysis of the total RNA obtained from each subject. For the RT reaction, 30 ng of RNA was used in a 20 µl reaction using Superscript II

(Superscript first-strand cDNA synthesis system, Invitrogen, Carlsbad, CA) using either 200 ng of random hexamers or 0.5 μg poly d(T) as primers for first-strand cDNA synthesis.

We carried out non-quantitative hot-start PCR to detect the presence of the CA isoenzyme transcripts. To normalize for possible variations in the amount of total RNA, we used GAPDH as an endogenous control (GAPDH is a housekeeping gene that is highly expressed in human tissues). Gene-specific primers for the 11 carbonic anhydrase genes (CA I, II, III, IV, VA, VB, VI, VII, IX, XII and XIV) and GAPDH were designed using Primer Express software (Perkin Elmer) based on sequencing data from the National Center for Biotechnology Information databases and purchased from BioSearch Technologies, Inc (Novato, CA). The sequences of these primers are shown in Table 2. Each PCR reaction (25 μl) contained 1 μl of the cDNA material from the RT reaction, 10 μM of the reverse and forward primer for each gene, 2.5 μl of 10 × Advantage2 PCR buffer (Clontech, Palo Alto, CA), 10 mM of each dNTP and 0.5 μl of Advantage Polymerase mix (Clontech). Amplification was carried out using the following thermal cycling conditions in a Tpersonal thermocycler (Biometra, Gottingen, Germany): 95°C for 2 min to inactivate the anti-Taq antibody, followed by 15 cycles of touchdown PCR [95°C for 45 s, 75°C (–1°C for each subsequent cycle) for 1 min] and 30 cycles of two-step PCR (95°C for 45 s, 60°C for 1 min). For analysis of the PCR products, 10 μl of the PCR reactions were loaded onto a 2% TAE (Tris acetate EDTA) agarose gel and subjected to electrophoresis. After staining with ethidium bromide (0.5 μg/ml), the gel was photographed.

For quantitation of CA isoenzyme gene expression, we carried out real-time PCR using TaqMan unlabeled PCR primers and a FAM (fluorescein) dye-labeled TaqMan MGB (minor groove binder) primer probes on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Inc., Foster City CA). The MGB probes have a minor groove binder at the 3′ end of the probe which increases the Tm of probes, allowing the use of shorter probes. TaqMan primers and MGB probes for carbonic anhydrase isoenzyme genes (CA I, II, III, VB, VI, VII, XII and XIV) were obtained from Assays on Demand (Applied Biosystems, Inc.). GAPDH TaqMan primers and MGB probe were obtained as Pre-Developed Assay Reagents from Applied Biosystems, Inc. CA isoenzyme genes (i.e. CA I, II, III, IV, VB, VI, VII, IX, XII, XIV) and the endogenous reference gene (GAPDH) were amplified by real-time PCR in separate tubes. For real-time PCR, cDNA from 3 ng of total RNA was used in 25 μl of TaqMan Universal Master Mix (Applied Biosystems, Inc.) with the TaqMan Primers and MGB probe. The PCR thermal cycling conditions were those specified by the manufacturer (Applied Biosystems, Inc.). All experiments were conducted with duplicate samples and C_T units obtained as the average of the results from the replicates. C_T is defined as the threshold cycle for PCR amplification that is detected by the ABI prism DNA sequence detector at a threshold value set at 0.45 for each run. Relative quantitation of the CA isoenzyme gene expression was done using the comparative C_T method. Relative quantitation computations were carried out as shown in User Bulletin 2 (Relative quantitation of gene expression, Applied Biosystems, Inc.).

To use the relative quantitation method, a validation experiment was performed to demonstrate that the efficiencies of the real-time PCR reactions for GAPDH and each of the CA isoenzyme genes were approximately equal. In the validation experiment, real-time PCR using the GAPDH and CA gene primers and probes was performed on four dilutions (1:1, 1:5, 1:10, 1:20) of cDNA synthesized from total RNA of one of the subjects. The ΔC_T value (computed as $\Delta C_T = \text{ave}C_{T,\text{gene X}} - \text{ave}C_{T,\text{GAPDH}}$) for each cDNA dilution was graphed against the log amount of cDNA. The absolute value of the slope of this graph for each of the CA isoenzyme

