The effect of changes in ambient oxygen concentration on the bioelectric properties of middle ear mucosa

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Mandell, David L., Daniel C. Devor, Joseph V. Madia, Chia-Yee Lo, Hilary Hake, and Patricia A. Hebda. The effect of changes in ambient oxygen concentration on the bioelectric properties of middle ear mucosa. Am J Physiol Cell Physiol 285: C618–C622, 2003. First published May 21, 2003; 10.1152/ajpcell.00553.2002.—The purpose of the present study was to compare the effect of 24 h of exposure to 7% O2 (normal middle ear physiological conditions) vs. 21% O2 (found in the middle ear after ventilation tube placement) on transepithelial Na+ absorption and Cl− secretion in cultured gerbil middle ear epithelial cell monolayers. Although no difference in apical Na+ absorption was identified, the UTP-induced stimulation of apical Cl− secretion in the presence of apical Na+ channel blockade with amiloride was significantly enhanced after exposure to 21% O2 compared with 7% O2 exposure. In the presence of a calcium-activated Cl− channel inhibitor, DIDS, UTP-induced stimulation of Cl− secretion after 21% O2 exposure was decreased, suggesting a role for calcium-activated Cl− channels in middle ear Cl− secretion in response to relative hyperoxia.

Ventriculation tube insertion through the tympanic membrane, a commonly performed procedure for patients with chronic otitis media with effusion (OME), results in a threefold increase in middle ear oxygen concentration, from 7% (PO2 = 43 mmHg) to nearly 21% (PO2 = 138 mmHg) (6). It is unknown whether this relative hyperoxia has any effect on middle ear mucosal ion transport and thus on mucosal absorptive or secretory properties. The primary determinant of baseline transepithelial potential difference in cultured gerbil middle ear epithelial cells is apical sodium (Na+) absorption through amiloride-sensitive Na+ channels (4, 9, 10). Compared with gerbil middle ear epithelial cells exposed to 21% O2, a significant, progressive decrease in apical Na+ absorption has been shown with up to 18 h of exposure to 0%, but not 5 and 10% O2 concentrations (14).

At baseline, a slight amount of apical Cl− secretion is present in gerbil middle ear epithelial cells (7), and it is Cl− secretion that is believed to account for residual current after middle ear epithelium apical Na+ channels are blocked with amiloride (4, 10). Stimulation of Cl− secretion can be achieved by applying UTP to either the apical or the basolateral surfaces of middle ear epithelial cells, possibly due to involvement of Ca2+-activated Cl− channels [Cl(Ca)] (5). The effect of changes in ambient O2 concentration on middle ear epithelial Cl− secretion is not known.

The purpose of the present study was to compare the effect of 24 h of exposure to 7% O2 (normal middle ear physiological conditions) vs. 21% O2 (middle ear conditions after ventilation tube placement) on transepithelial Na+ absorption and Cl− secretion in cultured gerbil middle ear epithelial cell monolayers.

MATERIALS AND METHODS

Cell culture. Middle ear epithelial cells from Mongolian gerbil (Meriones unguiculatus) that had been previously transformed with SV40 (middle ear cells derived by SV40 infection, MESV-40 cells) were a gift from Drs. Tran Ba Huy and Herman (Hôpital Lariboisière, Paris, France). All cell culture reagents were of the highest quality culture grade and were purchased from one of several American commercial suppliers. These stable MESV-40 cell lines were grown in culture according to Herman et al. (9) in Dulbecco’s modified Eagle’s medium-Ham F-12 medium (3:1), supplemented with 5% fetal bovine serum, 10% U/L penicillin, 10% µg/l streptomycin, 0.4 mg/l hydrocortisone, 1 µmol/l isoproterenol, and 10 µg/l epidermal growth factor. Cells were dissociated with 0.25% trypsin-EDTA and incubated at 37°C for 20 min, after which they were centrifuged at 2,000 rpm for 10 min and resuspended in medium. Cell viability was assessed using 0.4% trypan blue staining with cell counts performed to ensure 1 × 106 cells/ml. Cells were then seeded onto 24-well 0.35 cm2 polycarbonate Transwell filters, 0.4 µm-diameter pore size (Costar, Cambridge, MA), submerged in medium, and cultured in a humidified 5% CO2 incubator at 37°C. Cells were used between 4 to 7 days after seeding. Culture medium was changed every 3 days. Cultures were grown at the air-liquid interface for 2–3 days as follows: when cells reached confluence and exhibited a tight fluid barrier, culture medium was removed from the upper chamber (Transwell insert) and fresh medium was replaced in the lower chamber. This produced epithelial sheets with more consistent baseline electrophysiological readings, compared with

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submerged cultures, in an environment that more closely resembles the middle ear space.

