Alpha-adrenergic mRNA subtype expression in the human nasal turbinate

[Expression du sous-type d'ARN messager alpha-adrénergique dans le cornet nasal humain]

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Purpose: Alpha-adrenergic receptor (AR) agonist drugs (e.g., epinephrine) are commonly used for upper airway procedures, to shrink the mucosa, retard absorption of local anesthetic agents, and improve visualization by limiting hemorrhage. Decongestant therapy often also includes αAR agonist agents, however overuse of these drugs (e.g., oxymetazoline) can result in chronic rhinitis and rebound increases in nasal secretion. Since current decongestants stimulate αARs non-selectively, characterization of αAR subtype distribution in human airway (nasal turbinate) offers an opportunity to refine therapeutic targets while minimizing side-effects. We, therefore, investigated αAR subtype expression in human nasal turbinate within epithelial, duct, gland, and vessel cells using *in situ* hybridization.

Methods: Since sensitive and specific anti-receptor antibodies and highly selective αAR subtype ligands are currently unavailable, *in situ* hybridization was performed on sections of three human nasal turbinate samples to identify distribution of αAR subtype mRNA. Subtype specific ^{35}S-labelled mRNA probes were incubated with nasal turbinate sections, and protected fragments remaining after RNase treatment analyzed by light and darkfield microscopy.

Results: In non-vascular tissue α_{1d} AR mRNA predominates, whereas notably the α_{2c} is the only αAR subtype present in the sinusoids and arteriovenous anastamoses.

Conclusion: Combined with the current understanding that AR-mediated constriction of nasal sinusoids underpins decongestant therapies that minimize secretions and shrink tissues for airway procedures, these findings suggest that α_{2c} AR subtypes provide a novel selective target for decongestant therapy in humans.

***Objectif** : Les médicaments agonistes des récepteurs alpha-adrénergiques (AR) (par ex., l'épinéphrine) sont communément utilisés lors des interventions sur les voies aériennes supérieures, afin de rétrécir la muqueuse, de retarder l'absorption d'agents anesthésiques locaux et d'améliorer la visualisation en limitant l'hémorragie. Un traitement décongestionnant inclut également souvent des agents agonistes αAR ; toutefois, la surutilisation de ces médicaments (par ex., l'oxymétazoline) peut engendrer une rhinite chronique et l'augmentation rebond des sécrétions nasales lors de la cessation du traitement. Puisque les décongestionnants actuels stimulent les αAR de manière non-sélective, la caractérisation de la distribution des sous-types d'αAR dans les voies aériennes de l'homme (cornet nasal) offre la possibilité de perfectionner les cibles thérapeutiques tout en minimisant les effets secondaires. C'est pourquoi nous avons examiné l'expression des sous-types d'αAR au niveau du cornet nasal humain dans les cellules épithéliales, du canal, des glandes et des vaisseaux, à l'aide d'une hybridation in situ.*

***Méthode** : Étant donné que des anticorps anti-récepteurs sensibles et spécifiques ainsi que des ligands très sélectifs des sous-types d'αAR sont disponibles actuellement, l'hybridation in situ a été effectuée sur des sections de trois échantillons de cornet nasal humain afin d'identifier la distribution d'ARN messager des sous-types d'αAR. Des sondes d'ARN messager marquées au ^{35}S et spécifiques au sous-type ont été incubées avec des sections de cornet nasal, et les fragments protégés restants après le traitement à la ribonucléase ont été analysés par microscopie optique et sur fond noir.*

***Résultats** : Dans les tissus non-vasculaires, l'ARN messager AR α_{1d} est prédominant, alors que le α_{2c} est notablement le seul sous-type d'αAR présent dans les sinusoïdes et les anastomoses artérioveineuses.*

CAN J ANESTH 2007 / 54: 7 / pp 549–555

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Funding: This study was funded in part by NIH grant #HL49103 (DAS) and an educational grant from Proctor & Gamble. Tissue was provided by Drs. Rasmussen-Ortega and Stephen B. Liggett.

Accepted for publication February 16, 2007.

Revision accepted April 9, 2007.

