Modulation of Middle Ear Epithelial Function by Steroids: Clinical Relevance

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The efficacy of steroid therapy for the treatment of otitis media in children remains controversial, and a putative modulation of the middle ear epithelial function has to be demonstrated. Using the MESV cell line, short-circuit current (I_{sc}) technique was used to evaluate changes in ion transport induced by glucocorticoids. Dexamethasone (DXM) produced a dose- and time-dependent increase in I_{sc} in MESV cells. This effect was inhibited by specific glucocorticoid antagonist (RU-38486) and was related to a sodium transport, since the DXM-induced increase in I_{sc} could be prevented or abolished *i*) by apical addition of the specific Na^+ channel inhibitor benzamil; or *ii*) by substitution of sodium with N-Methyl-glucamine in the incubation medium. RNase protection assay revealed that DXM increased the expression of the α subunit sodium channel mRNA, which changes paralleled the modulation of ion transport. These data demonstrate that steroids up-regulate the trans-epithelial sodium transport in the middle ear epithelium. As far as these experimental data can be extrapolated to the *in vivo* situation, a component of the beneficial effect of steroid therapy for the treatment of otitis media may result from a corticosteroid-induced improvement in fluid clearance from the middle ear. **Key words:** short-circuit current technique, steroids, otitis media, fluid clearance.

INTRODUCTION

The middle ear epithelium has been found to possess active sodium transport, in primary cultures (1) as well as in SV40-transformed middle ear epithelial cells (2). The removal of solutes from apical fluid is of importance for the efficiency of the mucociliary clearance as well as for the dry-out of the middle ear cavities.

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MATERIALS AND METHODS

Cell culture

Techniques have been described elsewhere (2). Briefly, MESV cells were cultured in a humidified 5% CO_2 incubator at 37°C in a DMEM/medium 199 mixture supplemented with 5% fetal calf serum, hydrocortisone and growth factors. All experiments were performed after 4 days of steroid deprivation and 24 h serum and growth factor deprivation. For electrophysiological measurements, confluent MESV monolayers were trypsinized and plated (10^5 cells/cm²) onto 0.4 μ m-pored tissue culture-treated polycarbonate filters (Transwell, Costar, Cambridge, MA).

Bioelectric measurements

Cells were used 6 to 7 days after seeding. Filters were mounted into Ussing chambers perfused with a 95% O_2 -5% CO_2 gas-lifted Ringer's solution chambers were connected to a voltage-current clamp device (DVC1000, World Precision Instruments, New Haven, England). When ionic substitutions were performed, sodium was substituted with N-methyl-glucamine (NMGA), and chloride was substituted with gluconate.

Measurement of the ouabain-sensitive ^{86}Rb uptake

The ouabain-sensitive Rb influx was used as an indicator of Na^+, K^+ -ATPase activity (5). MESV cells were incubated for 24 h in the presence of dexa-

methasone (10^{-7} M) and/or benzamil (10^{-6} M) or as control. Uptake measurements were performed at 37°C in a solution derived from Eagle's Essential Medium. Uptake was performed for 5 min with ^{86}Rb in the basal compartment. Radioactivity was extracted by Triton X-100 (1%) and counted in a scintillation counter. Protein content of each filter was determined, and results were expressed as ng ^{86}Rb /mg prot.

RNase protection assay

RNase protection assay was performed as described earlier (6). MESV monolayers were incubated for 48 h in the presence of increasing concentrations of dexamethasone. Total RNA equivalent of 10^6 cells or yeast tRNA (tRNA, Boehringer) were hybridized with 5.10^5 CPM (for the human epithelial sodium channel, hENaC) and 5.10^4 CPM (for rat glyceraldehyde 3P dehydrogenase, GAPDH) radiolabeled probes at 50°C overnight, and RNase digestion (RNase A, 40 $\mu\text{g}/\text{ml}$ and T1, 2 $\mu\text{g}/\text{ml}$, from Boehringer) was performed at 30°C for 60 min. Then, digestion by proteinase K (125 $\mu\text{g}/\text{ml}$ Boehringer) was done at 37°C for 30 min. After phenol extraction and ethanol precipitation, protected fragments were separated by gel electrophoresis. Gels were analyzed with an Instant Imager (Packard Instrument Company, CT).