Table 2 Primer sequences used for non-quantitative RT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Fragment size
CA I	AAATGAGCATGGTTCAGAACATACA	ACTTTGCAGAATTCCAGTGAGCTA	83
CA II	TGGACTGGCCGTTCTAGGTATT	CCAGCACATCAACAACTTTCTGA	77
CA III	CACACCGTGGATGGAGTCAA	GTGTTATACTTCGGGTTCCAGTGA	68
CA IV	TCGGAGCACAGCCTCGAT	CTCGATGTCCCCTTCTCTTTCTC	77
CA VA	CACCTGGATCATCCAGAAGGA	ACAGGAGAGTACGAAATGCAGAGA	71
CA VB	GCCCTGCAGCCTCTATACTTGT	CACGCTCTCCCAGAGTGGAT	70
CA VI	GATGCGCCGGATGGTTT	GTAATAAGTGTTTTCAGGGTAATTCTTCAC	72
CA VII	GCTCTACATGGTCCGGTTCAA	GCAGGCAGGAGGCACTTG	72
CAIX	GAAAACAGTGCCTATGAGCAGTTG	TCCTGGGACCTGAGTCTCTGA	78
CA XII	ACTGCGGCAGGACTGAGTCT	CACAATACAGATGCCAAGAATGC	72
CA XIV	TCAGAACACCAGATCAACAGTGAA	CACTCAAGCTGTCATAGGAATCAGA	85
GAPDH	TCGGAGTCAACGGATTTGG	GCAACAATATCCACTTTACCAGAGTTAA	79

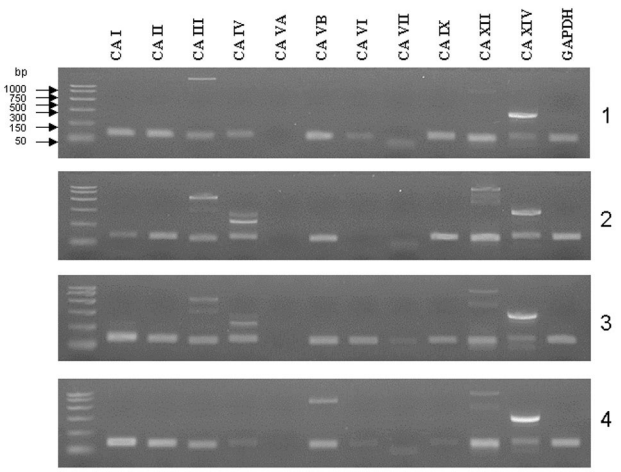

Figure 1 RT-PCR amplification of CA isoenzyme genes. cDNAs derived from nasal mucosal RNA of four subjects (1–4) were amplified with CA isoenzyme gene- and GAPDH-specific primers, run on agarose gels, and stained with ethidium bromide.

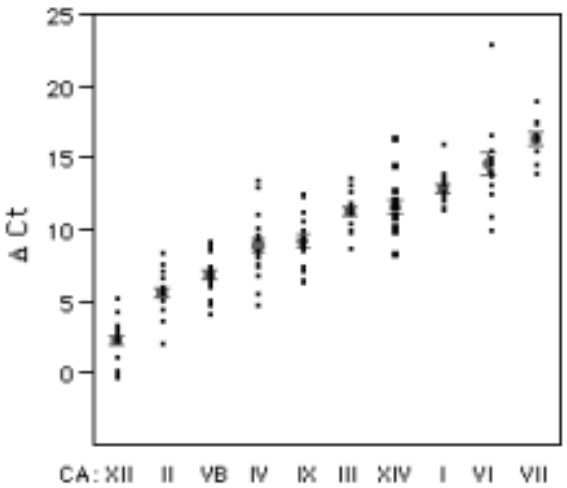

Figure 2 Relative expression (expressed in ΔCT) of the different CA isoenzyme genes in the 15 subjects arranged from the highest expressed (CA XII) to the lowest (CA VII). Averaged across subjects, there are significant differences in gene expression among the various CA isoenzymes ($P < 0.0001$).

genes was ≥0.5 which indicates that the PCR efficiencies for GAPDH and the CA isoenzyme genes were approximately equal.