Reagents. Amiloride (10⁻⁵ M) was obtained from Sigma and was made as a 1,000-fold stock solution in water. UTP (100 μM) was obtained from Calbiochem (La Jolla, CA) and was also made as a 1,000-fold stock solution in water. DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) (100 μM) was obtained from Sigma and was made as a 1,000-fold stock solution in dimethyl sulfoxide. All chemicals were of analytical grade.

Hypoxic and hyperoxic exposure. To achieve hypoxic exposure, 4–7 days after plating, culture dishes were placed in a humidified airtight incubation chamber with inflow and outflow valves (Billups-Rothenberg, Del Mar, CA). The desired gas mixture (either 7% O₂-5% CO₂-88% N₂ or 14% O₂-5% CO₂-81% N₂) was delivered at a constant flow rate (1 l/min) for about 30 min until equilibrated. The closed chamber was placed in a tissue culture incubator at 37°C for 24 h. Control normoxic cells (21% O₂-5% CO₂-74% N₂) were placed in a humidified chamber with the valves left open for the same time period. Before culture plates were removed from the chambers, the O₂ concentration was confirmed by using an oximeter (MiniOxI oxygen analyzer, Catalyst Research, Owings Mills, MD). The corresponding O₂ partial pressures for O₂ concentrations of 7, 14, and 21% were 53, 106, and 159 mmHg, respectively.

Other cultures were similarly exposed to hypoxic conditions of 50, 70, or 95% O₂, plus 5% CO₂, for 24 h. Under all experimental conditions of O₂, 5% CO₂ was used as part of the pH buffering of the cell culture medium.

Bioelectric measurements. Transwell cell culture inserts containing the cultured cells were mounted into an Ussing chamber (Jim’s Instruments, Dept. of Bioengineering, Univ. of Iowa, Iowa City, IA). The monolayers were continuously short-circuited via an automatic voltage clamp (Harvard Apparatus, Holliston, MA). Transepithelial short-circuit current (Isc) was continuously monitored with a 4-s, 5-mV pulse applied every 56 s to determine the resistance (Rt), which was calculated using Ohm’s law (R = V/I). The bath solution contained 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂, and 10 mM glucose. The pH of this solution was 7.4 when gassed with a mixture of 95% O₂-5% CO₂ at 37°C. A minimum of 10 min was allowed to elapse before determining baseline Isc and Rt measurements, after which pharmacological agents were added to the apical and/or the basal bath, with Isc and Rt measured immediately and at 10 min after drug addition.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using Student’s t-test. A P value of ≤0.05 was considered statistically significant. The value n refers to the number of replicate experiments performed.

RESULTS

Effect of oxygen concentration on Isc. Preliminary experiments confirmed the basic experimental model for bioelectric responses in primary cultures. Initially, MESV-40 cell monolayers were exposed to 24 h of 0% (n = 11), 7% (n = 38), 14% (n = 32), and 21% (n = 73) O₂ concentrations following 5 to 8 days of routine incubation. As demonstrated in Fig. 1, there were no significant differences in unstimulated, baseline Isc of cell monolayers exposed to 24 h of 7% O₂ (24.6 ± 1.3 μA/cm²), 14% O₂ (26.6 ± 1.2 μA/cm²), or 21% O₂ (23.7 ± 0.7 μA/cm²). Baseline Rt was also similar between cells exposed to all three oxygen concentrations. However, baseline Isc of cell monolayers exposed to 24 h of 0% O₂ (6.8 ± 1.3 μA/cm²) was significantly lower than baseline Isc of cell monolayers exposed to 7, 14, and 21% O₂.

Figure 2 demonstrates the effect of mucosal amiloride (10 μM) and UTP (100 μM) on Isc in MESV cell monolayer exposed to 24 h of 21% O₂ in a representative tracing.