Conclusion *: On considère maintenant que la constriction des sinus nasaux médiée par AR est à la base des thérapies de décongestion qui minimisent les sécrétions et rapetissent les tissus lors des interventions sur les voies aériennes. Ces résultats suggèrent donc que les sous-types d'AR α_{2c} fournissent une nouvelle cible sélective pour les traitements de décongestion chez les humains.*

ALPHA-ADRENERGIC receptors (αARs) are commonly used for upper airway procedures, to shrink the mucosa, retard absorption of local anesthetic agents, and improve visualization by limiting hemorrhage, and also play a key role in the pharmacologic treatment of nasal congestion, rhinorrhea, and epistaxis. Stimulation of α-receptors on the nasal erectile apparatus leads to constriction of arteriovenous anastomoses and collapse of venous sinusoids (Figure 1). The result is a decrease in the thickness of the nasal mucosa and so an increase in the nasal cross-sectional area of airflow within the bony confines of the nostrils. These effects are mediated *in vivo* by the sympathetic innervation, and therapeutically by oral and topical sympathomimetics. Effects on epithelial cell ciliary function and glandular exocytosis have also been reported.[1] Withdrawal of sympathetic effects leads to vasodilation of the arteriovenous anastomoses and engorgement of the venous sinusoids. The result is thickening of the nasal mucosa, reduction of cross-sectional area for airflow, and nasal obstruction. Prolonged use of topical sympathomimetics such as oxymetazoline and zylometazoline may lead to rebound vasodilation, ciliary dysfunction, and increased glandular secretion in the condition of *rhinitis medicamentosa*.[2–6]

Nasal αARs have been described previously. However, the specific α_1 subtypes (α_{1a}, α_{1b}, α_{1d}) and α_2 subtypes (α_{2a} - formerly α_2C10, α_{2b} - formerly α_2C2, α_{2c} - formerly α_2C4) that are present and their differential distribution on the epithelium, submucosal glands, arteriovenous anastomoses, post-capillary venules that regulate vascular permeability and leukocyte infiltration, and venous sinusoids have not been determined.[7–9] Currently available partially selective α_1- and α_2-agonists and endogenous norepinephrine are nonselective for these receptors. In addition, αAR subtype distribution is remarkably heterogenous among cell types in different tissues and mammalian species.[10–13] We hypothesize that understanding the distribution of each receptor subtype on nasal tissues may lead to more specific decongestant targets capable of providing benefit while minimizing worrisome side-effects.

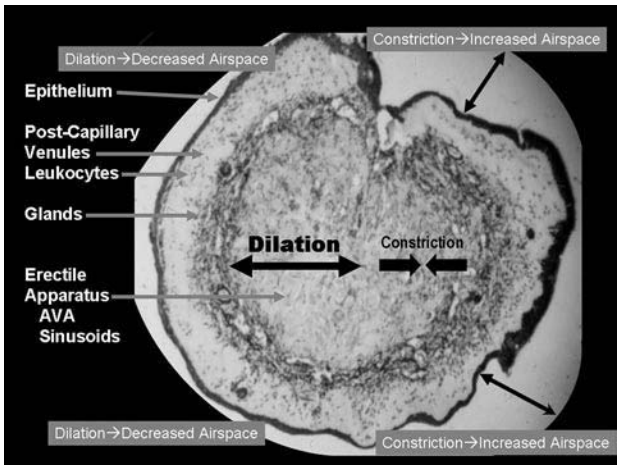

FIGURE 1 Diagrammatic representation of nasal turbinate cytoarchitecture. The nasal turbinates have concentric epithelial, superficial vascular, submucosal gland, and deep erectile tissues. Dilation of the venous sinusoids increases the thickness of the mucosa and decreases the cross-sectional area of airflow (left half of diagram). Vasoconstriction of the arteriovenous anastomoses and myoepithelial cells of the sinusoidal walls cause sinusoid collapse, thinning of the mucosa, and an increase in the cross-sectional area of airflow (right half of diagram).

Identifying cell specific distribution of αAR subtypes within nasal tissue should facilitate novel pharmacologic targeting directed at the common and vexing problem of nasal decongestion. Because highly subtype specific antibodies for αAR subtypes remain unavailable, we examined αAR subtype distribution using *in situ* hybridization on human nasal turbinate tissue.

