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Nasal polyps and middle turbinates epithelial cells sensitivity to amphotericin B

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SUMMARY

Hypothesis: *Intranasal application of the antimycotic agent amphotericin B (AmphoB) has been proposed as an effective treatment of chronic rhinosinusitis (CRS) with polyps. AmphoB is a sterol-binding agent known to modify cell membrane structure. The cytotoxic effects of AmphoB were studied on primary human nasal epithelial cells in vitro.*

Methods: *Human epithelial cells were isolated from nasal polyps and middle turbinates of patients suffering from CRS, and grown on collagen-coated polycarbonate filters with an air liquid-interface. After 15 days of culture, cells were exposed apically to 50 µM AmphoB during 4h daily for 5 days. Some cells were treated during 4 weeks. The bioelectric properties of cells were then studied in Ussing chambers. Integrity of the cell monolayers was assessed by measurement of the transepithelial resistance (R) and immunofluorescent localization of the tight junction protein occludin.*

Results: *Disruption of the epithelial monolayer integrity was observed in all of the nasal polyps cell cultures, as demonstrated by a 60% drop in R. Immunofluorescence microscopy showed significant loss in cell number and disruption in the distribution of occludin. Turbinate cell cultures elicited no change in R and expression of occludin after AmphoB treatment. However, the transepithelial potential, the basal short-circuit current and the amiloride-sensitive current were reduced by 70%.*

Conclusions: *AmphoB was cytotoxic for nasal polyp epithelial cells with disruption of the epithelium integrity and loss of tight junctions. In contrast, integrity of turbinate epithelial cells was conserved despite alterations in transepithelial ion transport. These observations may explain the beneficial effect of intranasal application of AmphoB on CRS observed in clinical trials.*

Key words: chronic rhinosinusitis, respiratory epithelial cells, ion channels, occludin

INTRODUCTION

Nasal polyposis (NP) is considered the ultimate stage of chronic rhinosinusitis (CRS), the most common chronic disease. However, the etiology and formation of nasal polyps have still not been elucidated (Larsen and Tos, 1991; Lund, 1995). Several theories have attributed nasal polyposis to a variety of causes including chronic inflammation secondary to a hypersensitivity disorder. Recent studies has suggested that hyperreactivity to fungi could be involved in the development of CRS (Ponikau et al., 1999; Braun et al., 2003). The suggested mechanism is of eosinophilic activation in response to the parasitic effect of fungi in susceptible individuals leading to the local release of potent inflammatory mediators, tissue damage and secondary bacterial infection. This fungus hypothesis has in turn been followed by the empirical topical application of the fungicide agent amphotericin B (AmphoB) therapy in patients suffering from NP (Ponikau et al., 2002; Ricchetti et al., 2002).

The ionic composition of both extra and intracellular fluids must remain at a specific concentration in order to maintain an osmotic equilibrium and cell viability (Guyton, 1986). In airway epithelial cells, Na^+ enters the cells via the amiloride-sensitive epithelial Na^+ channel (ENaC) located on the apical membrane, and is extruded by the Na^+-K^+-ATPase pump located on the basolateral membrane. Transcellular Na^+ movement then causes chloride and, subsequently water, to follow. Transepithelial cells electrolytes transport can be studied in vitro on human nasal epithelial cells monolayer cultured on permeable filter using Ussing chamber technique. Such method was applied to nasal polyp cultures and these cells have shown higher transepithelial potential differences and

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increased Na^+ absorption when compared to controls (Bernstein and Yankaskas, 1994). Because AmphoB, a cholesterol-binding agent, forms channels in lipid membranes that are permeable to ions, water and nonelectrolytes, we speculate that AmphoB might affect membrane fluidity and integrity and thus affect the properties and/or cell surface expression of ion channels/transporters, and as a consequence transepithelial ion transport. The goal of this study was therefore to investigate the possible effects of AmphoB on the bioelectric properties of human nasal epithelial cells obtained from polyps.

MATERIALS AND METHODS

Reagents

Amiloride, ATP, bumetanide and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Amphotericin B, 3-isobutyl-1-methylxanthine (IBMX) and forskolin were from Calbiochem (La Jolla, CA, USA). Vitrogen collagen was from Nutacon (Leimuiden, The Netherlands). Rabbit anti-occludin was purchased from Zymed Laboratories (San Francisco, CA, USA). Rhodamine conjugated goat anti-mouse IgG and FITC conjugated goat anti-rabbit IgG were obtained from Chemicon (Temecula, CA, USA).

