Modulation of sodium transport in cultured human middle ear epithelial cells by dexamethasone

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Modulation of sodium transport in cultured human middle ear epithelial cells by dexamethasone

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Finally, I hope that I will do an in-depth study for the next years without indolence and conceit, and that every person around me will encourage me to do so.

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ABSTRACT

Modulation of sodium transport in cultured human middle ear epithelial cells by dexamethasone.

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The efficacy of steroid therapy for the treatment of otitis media with effusion remains controversial, and a putative modulation of the middle ear epithelial function has yet to be demonstrated. The effect of glucocorticoids on sodium transport and fluid absorption capacity was investigated on cultured human middle ear epithelial cells. Dexamethasone produced a significant increase in amiloride-sensitive short-circuit

current (Isc). This effect was related to sodium transport because amiloride is the specific sodium channel inhibitor. Expression of mRNA for α−, β-, γ- subunits of ENaC was demonstrated in the presence and absence of glucocorticoids. Dexamethasone significantly increased expression levels of α- and β- subunits. In addition, the fluid absorption rate was significantly increased after dexamethasone treatment. These data demonstrate that steroids up-regulate the trans-epithelial sodium transport and fluid absorption rate. Extrapolating these experimental data to the in vivo situation, the beneficial effect of steroid therapy for the treatment of otitis media with effusion may result from a glucocorticoid-induced improvement in sodium transport and fluid absorption in the middle ear.

Key Words : glucocorticoid, fluid absorption, epithelial sodium channel

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I. INTRODUCTION

The physiology of the middle ear is primarily concerned with keeping the cavities air filled and fluid free to allow transmission of the sound vibration for the eardrum to the inner ear. The middle ear epithelial cells are believed to play a key role in this process by transporting sodium and water from the middle ear to the subepithelium.[1]

Various mechanisms involving Na^+ and Cl^- transport across cell membranes have been identified. These include electrogenic processes, such as epithelial Na^+ channel

(ENaC), and electroneutral processes such as Na^+/H^+ exchange. ENaC have been demonstrated to be the major determinant of baseline electrogenic ion transport in Mongolian gerbil middle ear epithelium.[2]

ENaC allow for passive gradient-driven diffusion of Na^+ into the cell, while the Na^+-K^+ ATPase, a Na^+ pump located on the basolateral aspect of the cells, actively exchanges intracellular Na^+ for extracellular K^+ using ATP as an energy source. The net result of these ion exchanges is establishment of a positive Na^+ gradient between the apical and basal surfaces of the epithelial cells which, in turn, modulates osmotically-driven water transport from the middle ear to the sub-epithelium. These ion transport mechanisms are responsible for controlling intracellular pH and cell volume, absorbing excess water from the tympanic cavity and maintaining the depth of the apical water layer that promotes effective mucociliary coupling and clearance. Impairment of this process is involved in the pathogenesis of otitis media with effusion, which is the main cause of acquired hearing loss.

Steroid administration is one of the useful medical treatment modality in otitis media with effusion. There are reports that steroids directly modulate sodium transport in

various epithelia through ENaC[3,4,5], but the molecular mechanisms by which steroids are achieved in human middle ear epithelium remains to be elucidated.

The purpose of the present study is to identify the effect of dexamethasone on the fluid absorption and the expression of ENaC in the cultured human middle ear epithelial cells.

II. MATERIALS AND METHODS

1. Cell culture

Primary cultures of normal human middle ear epithelial cells were prepared as described previously.[6] Passage-2 normal human middle ear epithelial cells were plated on a collagen-coated semipermeable membrane with a 0.45-μm pore size at a density of 1.0×10^4 cells/cm^2. The cells were maintained in 1:1 mixture of bronchial epithelial growth medium and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and all supplements.[7]

Confluence of passae-2 normal human middle ear epithelial cells (8-9 days after

seeding) was verified by measurement of transepithelial resistance (R_t; > 5000Ω/cm^2 at room temperature before treatment with dexamethasone) using endohm meter.

2. Measurement of the short circuit current

Cultured cells, grown at air-liquid interface (ALI) on Snapwell (1.13 cm^2 surface area) permeable supports (Costar Co., Cambridge, MA, USA) were mounted in modified Ussing chambers (World Precision Instruments, Sarasota, FL, USA). The epithelium was bathed on both sides with 5 ml of warmed (37°C) Krebs bicarbonate Ringer (KBR) solution circulated by gas lifts with 95% O_2-5% CO_2.