Materials and methods

Nasal turbinate tissue preparation and detection of αAR subtype mRNA

Institutional Review Board (IRB) approval and individual patient consent was obtained for access to explanted nasal turbinate tissue from three individuals undergoing endoscopic nasal surgery; in addition, IRB approval was obtained at the institution where laboratory studies were performed. Tissue samples were immediately placed in liquid nitrogen and stored at -70°C. Ten μm horizontal frozen sections were cut on a cryostate (Leitz Kryostat 1720 digital, Wetzlar, Germany) using a -20°C block, thaw mounted onto sialylated microscope slides, and stored at -70°C with desiccant until further use. Radiolabelled single strand-

ed sense (control) and antisense (specific) RNA probes were made using linearized cDNA constructs, [^{35}S]-αUTP (Dupont, NEN, Boston, MA, USA) and either SP6 or T7 RNA polymerase, as previously described; mRNA probes and controls for in situ experiments have been previously validated in our laboratory.[12–14]

In situ hybridization

Frozen slide mount sections were warmed to room temperature then rinsed for five minutes with 2 × SSC (1 × SSC = 0.15M NaCl, 0.04M Na citrate, pH 7.2). No permeabilization or prehybridization steps were performed. Hybridization buffer (0.02M DTT, 1x Denhart's solution (Sigma, St. Louis, MO, USA), 1 mg·mL^{-1} salmon sperm DNA (Sigma) heated to 80°C before use, 50 µg·mL^{-1} transfer RNA (Sigma), 2 × SSC, 50% formamide, 9% dextran sulfate), and 5000–7000 cpm·µL^{-1} linearized radiolabelled probe were applied to the slides. The slides were then incubated at 50°C overnight in sealed plastic containers lined with Whatman filter paper soaked with 50% formamide in 2 × SSC buffer to prevent evaporation of hybridization solution. To remove non-specific binding, slides were washed as follows: sequential immersion in 1 µL·mL^{-1} β-mercaptoethanol solutions in 2 × SSC (50°C, brief dip) and 50% formamide in 2 × SSC (50°C, ten then 20 min), followed by RNase treatment using 10 µg·mL^{-1} RNase in 2 × SSC (35°C, 30 min). Subsequent washes included the following: 2 × SSC (RT, five minutes), 50% formamide in 2 × SSC (50°C, five minutes), and 2 × SSC (ten minutes), followed by dehydration steps using two-minute immersions each in 0.3M ammonium acetate solutions containing 50%, 70%, and finally pure 100% ethanol. After air drying for 30–60 min, slides were then dipped in warm (40°C) autoradiography emulsion (Kodak NTB2, Rochester, NY, USA) in a darkroom illuminated with a Kodak safelight #2, dried for several hours in the dark, and placed in light-sealed slide boxes with desiccant at 4°C for four weeks. After warming for 90 min to room temperature, exposed slides were developed under a safelight by sequential immersion in fresh D19 developer (Kodak) mixed 1:1 with distilled water (dH_2O at 15°C, four minutes) followed by room temperature immersions in dH_2O (20 sec), fixer (Kodak) (five minutes), and dH_2O rinses (3 × five minutes). Slides were counterstained with hematoxylin and eosin, dehydrated in an ascending ethanol and xylene series, and coverslips added. Dry slides were examined and photographed under light and dark field microscopy using Lietz Leica (Wetzlar, Germany), WILD M420 (low power), and DMR (high power) microscopes at 100X.

TABLE I Darkfield and brightfield semiquantitative grading system to assess relative presence of αAR subtype mRNA within human nasal turbinate

Grade	*Darkfield*	*Brightfield*
0	background	background
+/-	very low density of individual grains	background
+	low density of individual grains	individual grains
++	moderate density of grains	patterns of individual grains
+++	high density grains and clusters	clusters of grains
++++	high density clusters	high density of grains

αAR = alpha-adrenergic receptor.

Controls

In order to ensure that detected signal represents specific probe hybridization, several positive and negative controls were performed. Since human nasal turbinate tissue was limited, each probe used in this study was carefully tested in experiments with human spinal cord using antisense human β-actin as a positive control.[12,13] Negative controls included verifying lack of signal with sense probes and demonstration of loss of signal in known positive samples exposed to excess RNase [*in situ* experiments using excess (50 µg·mL^{-1}) RNase in washes]. Controls for αAR subtype specificity of antisense probes included simultaneously performed *in situ* experiments with known positive and negative non-nasal turbinate tissues.