Patients

The patients who participated to this study received oral information and provided informed consent. A total of 29 patients were included in the present study which was conducted according to the declaration of Helsinki on biomedical research (HongKong amendment 1989). None of the patients studied were allergic, or affected by ASA intolerance. Patients with cystic fibrosis were excluded. Before surgical treatment was proposed, all patients had medical treatment for at least twelve months following diagnostic. Before polypectomy, all patients were treated by antibiotics for 10 days.

Cell culture

Human airway epithelial cells were obtained from different patients after surgical polypectomies, ethmoidectomies and partial middle turbinectomies. Cells were isolated by pronase (Roche, Mannheim, Germany) digestion as described (Karp et al., 2002). Freshly isolated cells were seeded at a density of $5x10^5$ cells/cm^2 onto 0.6-cm^2 collagen-coated Millicell polycarbonate filters (Millipore, Molsheim, France). Twenty hours after plating, the mucosal media was removed and the cells were allowed to grow at the air-liquid interface which will allow the cells to develop a morphological and functional phenotype that closely resembles in vivo airway epithelium. The culture media consists of a 1:1 mix of DMEM:F12 (Invitrogen, Life Technologies, Basel, Switzerland), 2% Ultroser G (Biosepra, Ciphergen Biosystems, Cergy-Saint-Christophe, France), 100 U/ml penicillin and 100 µg/ml streptomycin. After 15-20 days, the epithelial monolayers developed a transepithelial resistance of >500 ohm.cm^2.

Experimental procedure

One week treatment with AmphoB: polarized cells were treated apically with 50 µM Amphotericin B for 4h daily. After the treatment, AmphoB was removed, and the cells were further cultured in normal medium for 20h. The treatment was repeated during 5 days. The cells were then allowed to recover for 48h then mounted in an Ussing chamber for measurement of basal bioelectric characteristics.

One month treatment with AmphoB: the treatment described above was repeated during 4 weeks before measurement of basal bioelectric characteristics.

Ussing chamber experiments

The bioelectric properties of epithelial cell monolayers were studied by placing the filters in Ussing chambers (Jim's Instruments, Iowa City, IA, USA). The apical and basolateral chambers were filled with a Krebs bicarbonate that contained, in µM: 135 NaCl, 2.4 K_2HPO_4, 0.6 KH_2PO_2, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 5 glucose. The transepithelial potential difference was short-circuited with a voltage clamp connected to the apical and basolateral chambers via Ag-Ag electrodes and agar bridges. The transepithelial resistance R was determined using Ohm's law from the observed change in I_{SC} resulting from a 5s square-voltage pulse (5mV) imposed across the monolayer. After the voltage clamp condition was established, amiloride was applied to the apical solution to measure the fraction of the basal short-circuit current due to ENaC activity. The cAMP agonists, 10 µM forskolin and 50 µM IBMX were added to the mucosal solution to stimulate transepithelial Cl^- current through the CFTR Cl^- channel. To assess total Cl^- current, 100 µM bumetanide was added to the basolateral solution, and the change in current was measured.

Immunofluorescence

After bioelectric measurement, the cells were fixed with 100% methanol at -20°C for 10 min, then permeabilized with 0.5% Triton-X100 in PBS containing 1% BSA for 10 min at room temperature. Cells were then incubated with rabbit anti-occludin (1 µg/ml) overnight in PBS containing 1% BSA and 0.5% TritonX100. After washing, the cells were incubated with rhodamine-conjugated anti-rabbit IgG (1 µg/ml) for 1h in the dark at room temperature. After several washes, the filters were cut from their supports and mounted in polyvinyl alcohol mounting medium (Fluka). Cell nuclei were stained with DAPI (1 µg/ml). Stained cells were viewed with an Axioplan epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA).

Statistical analysis

The significance of the difference between groups ($p<0.05$) was determined using one way analysis of variance and Student's unpaired t-test using the Statview program for Windows (Version 5.0.1, SAS Institute Inc.). Results are given as means ± SEM.

RESULTS

Effects of AmphoB on the integrity of the epithelial monolayer

The apical treatment with AmphoB during one week and one month of cell cultures derived from polyps resulted in disruption of the integrity of the epithelial monolayer, as assessed by a significant drop in transepithelial resistance R. In contrast, cell cultures derived from middle turbinates remained intact as no change in R was observed (Figure 1).