Application of the Na⁺ channel blocker amiloride (1 × 10⁻⁵ M) resulted in a drop in baseline Isc of 89, 89, and 88% in cells exposed to 7% O₂ (2.7 ± 0.3 μA/cm²), 14% O₂ (3.0 ± 0.2 μA/cm²), and 21% O₂ (2.9 ± 0.1 μA/cm²), respectively (see Figs. 3 and 4). There were no
significant differences in postamiloride \( I_{sc} \) among cells exposed to the three \( O_2 \) concentrations 7, 14, or 21%. After amiloride application, \( R_F \) was similar between cells exposed to all three oxygen concentrations. In cells exposed to 0% \( O_2 \), application of amiloride resulted in a drop in baseline \( I_{sc} \) of 71% (2.0 \( \mu A/cm^2 \)), suggesting that even in the setting of low baseline \( I_{sc} \) in the presence of 0% \( O_2 \), the majority of transepithelial ion activity was still due to \( Na^+ \) channel activity.

With the cell monolayers in a hyperpolarized state following blockade of \( Na^+ \) absorption with amiloride, isolated \( Cl^- \) secretion in response to UTP was able to be studied (see Fig. 3). Application of 100 \( \mu M \) UTP to the apical surface of the cells resulted in an initial dramatic increase in \( I_{sc} \) that was greatest in cells exposed to 21% \( O_2 \) (31.3 ± 1.9 \( \mu A/cm^2 \)) and was significantly lower in cells exposed to 14% \( O_2 \) (20.5 ± 1.4 \( \mu A/cm^2 \)) and 7% \( O_2 \) (20.1 ± 1.6 \( \mu A/cm^2 \)). The initial increase in \( I_{sc} \) after UTP application was similar between cells exposed to 7% \( O_2 \) (20.1 ± 1.6 \( \mu A/cm^2 \)) and 14% \( O_2 \) (20.5 ± 1.4 \( \mu A/cm^2 \)). Initially after UTP application, \( R_F \) was similar among cells exposed to all three \( O_2 \) concentrations.

After the initial increase in \( I_{sc} \) following apical UTP application in the setting of amiloride blockade, \( I_{sc} \) decreased to a sustained level, lower than baseline \( I_{sc} \) (before any pharmacological intervention), but higher than the \( I_{sc} \) following amiloride application only (see Fig. 3). The sustained \( I_{sc} \) following apical UTP application was highest in cells that had been exposed to 21% \( O_2 \) (10.8 ± 1.0 \( \mu A/cm^2 \)) and was noticeably, but not significantly, lower in cells that had been exposed to both 14% \( O_2 \) (8.4 ± 0.5 \( \mu A/cm^2 \)) and 7% \( O_2 \) (8.5 ± 0.7 \( \mu A/cm^2 \)).

In another set of experiments, cell monolayers were hyperpolarized with amiloride and 100 \( \mu M \) UTP was then applied to the basolateral surface of the monolayers, yielding changes in \( I_{sc} \) that were similar to those observed after apical UTP application (see Fig. 4). The initial increase in \( I_{sc} \) following basolateral UTP administration was greatest in cells exposed to 21% \( O_2 \) (24.1 ± 1.9 \( \mu A/cm^2 \)) and was significantly lower in cells exposed to 14% \( O_2 \) (17.1 ± 1.7 \( \mu A/cm^2 \)) and 7% \( O_2 \) (14.8 ± 1.2 \( \mu A/cm^2 \)). \( R_F \) was similar among cells exposed to all three \( O_2 \) concentrations.

After the initial increase in \( I_{sc} \) following basolateral UTP application in the setting of amiloride blockade, \( I_{sc} \) decreased to a sustained level, lower than baseline \( I_{sc} \) (before any pharmacological intervention), but higher than the \( I_{sc} \) following amiloride application (see Fig. 4). This pattern was similar to that seen after apical UTP application. The sustained \( I_{sc} \) following basolateral UTP application was highest in cells that had been exposed to 21% \( O_2 \) (9.7 ± 0.6 \( \mu A/cm^2 \)), lower in cells that had been exposed to 14% \( O_2 \) (8.2 ± 1.0 \( \mu A/cm^2 \)), and least in cells that had been exposed to 7% \( O_2 \) (7.3 ± 0.8 \( \mu A/cm^2 \)). The difference in sustained \( I_{sc} \) between cells exposed to 7% vs. 21% \( O_2 \) was statistically significant; the differences between cells exposed to 7 vs. 14%, and 14 vs. 21%, were not significant.