Detection and analysis

Due to the thickness of the nasal turbinate specimens, a standard quantitative method previously used in our lab was found to be unreliable.[13] Instead, relative quantification of mRNA probe hybridization was recorded by two separate blinded observers using the presence of silver grains within specific nasal turbinate specific cell types - epithelium, ducts, glands, and/or vessels. Negative controls included sense probe experiments on the same tissue. Sections were scored by the intensity of silver grain density relative to background (Table I).

Results

There was excellent concordance (> 90%) between the two observers' independent scoring of relative intensities of silver grains for the different nasal turbinate samples. Furthermore, there was no evidence of non-specific signalling in positive control samples. Table II summarizes observations for specific cell types and all αAR subtype mRNAs. While all six AR subtype mRNAs are present in human nasal turbinate,

TABLE II Relative presence of αAR subtype mRNA within human nasal turbinate

αAR subtype	*Epithelium*			*Ducts*		*Glands*		*Vessels*		
	GC	*CC*	*BC*	*SD*	*MD*	*SG*	*MG*	*PCV*	*Sinsusoids*	*AVA*
α_{1a}	+	0	0	0	0	0	++	0	0	0
α_{1b}	0	+	0	+	0	++	+++	0	0	0
α_{1d}	+++	+++	+++	+++	+++	++++	++++	0	0	0
α_{2a}	0	+	0	++	+	+++	+	0	0	0
α_{2b}	0	+	0	++	+	+++	+	0	0	0
α_{2c}	+	++	+/-	++	++	+++	++	0	++	++

Presence of silver granules in cells resulting from *in situ* hybridization of αAR subtype mRNA probes scored by two independent observers. αAR = alpha-adrenergic receptor. Scoring: 0 = silver grain density similar to surrounding background tissue; +/- = very low density of silver grains in darkfield; + = silver grains seen only in darkfield; ++ = moderate density in darkfield, some silver grain apparent with brightfield; +++ = high density in darkfield, groups of silver grains seen in brightfield; ++++ = very high density silver grains in brightfield. GC = goblet cells, CC = ciliated cells, BC = basal cells, SD = serous ducts, MD = mucous ducts, SG = serous glands, MG = mucous glands, PCV = post-capillary venules, AVA = arteriovenous anastamosis.

individual subtype location tends to be restricted to specific cell types (Table II). Representative light and dark field images of nasal turbinate epithelium are presented in Figure 2. In non-vascular tissue, the overall predominant αAR subtype mRNA is the α_{1d}; this subtype is present in epithelium, ducts and glands. Less generalized presence of other α_1 mRNAs occurs in specific cell types such as goblet cells and mucous glands (α_{1a}) and ciliated cells, serous ducts and glands, and mucous glands (α_{1b}). Within the α_2AR family, the α_{2c} is present in all epithelial cells, ducts, and glands. In addition, α_{2c}ARs are present in sinusoids and arteriovenous anastamoses, where it is the only αAR present. In contrast, α_{2a} and α_{2b} signal is more restricted.

Discussion

In this study we describe, for the first time, αAR mRNA subtype distribution in ten cell types present in human nasal mucosa. By demonstrating cell and subtype specific expression using *in situ* hybridization, we confirm the hypothesis that αAR subtype distribution in the nasal mucosa is heterogenous. Such findings provide new mechanistic insight regarding potential roles of individual αAR subtypes in human nasal mucosa and suggest more selective targets for nasal decongestion therapy.