The relative expression of CA isoenzymes was normalized to the expression of GAPDH and expressed in ΔC_T units as described above. The relative abundance of each CA isoenzyme mRNA was averaged for all 15 subjects. A lower ΔC_T value indicates higher gene expression. The relative abundance of each CA isoenzyme mRNA was averaged for all 15 subjects.

Statistical analysis

Statistics were performed using either Excel (Microsoft Corp., Redmond, WA) or the JMP statistics package (SAS Institute, Cary, NC). Gene expression data were first checked for normality, then compared by isoenzyme or demographic markers using ANOVA. Cross-correlations between CA isoenzymes was done by least-squares regression. A P value of < 0.05 was regarded as statistically significant.

Immunocytochemistry

Tissue for immunocytochemical staining was collected from 4 of the 15 volunteers using a Rhinoprobe as described above. The tissue scrapings were fixed on glass slides with 4% paraformaldehyde for 30 min at room temperature, dried and processed for indirect immunofluorescence. Primary antibodies against carbonic anhydrase isoenzymes species: goat anti-human CA I (1:400, Chemicon), rabbit anti-human CA II (1:400, Chemicon) and rabbit anti-rat CA IV (1: 400, Drs W. Sly and A. Waheed, St Louis University) were incubated with tissue overnight at 4°C. Isoenzymes were detected using Cy3-labeled secondary antibodies (Jackson Labs donkey anti-rabbit or goat).

Results

Expression of carbonic anhydrase in human nasal mucosal cells

Figure 1 shows the RT-PCR amplification of the CA isoenzyme genes and the GAPDH housekeeping gene in total RNA extracted from the nasal mucosal tissue in four of the subjects. The primers were designed to amplify 70–80 bp products during the PCR reaction. PCR amplified products of the expected size for GAPDH, and most of the CA isoenzyme genes could be seen from the gels. We also observed variable intensity for the amplified PCR products for CAI, CA IV, CAVI and CA IX. Specifically we found that some individuals had no detectable expression of some of these CA genes. There was also very low expression for CAVII and no detectable expression of CA VA. None of the primer sets used amplified a band from human genomic DNA under the conditions described presumably because the forward and reverse primers for each CA gene spanned across an intron in the corresponding genes.

Results of quantification of the relative expression of the different CA isoenzyme genes (excluding CA VA, which was not detected) are shown in Figure 2 and Table 3. The relative abundance for the different CA isoenzyme genes, averaged across subjects is as follows: CA XII > CA II > CA VB > CA IV > CA IX > CA III > CA XIV > CA I > CA VI > CA VII. We also normalized the relative expression of each CA isoenzyme gene to the expression of CA VII, the isoenzyme with the lowest level of expression (Table 3). The results indicate that compared to CA VII, the average expression of the different CA isoenzyme genes in the nasal mucosa

Table 3 Relative expression of CA isoenzyme genes

CA gene	% Expression in subjects	$\Delta C_T \pm$ SD[a] ($C_{T,CA} - C_{T,GAPDH}$)	Fold variation (highest – lowest)/lowest	Expression relative to CAVII[b]
CA I	87	13.06±1.2	16.76	11.28
CA II	100	5.70±1.58	7.58	1842.57
CA III	100	11.40±1.32	12.85	35.68
CA IV	100	9.03±2.44	12.22	183.44
CA VA	0	ND[c]	ND	ND
CA VB	100	6.90±1.45	8.31	803.36
CA VI	87	13.99±1.93	15.86	3.63
CA VII	60	16.55±1.56	19.19	1.00
CA IX	100	9.31±1.91	11.59	151.74
CA XII	100	2.29±1.63	4 .42	19 708.09
CA XIV	100	11.69±1.95	15.43	29.09

[a]ΔC_T value is determined by subtracting the average GAPDH C_T value from the average CA gene C_T value from all 15 subjects.
[b]Computed using the expression $2^{-(\Delta CT, CA - \Delta CT, CA VII)}$
[c]ND, not detected.