Effects of AmphoB treatment on the expression of occludin

In the polyps cell cultures which showed significant loss of R after a one month treatment AmphoB, disruption of the distribution of occludin and significant decreased cell number were observed (Figure 2A). In the middle turbinates cell cultures that did not elicit a drop in R after AmphoB treatment, occludin immunolocalized as a continous ring identical to that observed in untreated cell cultures, but the cells were larger in size (Figure 2B).

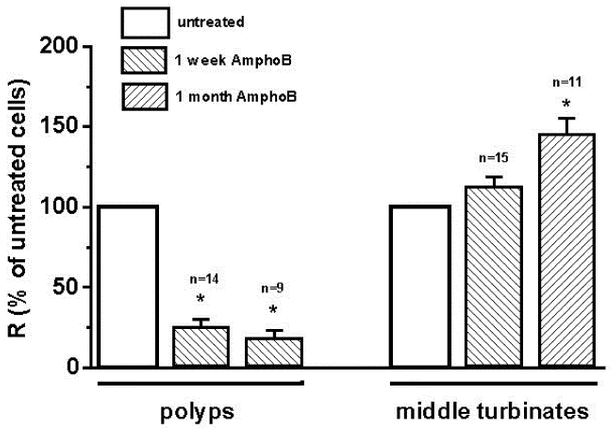

Figure 1. Effects of of AmphoB on the integrity of the nasal epithelial monolayers. In polyp cell cultures, Ampho B treatment for one week and one month induced disruption of the integrity of the epithelial monolayer, as shown by a significant drop in transepithelial resistance R. No toxicity was seen for the turbinates cell cultures. Results (means ± sem; n=number of independent experiments; * $p<0.05$) are expressed as percentage of R values of untreated cells (740 ± 99 ohm.cm^2) set as 100%.

Effects of AmphoB on bioelectric properties

Middle turbinate cell cultures that remained intact (no drop in R) after treatment with AmphoB for one month were mounted in Ussing chambers for measurement of the bioelectric properties. Figure 3A represents an original recording showing the effects of amiloride, forskolin and bumetanide on I_{SC} of untreated and AmphoB-treated cells. In untreated cells, the basal short-circuit current was largely inhibited by 100 µM amiloride, and subsequent apical addition of 10 µM forskolin plus 50 µM IBMX induced a biphasic increase in I_{SC} which was inhibited by addition of 100 µM bumetanide to the basolateral bathing solution. Untreated middle turbinate epithelial cells have a transepithelial potential (V) of -46 ± 4 mV, a short-circuit current I_{SC} of 110 ± 21 µA/cm^2 (n=7) corresponding to a transepithelial resistance (R) of 547 ± 57 ohm.cm^2. The magnitude of the amiloride-sensitive current (ΔI_{SC}(amil)), which represents the contribution of electrogenic Na+ absorption to transepithelial transport, was 109 ± 21 µA/cm^2. Middle turbinate cells that had been treated with AmphoB showed a 54% decrease in V, a 74% decrease in I_{SC} and a 80% decrease in ΔI_{SC}(amil) (Figure 3B). There was no significant change in Cl^- secretion in response to either forskolin (that increases cAMP) or ATP.

DISCUSSION

The present observations suggest that AmphoB produced overt cell injury in all the cell cultures derived from nasal polyps, associated with a significant loss of cells and a subsequent disruption of the integrity of the monolayers. In the middle turbinate cell cultures, AmphoB significantly decreased