The pH of the solution was maintained at 7.4. The epithelial culture was voltage clamped, i.e., short-circuit current (*I*sc), and R_t was measured with an automatic voltage clamp. Data were acquired and analyzed with Acquire and Analysis (AD instruments, Colorado springs, CO, USA) software. After 15-min equilibration, amiloride (100μM) was added to the luminal bath.

3. RT-PCR

Total RNA was extracted from both dexamethasone-treated and nontreated cultured human middle ear epithelial cells using TRIzol reagent according to user's manual, and the concentration was determined spectrophotometrically. Total RNA (2 μg) was converted into cDNA using 400 U Moloney murine leukemia virus RT (200 U/μl; Gibco, Carlsbad, CA, USA) reverse transcriptase in a buffer containing (in mM) 250 Tris-Cl, pH 8.3, 275 KCl, 15 $MgCl_2$, and 1 D-nucleotide triphosphate (final volume: 40 μl). The PCRs were performed with a Perkin Elmer Cetus DNA Thermal Cycler (Perkin Elmer, Branchburg, NJ, USA) according to manufacturer's recommendations. The main characteristics of the primers used to amplify the cDNA of the α-, β-, and γ-ENaC subunits are specified in Table 1. The cDNA segments were amplified from 1 μl of the RT product in a PCR buffer (in mM: 20 Tris-Cl, 50 KCl, 1.5 $MgCl_2$) and in the presence of 0.2 mM of dNTP and 1 U of Taq polymerase at 5 U/μl (Gibco, Carlsbad, CA, USA). Each PCR cycle comprised denaturation at 94 ℃ for 30 s, annealing during 30 s at variable temperature (Table 1), and elongation at 72 ℃ for 1 min. The reaction was stopped by a temperature decrease to 4℃. A 10-μl sample of the PCR product was run on a 2 % agarose gel with ethidium bromide. A 100-bp molecular

weight ladder permitted the verification of the length of the amplified fragments. For comparative analysis of mRNA levels, luminescent image analyzer LAS-1000plus (Fuji Photo Film Ltd., Tokyo, Japan) and TINA (Raytest, Staubenhardt, Germany) software were used.

Table 1. Primers used for PCR of α-, β-, and γ-ENaC subunits

PCR product/Primer Sequence	Segment Amplified, bp	Annealing Temperature, ℃	No. of cycles
ENaC-α			
S: 5'-CAGCCCATACCAGGTCTCAT-3'	220	55	25
AS: 5'-ATGGTGGTGTTGTTGCAGAA-3'			
ENaC-β			
S: 5'-GGGGTACTCGTGGATAAGCTT-3'	376	57	25
AS: 5'-GAGACAAGACGTGGAAAATCC-3'			
ENaC-γ			
S: 5'-ACCACCAGCCATGGTCTAAG-3'	209	54	29
AS:5'-CTTCAGGTCCCGGGATTTAT-3'			

4. Measurement of fluid absorption rate

To evaluate the fluid transport capacity across the cells, we measured the remaining volume after apical application of 100 μl of fluid. Briefly, cells were pretreated with dexamethasone for 24 hr at 37°C and 5% CO_2 in a humidified atmosphere. The luminal surfaces of cells at the air-liquid interface were washed with PBS three times and the remaining surface liquid was completely aspirated. 100 μL of KBR was then added to the cell surface. After an additional 24 hr of incubation, remained apical surface liquid was measured with micropipette.

5. Statistical analysis

The results of multiple experiments were presented as mean ± SD. Statistical analysis was performed by paired Student's t-test. A value of $p<0.05$ was considered statistically significant.

III. RESULTS

1. Effect of steroids on Isc

Dexamethasone (100nM) was added to apical side of cultured human middle ear epithelial cells. 12-hr incubation in the presence of dexamethasone increased Isc by 267% from 17.67±2.31 to 46.50±5.77 μA/cm^2 (*p=0.0182, n=3). The results of the time course of this effect showed that most significant increase was at 12 hr (Fig. 1). The kinetics of this process suggested a transcriptional effect followed by protein synthesis.