Table 4 Correlation coefficient of ΔC_T values of various CA genes[a]

Variable	ΔC_T CA I	ΔC_T CA II	ΔC_T CA III	ΔC_T CA IV	ΔC_T CA VB	ΔC_T CA VI	ΔC_T CA VII	ΔC_T CA IX	ΔC_T CA XII	ΔC_T CA XIV
ΔC_T CA I	1	0.0526	0.6648**	–0.1684	0.7936**	–0.2798	–0.4459	0.8108***	0.5540*	0.6708***
ΔC_T CA II		1	0.4252	0.5934*	0.1298	0.5725*	0.3105	0.2981	0.5485*	0.1971
ΔC_T CA III			1	0.086	0.7642***	0.1815	–0.3075	0.8267***	0.9087***	0.8814***
ΔC_T CA IV				1	–0.0212	0.4552*	0.1938	0.0913	0.244	–0.1312
ΔC_T CA VB					1	–0.0069	–0.5455*	0.8547***	0.7641***	0.8295***
ΔC_T CA VI						1	0.1739	–0.0105	0.2993	–0.0718
ΔC_T CA VII							1	–0.2949	–0.2757	–0.4012
ΔC_T CA IX								1	0.8688***	0.8318***
ΔC_T CA XII									1	0.8395***
ΔC_T CA XIV										1

[a]ΔC_T value is determined by subtracting the average GAPDH C_T value from the average CA gene C_T value from all 15 subjects.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

ranged from 4-fold (CAVI) to almost 20 000-fold (CA XII) greater.

Figure 2 shows the relative expression of the different CA isoenzyme genes in each of the 15 subjects. Analysis of variance of this data indicates that, averaged across individuals, there is significant inter-gene difference in transcript levels ($P < 0.0001$). Further, 13% of the subjects (2 out of 15) showed no detectable expression of CA I and CA VI, while 40% of the subjects (6 out of 15) did not have detectable expression of CA VII. Inter-individual variation in relative gene expression was lowest for CA XII (4.4% variation; Table 3) and highest for CA I, CA VI, CA VII and CA XIV (15- to 20-fold variation; Table 3). We also examined the correlation coefficients of the relative expression of the CA isoenzyme genes with each other using mean data from all 15 subjects. The results shown in Table 4 indicate that there is a high correlation for the relative expression for subsets of CA isoenzyme genes. For instance, subjects with high expression of CA VB also have high relative expression of CA I, CA III, CA IX, CA XII, and CA XIV ($r \geq 0.8$; $P < 0.01$). While the subjects' relative expression for CA II, CA IV and CA VI show a more modest correlation ($r \geq 0.57$; $P < 0.05$). On the other hand, CA VII expression appears to negatively correlate with the expression of other CA isoenzyme genes, notably CA VB ($r = –0.54$; $P < 0.05$) and CA I ($r = –0.46$; $P < 0.10$).

To better assess individual variation in CA isoenzyme expression, we compared relative expression of CA

Table 5 Relative gene expression (ΔC_T) of CA isoenzyme genes by subject characteristic[a]

CA gene	Ave ΔC_T male	No.	Ave ΔC_T female	No.	Ave ΔC_T male–female	Ave ΔC_T AR	No.	Ave ΔC_T NR	No.	Ave ΔC_T AR–NR	Slope ΔC_T vs. age
CA I	13.35	7	12.71	6	0.64	13.86	5	12.55	8	1.31*	0.0173
CA II	5.18	9	6.49	6	–1.31	5.64	6	5.75	9	–0.11	–0.0019
CA III	11.77	9	10.84	6	0.93	11.95	6	11.02	9	0.93	–0.0276
CA IV	8.20	9	10.25	6	–2.03	9.32	6	8.84	9	0.48	0.0041
CA VB	7.35	9	6.24	6	1.11	7.82	6	6.29	9	1.53**	–0.0170
CA VI	15.20	9	13.55	4	1.64	13.96	6	15.32	7	–1.36	0.1523**
CA VII	16.09	6	17.48	3	–1.39	15.92	4	17.06	5	–1.14	0.0704
CA IX	9.75	9	8.64	6	1.11	10.41	6	8.57	9	1.83	–0.0226
CA XII	2.47	9	2.01	6	0.46	3.00	6	1.81	9	1.20	–0.0134
CA XIV	12.19	9	10.94	6	1.25	12.77	6	10.97	9	1.80	–0.0067

[a]ΔC_T value is determined by subtracting the average GAPDH C_T value from the average CA gene C_T value from a subset of the 15 subjects exhibiting the subject characteristic.
*$P = 0.05$; **$P > 0.05$.