cRNAs probes

Preliminary experiments were performed to validate the cross-reactivity between the human epithelial sodium channel probe and gerbil mRNA. A 110 bp fragment was recovered. Antisense RNA probes were synthesized from the translated region of the α subunit (1036–1259) of hENaC. The cRNA synthesis (Promega kit) was done using ^{32}P -UTP (Amersham, specific activity >15 TBq/mmol). The ^{32}P -cRNA probe was 307 base pairs and the protected fragment in gerbil was approximately 110 base pairs. Rat glyceraldehyde 3P dehydrogenase (GAPDH) mRNA was used for standardization.

α -hENaC subunit cDNA was a gift from Richard Boucher (Chapel Hill, NC) and GAPDH cDNA from C. Dani (Nice, France).

Reagents

Hydrocortisone, transferrin, triiodothyronine, insulin, EGF, ouabain, N-Methyl-glucamine, sodium gluconate, EDTA, were purchased from Sigma Chemical (St. Louis, MO). DMEM, medium-199, penicillin/streptomycin, HEPES and glutamine were from TechGen (Les Ulis, France). FCS was purchased from GibcoBRL (Life Technologies, UK). Benzamil was from Research Biochemical Incorporated (Nat-

ick, MA). RU-38486 was a gift from Roussel UCLAF Pharmaceuticals (Romainville, France). All other chemicals were of analytical grade.

Statistical analysis

Results were expressed as mean $\pm$ S.E.M. of n separate experiments. Comparisons of means were performed by using one or two-way analysis of variance (as appropriate) followed by Fisher's least significant difference or Dunnett's test for comparison from control. Differences were considered significant at $p < 0.05$.

RESULTS

Effect of steroids on I_{sc}

Incubation for 48 h in the presence of dexamethasone (10^{-7} M), a glucocorticoid agonist, increased I_{sc} from 0.71 ± 0.05 $\mu\text{A}/\text{cm}^2$ to 2.06 ± 0.07 $\mu\text{A}/\text{cm}^2$ ($p < 0.01$, $n = 6$, Fig. 1A). The time-course of this effect evi-

Fig. 1. Time-response relationship of DXM on I_{sc} of MESV monolayers. (A) Steady-state I_{sc} was measured in control (CTL, empty bars) and in monolayers exposed to dexamethasone (DXM, 10^{-7} M, hatched bars). (B) In the same conditions, the benzamil-sensitive I_{sc} is reported, calculated as the difference between steady-state I_{sc} and the value measured after addition of benzamil (10^{-6} M) to the apical compartment.

* Significantly different from control ($p < 0.05$, $n = 6$).

Fig. 2. Dose-response relationship of DXM on short-circuit current of MESV monolayers. MESV monolayers were incubated for 24 h in the presence of increasing concentrations of DXM (from 10^{-10} to 10^{-5} M) before measurement of the steady-state I_{sc} . Values are means $\pm$ S.E.M. ($n = 4-8$).

Fig. 3. Incidence of ion substitutions on the effect of DXM on I_{sc} . MESV monolayers were incubated for 24 h as control (empty bars) or in the presence of DXM (10^{-7} M, hatched bars). Subsequently, I_{sc} was measured either in a modified Ringer solution (NaCl), or in the same solution in which sodium was substituted with a non-permeant cation (NMGA Cl) or chloride with a non-permeant anion (Na gluconate). * No significant difference between control and DXM-treated monolayers.

denced a significant increase starting at 12 h. Increasing concentrations of dexamethasone from 10^{-10} to 10^{-5} M for a 24 h incubation induced a concentration-dependent increase in I_{sc} , from $0.84 \pm 0.09 \mu\text{A}/\text{cm}^2$ for control up to 5.97 ± 0.77 in the presence of a

Table II. Effect of dexamethasone on the ouabain-sensitive ^{86}Rb uptake

The Na^+, K^+ -ATPase activity was determined as the ouabain-sensitive rubidium uptake in monolayers treated or not with DXM (10^{-7} M) for 24 h, without (BZ-) or with benzamil (10^{-6} M; BZ+) in the bath during the incubation period.