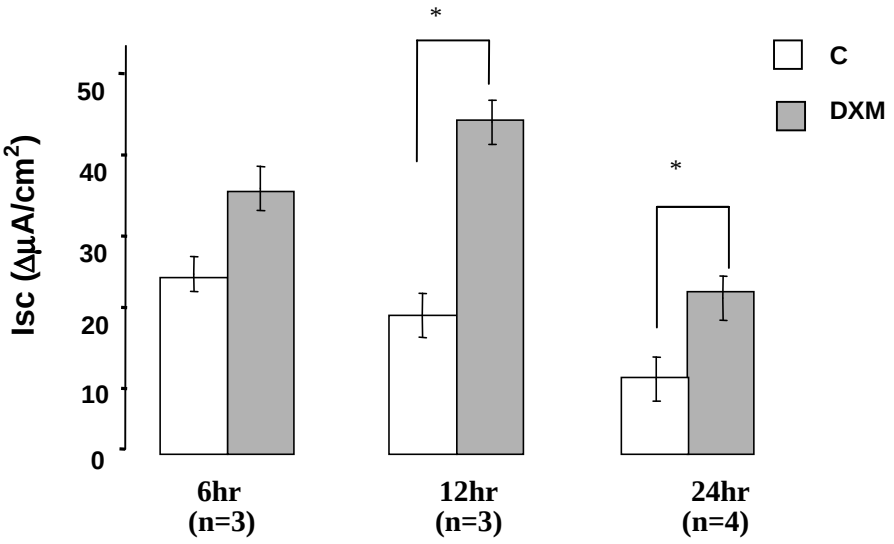


Fig. 1. Time course of activation of amiloride-sensitive short-circuit current (Isc) after dexamethasone treatment

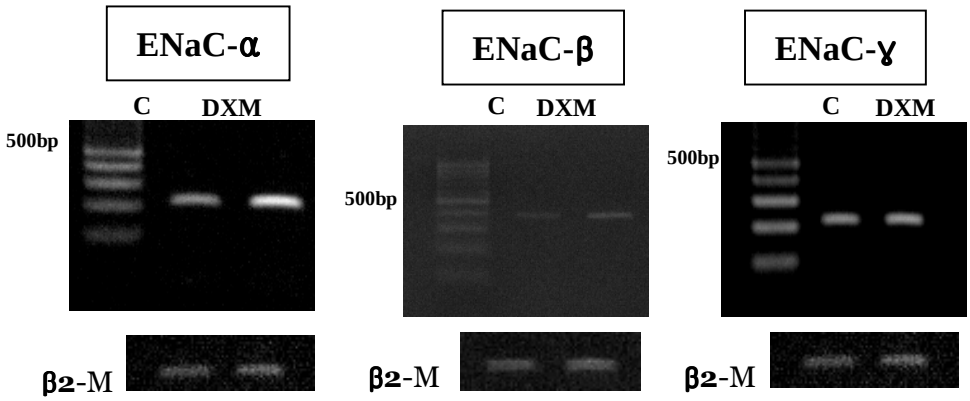
The amiloride-sensitive Isc is reported, calculated as the difference between steady-state Isc and the value measured after addition of amiloride (100nM) to the apical compartment. C, control; DXM, dexamethasone.

2. Expression of ENaC genes

Transcripts encoding α-, β-, γ- subunits of ENaC were detected by RT-PCR in cultured human middle ear epithelial cells in both the absence and presence of dexamethasone (100nM; 12h) treatment (Fig. 2). Bands corresponding to the expected lengths for each subunit fragment were observed. No contamination of genomic DNA was observed in reverse transcription-negative controls (-RT) amplified by PCR. As shown in Fig 2A and 2B, the expression levels of ENaC-α and ENaC-β significantly increased after dexamethasone treatment compared with untreated control. mRNA levels of the α-subunit were increased by 176% ($p<0.05$, $n=3$) after dexamethasone treatment. Levels of β-subunit mRNA were increased by 221% ($p<0.05$, $n=3$) after dexamethasone treatment. Despite significant increase in the expression levels of both α- and β-ENaC mRNA, the expression levels of γ-subunit were not statistically different after

dexamethasone treatment (p=0.532).