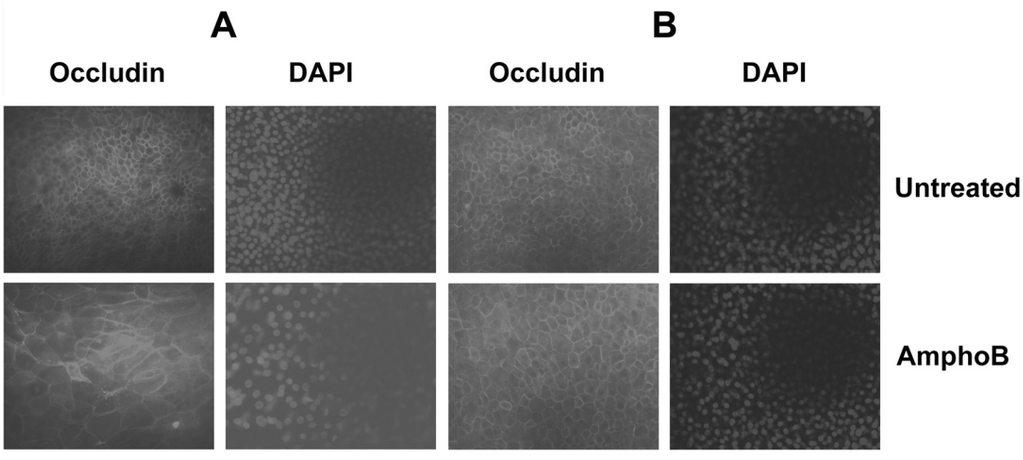

Figure 2. Expression of the tight junction protein occludin in untreated and AmphoB-treated nasal epithelial cells. Immunocytochemistry localization of occludin was performed in cell cultures derived from polyps (A) or middle turbinates (B), left untreated or treated with AmphoB for one month. Cell nuclei were stained with DAPI to assess the number of adherent cells.

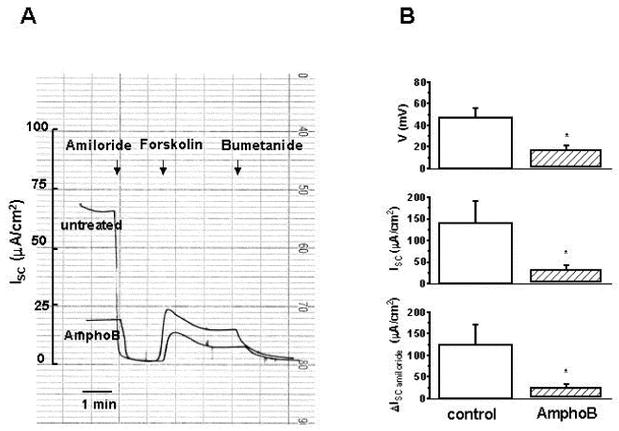

Figure 3. Effects of an apical treatment with AmphoB for one month on the bioelectric properties of nasal epithelial cells derived from middle turbinates. A: Representative I_{SC} traces of cells left untreated or apically treated with AmphoB in response to 100 µM amiloride, 10 µM forskolin and 100 µM bumetanide. B: Effects of AmphoB on transepithelial potential V, short-circuit current I_{SC} and amiloride-sensitive I_{SC} (means ± sem; n=7; *$p<0.05$).

Na^+ absorption without affecting transepithelial resistance. According to recent clinical observations, intranasal application of Ampho B induced disappearance of NP in about 50% of the patients studied (Ricchetti et al., 2002). A possible relationship between the present in vitro observations and the reported clinical effects on NP could be due to several mechanisms.

AmphoB, an amphipathic polyene macrolide, is a natural product derived from *Streptomyces nodosus* commonly used to treat systemic fungal infections. There is a relationship between polyene susceptibility and the presence of sterols in the plasma membrane of cells. The higher affinity of AmphoB toward ergosterol (the dominant sterol in fungal cells) than cholesterol (mammalian sterol), and the greater stability of the AmphoB channel in the ergosterol containing membranes are the essential factors on which antifungal chemotherapy is based. The interaction of the fungicide agent with membrane sterol results in the formation of aqueous pores consisting of eight AmphoB molecules linked hydrophobically to the membrane sterols (Ghannoum and Rice, 1999). This leads to an increase in membrane permeability to small ions, with an inward leak of Na^+ and an outward leak of K^+, that might be involved in modifications of membrane bioelectric properties. Indeed, earlier studies have shown changes in membrane potential in human leukocytes (Jullien et al., 1991). Here we show that, in epithelial cell cultures derived from nasal middle turbinates, AmphoB markedly decreased the transepithelial potential and inhibited Na^+ absorption without affecting cell viability. A feedback inhibition mechanism could be responsible for this phenomenon. Feedback inhibition describes an inhibition of the Na^+ channels that is secondary to an increase in the intracellular Na^+ concentration $[Na^+]i$. The entry of Na^+ through the pores formed by AmphoB led to an increase in $[Na^+]i$. This rise in $[Na^+]i$ in turn downregulates the activity of the apical Na^+ channels to limit Na^+ entry. Since AmphoB also acts as a Ca^{2+} ionophore (Sabra & Branch, 1992), a decrease in Na^+ conductance subsequently to an increase in $[Ca^{2+}]i$ as shown in numerous studies can not be excluded (Silver et al., 1993). We also observed that cells that had been treated with AmphoB were larger in size. This suggests imbalance of intracellular osmolarity paralleled by respective water movement across cell membranes and subsequent alterations of cell volume (Lang et al., 1998).