To determine whether the enhanced UTP-induced stimulation of \( Cl^- \) secretion in the setting of 21% \( O_2 \) exposure involved \( Ca^{2+} \)-activated \( Cl^- \) channels, cell monolayers exposed to 21% \( O_2 \) were hyperpolarized with amiloride and then 100 \( \mu M \) DIDS (a \( Cl^- \) channel inhibitor) was applied to the apical surface (see Fig. 5). In the presence of DIDS, basal application of UTP generated an initial increase in \( I_{sc} \) (12.2 \( \mu A/cm^2 \)) that was of significantly less magnitude compared with the initial spike in \( I_{sc} \) seen with UTP application in the absence of DIDS under otherwise similar conditions (24.1 \( \mu A/cm^2 \)). However, as \( I_{sc} \) decreased to a new sustained level after the initial response to UTP, the difference in sustained \( I_{sc} \) was similar between cells that were (10.9 \( \mu A/cm^2 \)) and were not (9.7 \( \mu A/cm^2 \)) exposed to DIDS in the setting of 21% \( O_2 \) exposure.

**DISCUSSION**

Although no difference in \( Na^+ \) absorption was identified, the UTP-induced stimulation of \( Cl^- \) secretion in the presence of apical \( Na^+ \) channel blockade with amiloride was significantly enhanced after exposure to 21% \( O_2 \) compared with 7% \( O_2 \) exposure. The gas composition of the normal human middle ear is in equilibrium with that of local venous blood (PO2 = 43 mmHg, PCO2 = 50 mmHg) (6) and is unchanged in the presence...
of a serous middle ear effusion (6). Insertion of a ventilation tube in the tympanic membrane leads to a threefold increase in O₂ tension (PO2 = 138 mmHg, PCO₂ = 5 mmHg), approximating atmospheric gas composition (6).

The effect, if any, of relative middle ear hyperoxia after ventilation tube placement on mucosal ion transport and thus on mucosal absorptive or secretory properties is not known. Given the paucity and inaccessibility of human middle ear mucosal specimens, most studies of middle ear mucosa have utilized transformed cell cultures derived from the Mongolian gerbil (Meriones unguiculatus) (9), because in this species the distribution of middle ear epithelial cells is similar to that of the human (3).

Amiloride-sensitive apical Na⁺ channels have been demonstrated to be the major determinant of baseline electrogenic ion transport in Mongolian gerbil middle ear epithelium, accounting for 62 (4) to 90% (9) of baseline Isc.

At baseline, a slight amount of transepithelial Cl⁻ secretion is present in gerbil middle ear cells (7). Residual current after blocking apical Na⁺ channels with amiloride is believed to be due to passive transepithelial Cl⁻ secretion (4, 10). Stimulation of Cl⁻ secretion can be achieved by applying UTP to either the apical or the basolateral surfaces of middle ear cells, possibly due to activation of Cl(Ca) channels (5). UTP is known to activate a Cl(Ca) channel, resulting in stimulation of Cl⁻ secretion (12). In the present study, the initial marked increase in Isc seen in response to UTP at 21% O₂ concentration was significantly less robust in the presence of DIDS, a Cl(Ca) channel blocker, suggesting that Cl(Ca) channels may play an important role in fluid secretion in the middle ear.

In transformed cultured Mongolian gerbil middle ear epithelium, it has been shown that 6–18 h of exposure to 0% O₂ concentrations results in a progressive decrease in apical Na⁺ absorption compared with cells exposed to 21% O₂ (14). Similarly, in cultured rat type II alveolar epithelial cells exposed to 3–28 h of 0% O₂ concentration, a time-dependent decrease in apical Na⁺ channel activity was noted (13). Exposure to 3% O₂ also resulted in a decrease in Na⁺ channel activity, whereas exposure to 5% O₂ did not. In another study, fetal distal lung epithelial cell monolayers grown for 3 days in 20% O₂ demonstrated significantly higher levels of total and amiloride-sensitive Isc compared with cells exposed to 3 days of 2.5 to 5% O₂ (16).