isoenzyme genes as a function of the subjects' demographic factors such as age, gender and allergy status. The results of our analysis are shown in Table 5. There was a statistically significant association between the relative gene expression of CA I and CA VB and the allergy status of the subjects. Subjects with no allergies had higher expression of CA I and CA VB than subjects with allergies ($P \leq 0.05$; Table 5). There was also a trend toward lower expression of CA IX and CA XIV ($P < 0.1$; Table 5) for subjects with no allergies. With regards to age, the only statistically significant association was with the relative gene expression for CA VI ($P < 0.05$; Table 5) with older subjects having lower gene expression than younger subjects. There was no statistically significant association between subject gender and the expression of any CA isoenzyme gene (Table 5).

Immunocytochemistry of CA proteins

From each subject, a confirmatory slide was stained and examined to verify that the target area was sampled. The inferior turbinate of the nasal cavity is covered by a ciliated, pseudocolumnar epithelium, whereas the anterior nares are covered by squamous epithelium and an intermediate morphology ('transitional epithelium') overlies the turbinates' anterior tip. As expected, cytologic examination of our nasal scrapings indicate columnar epithelial cells with very few squamous cells (data not shown). We performed immunofluorescence studies on nasal smears from the four subjects represented in Figure 1 using antibodies against CA I, CA II and CA IV. The qualitative immunofluorescence results were in accord with our expression data for these three CA isoenzyme genes. We found CA II and CA IV immunoreactivity (IR) in nasal mucosal cells and no staining above background for CA I (Figure 3). In addition, we found that there was a difference in which types of cells expressed CA II and CA IV. CA II-IR was found predominantly in columnar epithelial cells, which expressed no CAIV-IR. Conversely, we found CA IV-IR and little CA II-IR in a non-ciliated cell type. Thus there appears to be differential distribution of CA isoenzymes among the different types of nasal mucosal cells.

Discussion

This study provides the first systematic survey of relative gene expression for the catalytically active CA isoenzyme genes in the human nasal mucosa. Further, we applied the real-time PCR technique to provide a precise relative quantitation of CA isoenzyme trancripts. Our results indicate that all of the catalytically active CA isoenzymes except CA VA are expressed in the nasal mucosa. We also found that there are variations in individuals' gene expression for various CA isoenzyme genes and that some of these individual variations in CA gene expression correlate with the allergy status and age of the subjects.

Carbonic anhydrase has been implicated to have various roles in the nasal mucosa. CA is believed to play a role in pH regulation of nasal secretions, in electrolyte transport through the epithelium (Cavaliere *et al.*, 1996) and in the nasal trigeminal response to CO_2 irritation (Bryant, 2000). While CA histochemical activity has been demonstrated in various regions of the nasal cavity (Brown *et al.*, 1984; Cavaliere *et al.*, 1996; Okamura *et al.*, 1996; Coates *et al.*, 1998), it is not known which specific CA isoenzymes are functional and responsible for the activity. This study provides some clues in this regard. The high level of expression of CAXII, CA II and CA VB may indicate their importance in general CA activity in the nasal mucosa. These three CA isoenzymes are widely expressed in many tissues, have moderate to high CA activity, but are expressed in different cellular compartments (Chegwidden and Carter, 2000). CAII is an intra-