Ouabain-sensitive ^{86}Rb uptake (pg/mg prot)	CTL	DXM
BZ-	35.10 ± 5.10	$54.50 \pm 6.20^*$
BZ+	34.40 ± 5.90	34.20 ± 6.40

* Significant difference between DXM-treated and control monolayers ($p < 0.05$, $n = 5$).

10^{-6} M concentration of DXM ($n = 6$). The resulting concentration of half maximal stimulation (EC_{50}) was 2.68×10^{-8} M (Fig. 2).

Addition of benzamil (10^{-6} [7]), a specific sodium channel blocker to the apical side of MESV monolayers blunted the effect of DXM (Fig. 1B). The DXM-induced increase in I_{sc} was further characterized by ionic substitutions with non-permeant ions. The DXM effect on I_{sc} was preserved when chloride was substituted with gluconate; on the contrary, replace-

Fig. 4. Modulation by dexamethasone of the expression of transcripts of the Na^+ channel. RNase protection assay measured the amount of mRNA encoding the α subunit of the epithelial sodium channel on MESV monolayers incubated in the presence of increasing concentrations of dexamethasone (arbitrary units).

Table I. Effect of RU-38486 on the DXM effect on I_{sc}

Cells were grown to confluence on Transwell filters, and incubated as control (CTL) or in the presence of dexamethasone (DXM, 10^{-7} M) for 24 h, with or without increasing concentrations of the glucocorticoid antagonist RU-38486.

Incubation	CTL	DXM	DXM + RU (10^{-8} M)	DXM + RU (10^{-7} M)	DXM + RU (10^{-6} M)	DXM + RU (10^{-5} M)
I_{sc} ($\mu\text{A}/\text{cm}^2$)	$0.68 \pm 0.02^*$	1.22 ± 0.06	1.21 ± 0.06	$1.00 \pm 0.04^*$	$0.86 \pm 0.06^*$	$0.90 \pm 0.04^*$

* Significantly different from the DXM condition ($p < 0.05$, $n = 6$).

ment of sodium with N-Methyl-glucamine blunted the increase in I_{sc} (Fig. 3). The specificity of the glucocorticoid effect was assessed by inhibition of the DXM effect by increasing concentrations of the selective antagonist RU-38486 (Table I). MESV monolayers were incubated for 24 h in the presence of DXM (10^{-7} M) and increasing concentrations (10^{-8} M to 10^{-5} M) of RU-38486, which resulted in a dose-dependent decrease in the effect of DXM.

Measurement of ouabain-sensitive ^{86}Rb uptake

The effect of DXM was investigated on intact cells by the ouabain-sensitive ^{86}Rb uptake. Incubation with DXM (10^{-7} M for 24 h) increased the ouabain-sensitive rubidium uptake from 35.1 ± 5.1 pg/mg prot/5min in control to 54.5 ± 6.2 pg/mg prot/5min ($p < 0.05$; $n = 5$). Coincubation with benzamil (10^{-6} M) prevented this effect since the ouabain-sensitive rubidium uptake was 34.4 ± 5.9 pg/mg prot in control and 34.2 ± 5.4 pg/mg prot/5min in DXM-treated cells (Table II).

Effect of steroid treatment on mRNA level of the α subunit of the Na^+ channel

The ^{86}Rb uptake experiments suggested that the apical sodium entry was the primary target of the steroid effect. Therefore we investigated whether steroids might directly modulate the transcription of apical transporters. The level of expression of the mRNA encoding the α subunit of the sodium channel paralleled the increase in the concentration of DXM. The kinetics of this process was sigmoidal. The resulting concentration of half-maximal stimulation was 1.13×10^{-8} M (Fig. 4, $n = 4$).