A



B

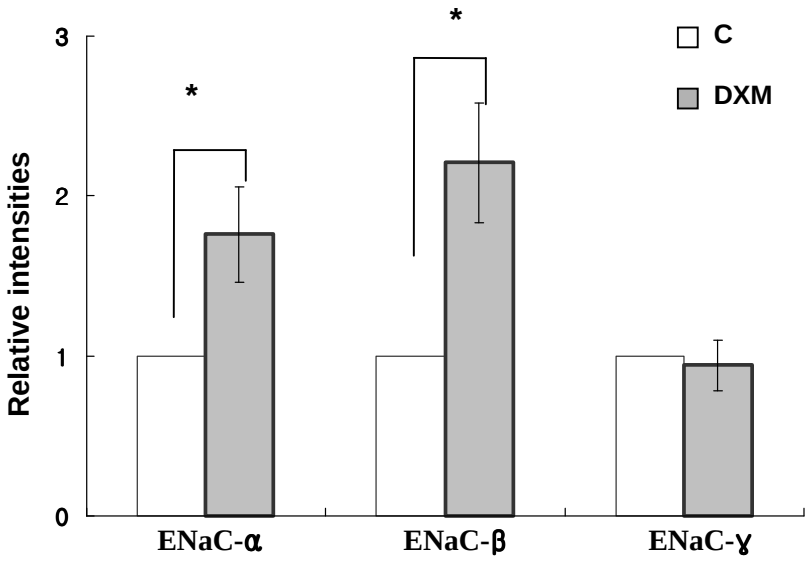


Fig. 2. Expression of ENaC genes

A: Detection of α-, β-, γ- ENaC subunit mRNA by RT-PCR in cultured human middle ear epithelial cells without and with dexamethasone treatment (100nM; 12hr). A single band was visualized for α-, β-, γ- ENaC subunit mRNA at expected sizes (+RT); no signal was observed in negative RT controls (-RT). C, control; DXM, dexamethasone; β2-M, β2-microglobulin. B: Effect of dexamethasone treatment (100nM; 12hr) on α-, β-, γ- ENaC subunit mRNA. Dexamethasone significantly increased ENaC-α (*$p<0.05$, n=3) and ENaC-β (*$p<0.05$, n=3). C, control; DXM, dexamethasone.

3. Fluid absorption rate

In order to evaluate the effect of dexamethasone in fluid absorption, the absorption rate was measured after application of 100μl of fluid to the luminal cell surface. Dexamethasone (100nM; 24hr) increased the fluid absorption rate from 2.85±0.22 μl/cm^2/hr to 3.28±0.15 μl/cm^2/hr (*$p=0.0139$, n=5).

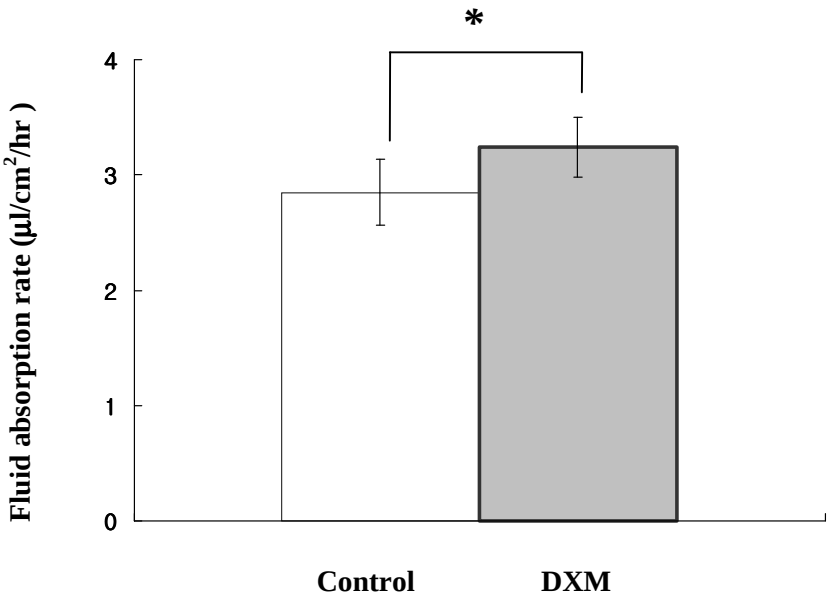


Fig. 3. Effect of dexamethasone on fluid absorption rate

The fluid absorption rate was measured after application of 100μl of KBR on the luminal surface of cultured human middle ear epithelial cells. Dexamethasone stimulates the fluid absorption rate (*$p<0.05$, n=5). DXM, dexamethasone.