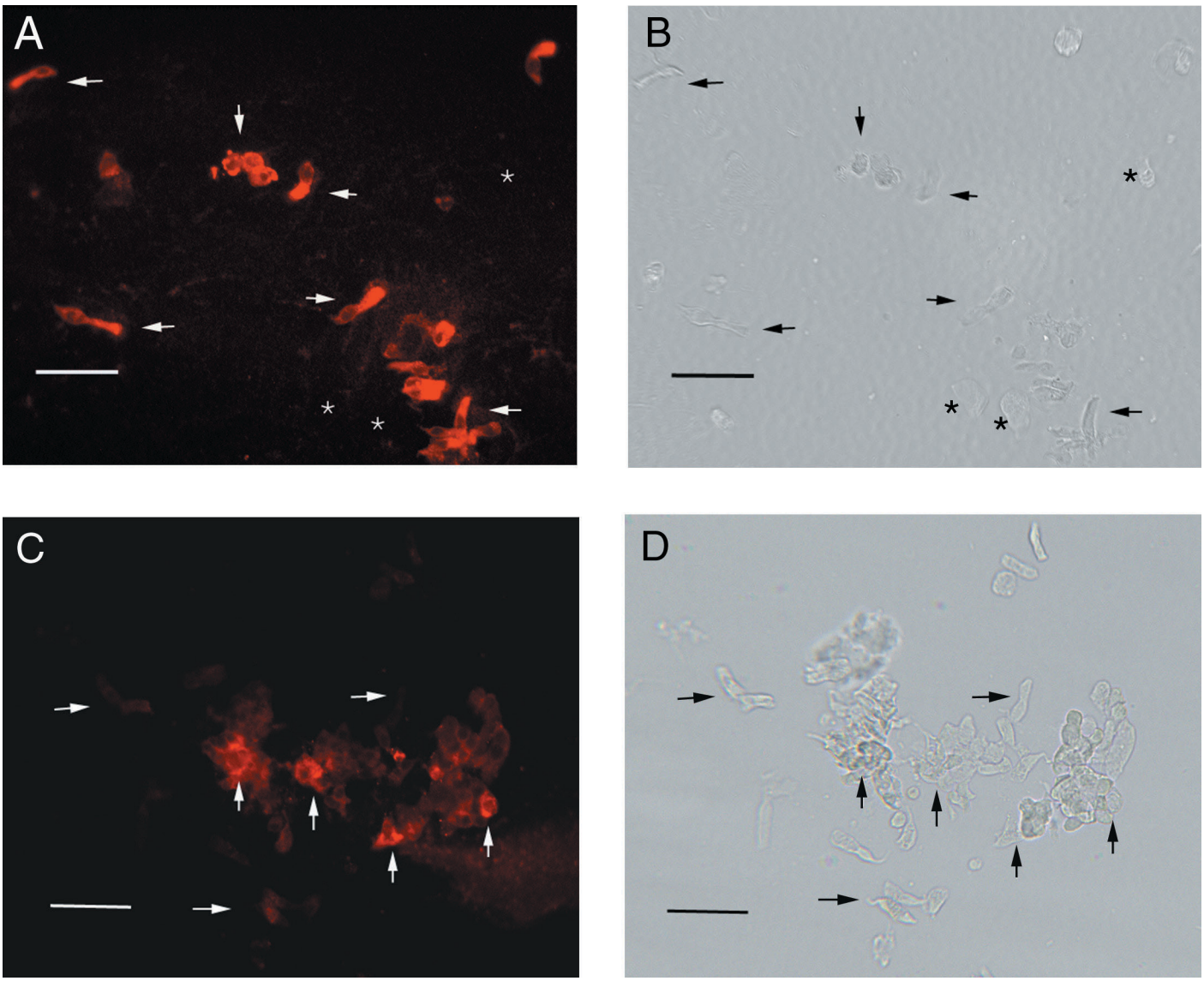

Figure 3 Fluorescence **(A, C)** and brightfield images **(B, D)** of nasal cytological smears obtained from subjects whose CA gene expression is PCR profiled in Figure 1. CA II immunoreactivity is seen in ciliated columnar epithelial (A, horizontal arrows) as well as some non-ciliated cells (A, vertical arrows). Cells without immunoreactivity are also found (*). CA IV immunoreactivity is absent from ciliated columnar epithelial cells (C, horizontal arrows). Other types of cells exhibit variable CA IV immunoreactivity (C, vertical arrows). Scale bar is 50 μm.

cellular, cytoplasmic enzyme; CAVB is also intracellular but localized in the mitochondria, while CA XII is an extracellular, transmembrane enzyme (Table 1). Two other extracellular CAs (CA IV and CA IX) are expressed at moderate levels in the nasal mucosa (Table 3). The low and inconsistent expression for the other CA isoenzymes (i.e. CA I, CA VI and CA VII) could reflect low level-constitutive expression in nasal epithelial cells or alternatively confounding by 'auxiliary' cell types incidentally collected during sampling (such as nerve endings, leukocytes and submucosal cells). For instance, some of the CA I expression may be from reticulocytes (0.5–1.5% of total circulating erythrocytes) that could potentially be sampled with the nasal scraping (Chegwidden and Carter, 2000).