DISCUSSION

This work supports the use of glucocorticosteroids in the treatment of otitis media. Steroids directly modulate sodium transport across the middle ear epithelium, through an increase in the transcription of sodium channels. This process might allow dry-out of the middle ear cavities and enhance the healing process in the course of chronic otitis.

Steroids enhance sodium transport across the middle ear epithelium

Previous experiments demonstrated the role of apical amiloride-sensitive sodium channels and basolateral Na^+, K^+ -ATPase in the genesis of a trans-epithelial sodium transport, which in turn drives a trans-epithelial water flow (1). Incubation in the presence of dexamethasone, a major glucocorticosteroid agonist, increased dramatically the electrogenic ion transport across the middle ear epithelium (Fig. 1A). Sodium

transport mainly accounts for this increase since (i) the increase in I_{sc} was primarily related to a benzamil-sensitive current (Fig. 1B); and (ii) replacing sodium on both sides of the epithelium by a non permeant cation abolished the steroid effect (Fig. 3), which was preserved when chloride was substituted with gluconate.

The trans-epithelial ion transport rate was measured by the ouabain-sensitive ^{86}Rb uptake, which indirectly reflects sodium extrusion across the basolateral membrane. As shown in Table II, while DXM increased the ouabain-sensitive Rb uptake, this increase was blunted in the presence of benzamil, which precludes a primary modulation of Na^+, K^+ -ATPase. On the contrary, the effect of benzamil suggests an intrinsic effect of glucocorticosteroids on the apical sodium entry, followed by a secondary increase in Na^+, K^+ -ATPase activity.

The increase in sodium transport is related to an increase of the expression of transcripts encoding for the α subunit of the epithelial sodium channel

The epithelial sodium channel has recently been cloned and consists of three separate subunits designated as α , β , γ which combine to form a highly selective pore (for review, see [8]). Sodium channel regulation may occur through alterations in channel kinetics, channel number, or both. In our experiments, the DXM-induced increase in mRNA encoding the α subunit of the epithelial sodium channel strictly paralleled the effect observed on short-circuit current, which favors a direct effect on the number of channels. Likewise, recent studies have evidenced in the pulmonary epithelium that increased Na^+ transport is related to increased steady-state levels of Na^+ channel mRNAs and likely channel number (8, 7).

The putative modulation of β and γ subunits was not investigated in this study. However, the α -subunit of the sodium channel exhibits, when expressed in oocytes, all the characteristics of the highly selective channel (10), while β - and γ -subunits allow maximal activity of active sodium channels. The intensity of the modulation of sodium transport by dexamethasone in MESV cells suggests that either β - and γ -subunits are constitutively expressed at a high level, or that these subunits are also modulated by steroids (11).

Altogether, our data suggest that cell sodium entry through the amiloride-sensitive Na channel is enhanced by corticosteroids, while cell Na^+ extrusion by Na^+, K^+ -ATPase is not regulated. Indeed, it has been shown in other tight epithelia that the rate-limiting step for trans-epithelial sodium reabsorption is Na entry, while Na^+, K^+ -ATPase adapts its activity to maintain a low intracellular sodium concentration.

Incidence of the epithelial effect of steroids on the management of otitis media

Because of its ontogeny and morphological feature, the middle ear epithelium may be considered as a respiratory epithelium. Our data demonstrate, as for the case of lung epithelium, that steroids up-regulate the trans-epithelial sodium transport in the middle ear epithelium. These data were obtained on a middle ear cell line and require obviously a complementary assessment on primary culture and *in vivo*. However, as far as these experimental data can be extrapolated to the *in vivo* situation, a component of the beneficial effect of steroid therapy for the treatment of otitis media (3) may result from a corticosteroid-induced improvement in fluid clearance from the middle ear.

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