In spite of the importance of nasal tissue as a pharmacologic target, little information is available regarding αAR distribution in this tissue. For many years investigators have assumed beneficial effects of αAR agonists on nasal tissues resulted from vasoconstrictor effects, essentially dampening fluid flow through this tissue. [2–4] Indeed, nasal mucosa is highly vascular. Nasal vessels include arteries and arterioles (resistance vessels), arteriovenous connections, subepithelial and periglandular capillary networks which drain into collecting veins and venous sinusoids (capacitance vessels), and finally the sphenopalatine vein.[15,16]

Initially investigating αAR effects on vascular tone, Ichimura *et al.*[17] demonstrated the presence of postsynaptic α_1ARs as well as pre- and postsynaptic α_2ARs in canine nasal vascular smooth muscles using pharmacologic approaches. These findings suggested that stimulation of either α_1 or α_2ARs would be expected to provide vascular smooth muscle contraction. Using guinea pig nasal mucosa, Tanimitsu *et al.*[18] suggested that activation of α_{1a}ARs result in contraction of nasal mucosa vasculature. This is in contrast to pharmacologic studies in dogs suggesting both α_1 and α_2AR mediate vasoconstriction.[19] This disparity between guinea pig and dog in nasal turbinate αAR distribution is not surprising, given the marked species variability in overall expression of AR subtypes, and highlights the importance of studies using human tissue.

Studies in humans suggest the primary mechanism underlying nasal decongestion appears to be venous constriction of the collecting veins and sinusoids;[15,16,20–23] such constriction decreases engorgement of nasal mucosa, attenuating alterations in nasal anatomy that have been shown to alter nasal

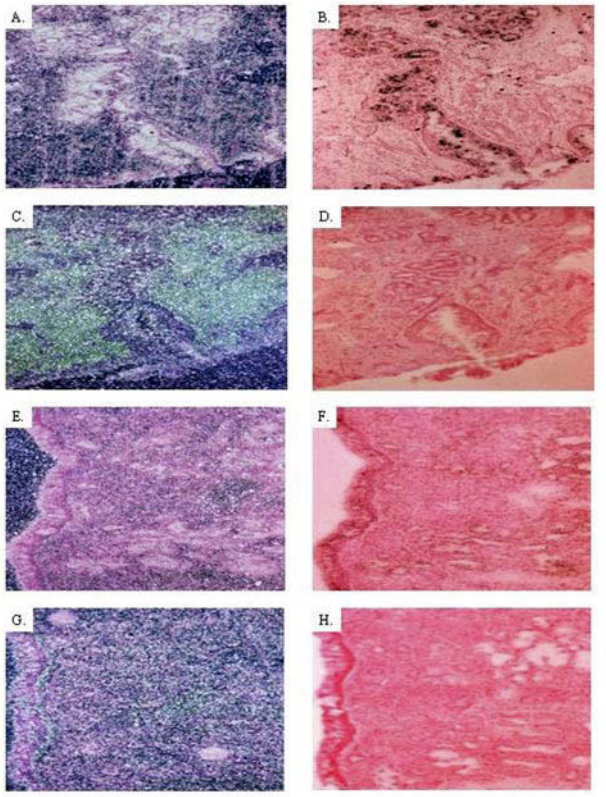

FIGURE 2 Representative examples of *in situ* hybridization experiments in human nasal turbinate tissue. The α_{1d}-antisense probe demonstrates a high density signal by darkfield (A, whitish silver grains in glands, ducts, and epithelial cells) and lightfield (B, black grains) microscopy. High density signal is absent in equivalent experiments in adjacent tissue sections (C and D, respectively) using the α_{1d}-sense (control) probe. Similar experiments using α_{2c}-antisense probe demonstrate more moderate density signal by darkfield (E) and lightfield (F, light brown) microscopy, which is absent in equivalent experiments (G and H, respectively) using the α_{2c}-sense (control) probe. Cell type specificity in α_1 and α_2 signalling is evident in epithelial, duct and glandular regions, and is also visible in vascular tissues at higher levels of magnification (see Table II).

air flow.[22,24,25] Our results are the first to address this question in humans at an αAR subtype level, revealing presence of only α_{2c}AR mRNA in human nasal sinusoids and arteriovenous anastamoses. In the αAR family, α_2ARs are primarily responsible for venous (rather than arterial) constriction. This is true for a wide range of venous capacitance beds such as saphenous vein,[26–28] dermal veins,[28,29] and nasal mucosa.[15,16,20,21,30] Our findings suggest an important and unique role for the α_{2c} subtype in nasal sinusoids, making this receptor a new subtype selective target for decongestion.