In the present study, exposure of transformed cultured Mongolian gerbil middle ear epithelial monolayers to 24 h of 0% O₂ also resulted in decreased amiloride-sensitive Isc compared with similar monolayers exposed to higher O₂ concentrations, similar to other studies. However, a concentration of 0% O₂ is experimental and does not reflect the O₂ concentration that is found in either the normal middle ear or the ear with a serous middle ear effusion (7%, PO₂ = 43 mmHg) in humans (6). In the present study, exposure of cultured gerbil middle ear epithelial monolayers to 7% O₂ resulted in no change in Na⁺ channel activity compared with similar cells exposed to 21% O₂ concentration. Therefore, our results suggest that changes in Na⁺ transport cannot account for alterations in fluid clearance from the middle ear after tympanostomy tube placement.

In the present study, transepithelial Cl⁻ secretion was isolated by blocking apical Na⁺ channels with amiloride. Then, Cl⁻ secretion was stimulated using UTP, applied to either the apical or basolateral cell monolayer surface. The typical response of Isc after UTP application (an initial marked increase, or spike, in Isc followed by a sharp decrease and new sustained level of Isc) occurred in cells exposed to both 7 and 21% O₂. However, in cells that had been exposed to 21% O₂, the initial increase in Cl⁻ secretion was significantly greater than that seen in cells that had been exposed to 7% O₂. The subsequent drop and final sustained level of Isc, which represented ongoing Cl⁻ secretion at a level above that seen before UTP application, was still greater in cells exposed to 21% O₂ compared with cells exposed to 7% O₂, although the difference was only significant with basolateral UTP application. Another study has shown, using a cultured canine kidney cell line, that exposure to relative hyperoxia (20%) results in an increase in forskolin-stimulated Cl⁻ secretion compared with exposure to lower O₂ tensions (as low as 2.5%) (2).

We theorize that the increase in UTP-stimulated transepithelial Cl⁻ secretion seen in middle ear mucosal cells exposed to 21 vs. 7% O₂ will result in a relatively small amount of apical water secretion via osmosis. Because Cl⁻ secretion constitutes a relatively small amount of Isc (~10%), the amount of water that is secreted into the middle ear space may not result in the development of an effusion but, rather, may contribute to the middle ear space in a more subtle manner.

Ciliated pathways leading to the Eustachian tube have been identified in both human (8) and Mongolian gerbil (3) middle ear epithelium. Secretions propelled by ciliated respiratory epithelium consist of two layers: a viscous mucus (gel) layer above the cilia that is propelled via intermittent contact with the tips of the cilia, and a serous periciliary (sol) layer within which the cilia beat (1, 15). The origin of the periciliary fluid is not known but has been proposed to originate via transepithelial osmosis as governed by mucosal ion transport mechanisms (15).

If the periciliary fluid layer is reduced, the cilia may become entangled in the mucus and thus beat less efficiently (1, 15). A decrease in periciliary fluid has been demonstrated in middle ear mucosal specimens from humans with otitis media with effusion (11). The pathophysiology of this decrease in periciliary fluid is unknown and is unlikely to be due to changes in middle ear O₂ concentration, because O₂ concentration is similar in nontubulated ears with and without a serous effusion (6).

Irrespective of the cause of decreased middle ear periciliary fluid in patients with otitis media with effusion, we theorize that this periciliary fluid deficiency

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may be overcome by enhancement of apical Cl\(^-\) secretion after ventilation tube placement due to a resultant increase in middle ear O\(_2\) concentration from 7 to 21%.

Although the middle ear cells in our study are not ciliated, periciliary fluid has been found to be present on the surface of both ciliated and nonciliated human middle ear cells (11), suggesting that even nonciliated middle ear mucosal cells may regulate sol layer fluid production via their microvilli.

Transepithelial chloride secretion requires the coordinate action of both apical chloride channels and basolateral potassium channels (5). Future work is planned to further elucidate the effect upon these channels of an increase in ambient O\(_2\) concentration from 7 to 21%.

DISCLOSURES

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