The most striking observation in this study is that AmphoB appears to produce two important types of changes: an increase in cell permeability leading to disruption of cell monolayer integrity and ultimately cell death in nasal polyp epithelial cells, and alterations in transepithelial ion transport without change in paracellular permeability in middle turbinate epithelial cells. There could be several explanations for the observed difference in the sensitivity of the different cell cultures to lethal effects of AmphoB. Since there is a correlation between sterol content and AmphoB potency, quantitative or qualitative differences in the sterol content of the polyps and turbinates epithelial cell membrane resulting from inadequate metabolic turnover of cholesterol, may affect the interactions of the drug with the bilayer. Decrease in total ergosterol content of the cell, replacement of some or all of the polyene-binding sterols by ones which bind polyene less well (e.g. substitution of ergosterol, cholesterol or stigmasterol by a 3-hydroxy or 3-oxosterol), or reorientation of ergosterol so that binding with polyenes is sterically or thermodynamically less favored, have been observed in organisms that are resistant to polyenes (Ghannoum and Rice, 1999). Alternatively, inadequate mechanisms to repair membrane damage that result from either pore formation or oxidative stress caused by polyenes (Sokol-Anderson et al., 1986) may be responsible for the increased susceptibility of nasal polyps epithelial cells to lethal effects of AmphoB.

In conclusion, we have observed in vitro a direct cytotoxic effect of AmphoB on human nasal polyps epithelial cells. Our findings suggest that modifications of the nasal mucosa epithelial cells induced by AmphoB could contribute to the disappearance of NP observed in clinical trials.

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REFERENCES

1. Bernstein JM, Yankaskas JR (1994) Increased ion transport in cultured nasal polyp epithelial cells. Arch Otolaryngol Head Neck Surg 120: 993-996.
2. Braun H, Buzina W, Freudenschuss K, Beham A, Stammberger H (2003) 'Eosinophilic Fungal Rhinosinusitis': a common disorder in Europe? Laryngoscope 113: 264-269.
3. Ghannoum MA, Rice LB (1999) Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev 12: 501-517.
4. Guyton AC. Transport through the cell membrane. In: Textbook of Medical Physiology. Philadelphia, WB Saunders, pp 88-100.
5. Jullien S, Capuozzo E, Salerno C, Crifo C (1991) Effects of polyene macrolides on the membrane potential of resting and activated human leukocytes. Biochem Int 24: 307-319.
6. Karp PH, Moninger TO, Weber SP, Nesselhauf TS, Launspach JL, Zabner J, Welsh MJ (2002) An in vitro model of differentiated human airway epithelia. Methods for establishing primary cultures. Methods Mol Biol 188: 115-137.
7. Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D (1998) Functional significance of cell volume regulatory mechanisms. Physiol Rev 78: 247-306.
8. Larsen PL, Tos M (1991) Origin of nasal polyps. Laryngoscope 101: 305-312.
9. Lund VJ (1995) Diagnosis and treatment of nasal polyps. BMJ 311: 1411-1414.
10. Ponikau JU, Sherris DA, Kern EB, Homburger HA, Frigas E, Gaffey TA, Roberts GD (1999) The diagnosis and incidence of allergic fungal sinusitis. Mayo Clin Proc 74: 877-884.
11. Ponikau JU, Sherris DA, Kita H, Kern EB (2002) Intranasal antifungal treatment in 51 patients with chronic rhinosinusitis. J Allergy Clin Immunol 110: 862-866.
12. Ricchetti A, Landis BN, Maffioli A, Giger R, Zeng C, Lacroix JS (2002) Effect of anti-fungal nasal lavage with amphotericin B on nasal polyposis. J Laryngol Otol 116: 261-263.
13. Sabra R, Branch RA (1992) Effect of amphotericin B on intracellular calcium levels in cultured glomerular mesangial cells. Eur J Pharmacol 226: 79-85.
14. Silver RB, Frindt G, Windhager E E, Palmer LG (1993) Feedback regulation of Na channels in rat CCT. I. Effects of inhibition of Na pump. Am J Physiol 264: F557-F564.
15. Sokol-Anderson ML, Brajtburg J, Medoff G (1986) Amphotericin B-induced oxidative damage and killing of Candida albicans. J Inf Dis: 154, 76-83.

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