IV. DISCUSSION

ENaC, which consists of three homologous subunits, is expressed in a wide variety of epithelia including sweat glands and lung.[8] ENaC is the rate-limiting structure in inward transport of Na^+ and plays a vital role in the absorption of periciliary or alveolar fluid in airway epithelium.[9,10] The basic function of ENaC is to allow vectorial transcellular transport of Na^+ by electrochemical gradient coupled with the Na^+-K^+ ATPase of basolateral membrane and the apical entry of Na^+ is blocked by submicromolar concentrations of amiloride.[8] Its mutational defect causes pseudohypoaldosteronism, whose symptoms include watery rhinorrhea and increased bronchial secretion.[11] Since the middle ear epithelium is an extension of the airway epithelium, ENaC may have an important role in the middle ear. In addition, amiloride-sensitive current has been shown to be a main contributor of basal current in Mongolian gerbil middle ear epithelia.[2] Also, the observations that patients with pseudohypoaldosteronism present with frequent episodes of otitis media as well as lower airway hypersecretion, support the importance of ENaC function in the middle ear.[12]

Clinical evidence that glucocorticoids have beneficial effect in the treatment of otitis media with effusion is abundant. Glucocorticoids stimulate fluid absorption by up-regulating Na^+ transport such as ENaC in airway epithelial cells.[3,4] In addition, there are reports that glucorcoticoids could stimulate sodium transport in the gerbil middle ear epithelium[13]. In this study, dexamethasone directly modulates sodium transport across the middle ear epithelium, thereby enhancing fluid absorption. The action of glucocorticoids in middle ear epithelium is through genomic regulation because the time course is on the order of hours; nongenomic action would be expected to occur on a much shorter time scale.[14]

ENaC consists of three homologous subunits, α, β, and γ, which share 35% identity in amino acid sequence.[9,15,16] Sodium channel regulation may occur through alterations in channel kinetics, channel number or both. In this experiment, dexamethasone induced significant increases in ENaC-α and ENaC-β mRNA, but not in ENaC-γ mRNA. It has been observed that when corticosteroids bind to their receptors, they can trans-activate the GRE and stimulate ENaC-α transcription.[17].However, this is probably not the only mechanism of ENaC induction by steroids, because ENaC-α responds to

corticosteroids depending on the cell type. For example, treatment of intestinal epithelium with corticosteroids does not significantly induce α subunit mRNA, while increasing ENaC-β, and ENaC-γ gene expression[18,19,20,21], suggesting that tissues specifically express transcription factors that are required for steroid regulation of ENaC subunits.

In summary, dexamethasone, a major glucocorticoid agonist, increased dramatically both the ENaC-dependent Isc and the fluid absorption rate in cultured human middle ear epithelium. Such up-regulated ENaC function by glucocorticoids might allow dry-out of middle ear cavities and enhance the healing process in the course of otitis media with effusion.

V. CONCLUSION

This study demonstrates that steroids up-regulate the trans-epithelial sodium transport and thereby fluid absorption rate in the human middle ear epithelium. Extrapolating these experimental data to the in vivo situation, 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.

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국문요약

배양된 인간중이상피세포에서의 dexamethasone에 의한 sodium 이동의 조절

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의학과

(지도교수 : 김 희 남)

삼출성 중이염에 있어서 스테로이드 치료는 아직도 논란의 대상이 되고 있어, 스테로이드가 중이상피세포의 기능을 어떻게 조절하는지에 대해 연구가 필요한 실정이다. 본 연구에서는 배양된 인간중이세포에서 스테로이드가 fluid absorption과 Na^+ 이동에 미치는 영향을 살펴보았다. 스테로이드는 amiloride-sensitive short-circuit current (Isc)과 ENaC mRNA의 발현, 그리고 fluid absorption rate를 현저히 증가시켰다. Amiloride는 Epithelial Na^+ channel (ENaC)의 선택적 길항제이므로, 이러한 결과는

ENaC의 발현 증가에 기인한다고 볼 수 있다. 이러한 결과들은 스테로이드가 ENaC의 발현을 증가시킴으로써 Na^+의 이동을 증가시키고, fluid absorption을 향상시킨다는 보여준다. 그러므로, 삼출성 중이염에 있어서 스테로이드의 사용의 근거가 될 수 있을 것으로 사료된다.

핵심되는 말 : glucocorticoid, fluid absorption, epithelial sodium channel