In addition to sinusoidal constriction as the main mechanism for decongestant action, other mechanisms have been shown to contribute to a lesser extent; such decongestant targets include α_1AR-mediated arteriolar constriction.[15,20,30] Andersson and Bende[2] studied specific αAR effects using topical application of αAR agonists and measurement of Xenon washout in the nasal mucosa of 43 healthy subjects. Findings from this study prompted the authors to conclude that vasoconstrictor actions of phenylephrine are likely due to preferential action on α_1ARs whereas oxymetazoline action is due to α_2AR activation.

While vasoconstriction is thought to be the major effect of topical αAR agonists,[3] other functional targets are possible. For example, α_1AR-mediated slowing of nasal ciliary beat frequency,[31] as well as effects on mucosal gland functioning,[32] provides two alternative targets. Alpha AR agonists have been shown to decrease the secretion of serous cell products from human nasal mucosa although underlying mechanisms currently remain unknown.[2,4] The strong presence of α_{1d} mRNA in epithelial cells, serous, and mucous cells of glands and ducts suggests this adrenergic receptor subtype may play a role in nasal secretions from these cells. Supporting this contention, α_1AR antagonists (often administered to treat prostate disease) tend to increase nasal secretions.[33] Since the $\alpha_{1a/d}$ selective antagonist tamsulosin also has this effect, this limits the possible subtypes involved to the α_{1a} or α_{1d}. Our demonstration that only α_{1d} mRNA is present on epithelium, ducts, and glands strongly suggests that the α_{1d}AR subtype is important in nasal secretion. This pathway of exocytosis represents a secondary mechanism (beyond simple vasoconstriction) for the efficacy of non-subtype selective α_1AR agonists currently being marketed as nasal sprays and oral therapies. However, since rhinnorhea is a common side-effect of overuse of oxymetazoline nose sprays, one might hypothesize that avoiding α_{1d}AR effects might minimize this possible complication.

In spite of our novel findings, some limitations to our study exist. Because 10 μm thick nasal turbinate slices cut through multiple cell layers, precise determination of exact amounts of tissue mRNA was precluded. Instead, we relied on two blinded expert observers; while there was excellent concordance between observers, it should be remembered that our findings represent relative, and not absolute, quantitation. Another possible limitation is that nasal turbinate explants came from patients undergoing functional endoscopic surgery, a surgery often employed for chronic sinusitis. These patients may have been taking medications that may have altered levels of receptor

expression and cellular distribution. Countering this possibility are previous studies suggesting no major changes occur in expression of nasal turbinate αARs (α_1 *vs* α_2) with chronic sinusitis.[7] Obtaining fresh human nasal tissue from individuals without disease is ethically challenging, making the optimal control study almost impossible. Further, it should be remembered that decongestants are most often utilized by individuals with diseased nasal mucosa; therefore, our findings are important for the target patient population in any case. Finally, because of lack of monoclonal and/or highly specific polyclonal antibodies against specific αARs, we cannot be entirely sure the presence of mRNA correlates with the presence of receptor protein at the cell surface. However some of our key findings suggest the presence of only one (with demonstrated absence of the other five αAR subtypes) αAR mRNA species. In these cases, general supportive pharmacologic data (α_1 *vs* α_2) exist, suggesting the mRNA is expressed at a protein level and functional. Hence, we have confidence in our major key findings that only α_{2c}AR mRNA is noted in nasal sinusoids, the precise tissue thought key in decongestant activity. Since human tissue samples were quickly frozen after explantation and clear αAR subtypes are present in each sample (documented by each observer), it is highly unlikely that our negative findings for some subtypes resulted from RNA degradation. Further studies evaluating specific receptor protein expression and functional effects (using antibodies and/or more highly selective ligands as they become available) will be helpful in confirming our findings.

In summary, this is the first study to report cell type-specific distribution of αAR mRNAs. We confirm that α_1 and α_2AR mRNA is present in human nasal turbinate epithelium, ducts, glands, and vessels, with selectivity for distinct subtypes limited to specific cell types. For instance, α_{2c}AR mRNA is the only αAR mRNA present in nasal venous sinusoids. These findings suggest that specific α_{2c}AR agonists might provide targeted decongestant therapy with fewer side-effects.

Acknowledgement

The authors acknowledge with thanks the editorial assistance of Cheryl J. Stetson.

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