A potential limitation of our study is the lack of a 1:1 correspondence between transcriptional message and catalytically active protein. Not only can protein translation be uncoupled from transcription, but also additional factors, such as enzyme stability can alter the ratio of transcript to active enzymes. For this reason we are currently conducting immunocytochemical studies of the nasal mucosa to localize the CA protein expression in specific cell types. Our immunofluorescence results indicate differential expression of CA IV and CA II with CA IV immunofluorescence not being

detected in ciliated epithelial cells. This result is similar to the observation in eye tissue where CA IV is not detected in ciliated epithelial cells (Hageman *et al.*, 1991). We plan to extend our immunostaining studies to include additional isoenzymes as well as intact tissue specimens; the latter should enable us to identify with greater specificity the cell types (basal, ciliated columnar, nonciliated columnar and goblet) expressing specific isoenzymes.

Previous studies have documented CA activity in the mammalian nasal mucosa. Cavaliere *et al.* (1996) performed histochemical studies in human nasal mucosa and identified CA activity in columnar ciliated respiratory epithelium, which was absent in the adjacent stratified squamous epithelium. Coates *et al.* (1998) performed an immuhistological study of the localization of CA activity in the rat nasal cavity and found that CA activity is present in many olfactory neurons and some regions of the respiratory epithelium. Okamura *et al.* (1996) demonstrated the presence of cells with CA activity in both olfactory and respiratory epithelium in the guinea pig nose.

An interesting observation from this study is the high correlation of expression of subsets of CA isoenzyme genes across subjects (Table 4). This may indicate a possible coordinated regulation of these genes, particularly CA I, CA III, CAVB, CA IX, CA XII and CA XIV in the nasal mucosa. The molecular basis of such a coordinated regulation in these CA isoenzymes is not known but could be relevant to the functional significance of these CA isoenzymes in this tissue.

An overall objective of our studies is to determine whether differences in CA gene expression may contribute to individual differences in CO_2 chemosensitivity. Previous studies have indicated that increased CO_2 chemosensitivity is observed among females and younger subjects (Shusterman *et al.*, 2001). Thus, if observed differences in individual CO_2 thresholds are mediated through mucosal CA expression and activity, we would predict that there would be higher CA gene expression in females and younger subjects. Examining the trends in gene expression for each CA isoenzyme gene summarized in Table 5 shows that the expression of CA VI follows this pattern, although only the association of CA VI gene expression with the age of the subjects is statistically significant. If nasal CA VI, like its salivary and lacrimal counterpart (see below) is of glandular origin, then it is possible that our superficial sampling technique may have underestimated relative gene expression by omitting submucosal glands.

CA VI is a secretory type isoenzyme that is primarily expressed in the salivary glands as well as other exocrine glands such as the lacrimal and mammary glands of animals (Kivela *et al.*, 1999; Karhumaa *et al.*, 2001; Ogawa *et al.*, 2002). CA VI has been implicated in the neutralization of excess organic acids produced by microbial flora on dental and epithelial surfaces, and forms a mutually complementary system with cytosolic CA for pH regulation in the upper alimentary tract of humans (Kivela *et al.*, 1999). However our results indicate that CA VI has very low expression in nasal mucosal tissue (Table 3). As noted above, however, this low expression may be due to sampling artifact; a possibility that could be addressed by applying *in situ* hybridization and/or immunohistochemical techniques to intact biopsy or excisional specimens. A splice-variant form of CAVI that is intracellular and synthesized in response to stress has recently been described in mouse fibroblasts (Sok *et al.*, 1999). Our data do not indicate which of these two CA VI isoforms (secreted or intracellular) are expressed in the nasal mucosal tissue.

Does CA act extracellularly or intracellularly in the CO_2 irritation pathway? Although our laboratory has documented acute pH changes in superficial nasal mucus coincident with CO_2 stimulation (Shusterman and Avila, 2003), parallel pH changes could also be taking place within the mucosa in interstitial fluid and/or intracellularly. Exploration of this phenomenon may require the application of animal models and/or supravital dye techniques to correlate micro-environmental changes with sensory activation.

In summary, we have found that most catalytically active CA isoenzymes are expressed in the normal and allergic human nasal mucosa. Significant variability in gene expression is apparent both between CA isoenzymes and between individuals. The functional significance of this variability is highly speculative at this time.

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