Original Article
Effects of glucocorticoids on lipopolysaccharide-induced inflammatory responses in human middle ear epithelial cells and its mechanisms

Hong-Zhou Ge1,2*, Yu-E Han3*, Zhen Li4, Xiao-Yu Chen2, Sheng-Lin Liu2, An-Ying Xu1

1Department of Otorhinolaryngology, The Second Hospital of Shandong University, Jinan, Shandong, China; 2Department of Otorhinolaryngology, The Affiliated Qingdao Hiser Hospital of Qingdao University, Qingdao, Shandong, China; 3Department of Otorhinolaryngology, Qilu Hospital of Shandong University (Qingdao), Qingdao, Shandong, China; 4Department of Otorhinolaryngology, Yantaishan Hospital Affiliated to Medical College of Taishan, Yantai, Shandong, China. *Equal contributors.

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Abstract: This study aimed to investigate the effects and mechanisms of glucocorticoids on lipopolysaccharide (LPS)-induced inflammatory responses in human middle ear epithelial cells (HMEECs). HMEECs were incubated with LPS and then maintained with glucocorticoids. Inflammatory mediators including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and cyclooxygenase-2 (COX-2) were analyzed. The cell viability at 24 h, 48 h, and 72 h and cell apoptosis were determined, together with apoptosis-related factors Bax and B-cell lymphoma (Bcl-2) and nuclear factor kappa B (NF-κB) pathway related proteins I-kappa-B-alpha (IκB-α) and p-NF-κB. Moreover, the activator of NF-κB, phorbol 12-myristate 13-acetate (PMA), was added to the cells, and then the expression levels of inflammatory mediators were analyzed again. The results showed that the relative expression of TNF-α, IL-6 and COX-2 was significantly increased by LPS, and while statistically decreased by glucocorticoids (P < 0.05 or P < 0.01). Besides, we found that LPS could dramatically lowered the cell viability and elevated the cell apoptosis (P < 0.05 or P < 0.01), whereas, application of glucocorticoids reversed the results. Moreover, glucocorticoids remarkably decreased the expression levels of Bax and p-NF-κB but increased the levels of Bcl-2 and IκB-α (P < 0.05 or P < 0.01). Additionally, the effects of glucocorticoids on expression levels of inflammatory mediators were prominently inhibited by administration of PMA (P < 0.05). Our results suggest that glucocorticoids may reduce LPS-induced inflammation responses in HMEECs by inhibiting the NF-κB pathway.

Keywords: Glucocorticoids, lipopolysaccharide, otitis media, inflammatory responses, nuclear factor kappa B

Introduction

Otitis media (OM) is a complex and multifactorial condition that can affect the middle ear, including acute otitis media (AOM), otitis media with effusion (OME), and chronic otitis media with effusion (COME) [1]. Patients with different subtypes of OM present a significant difference in clinical manifestations, associated complications, and treatments [2]. It is a leading cause of health care worldwide and antibiotic prescription. The common complications of OM include conductive and sensorineural hearing loss, perforation of the ear drum, mastoiditis, and infrequent complications are intracranial complications such as bacterial meningitis, brain abscess, or dural sinus thrombosis [3, 4]. Although the overall incidence of the complications has been decreasing significantly due to the introduction of antibiotics, a very high mortality rate still exists if not treated properly [3]. It has been estimated that 28,000 people die every year due to complications of OM [5]. Therefore, there is an urgent need to discover alternative novel therapeutic agents with higher efficacies.

Glucocorticoids are a class of corticosteroids, which play important roles in regulation of carbohydrate, protein and lipid metabolism, immune stress, and inflammatory reactions [6, 7]. Glucocorticoids are potent anti-inflammato-
ry and immunosuppressive agents, regardless of the inflammation’s cause. Glucocorticoids have been proven to mediate cell inflammation by suppressing cell proliferation and cytokine production [8]. It has been well acknowledged that synthetic glucocorticoids are widely prescribed drugs for a variety of medical conditions including rheumatoid arthritis [9], asthma [10], chronic obstructive pulmonary disease (COPD) [11], and sepsis [12]. The effects of glucocorticoids on OM have been extensively studied, and good outcomes have been confirmed [13-15]. However, the exact mechanism by which the action of glucocorticoids in the treatment of OM remains largely unexplored and unknown.

Therefore, in the present study, we aimed to explore the effects and mechanisms of glucocorticoids on OM. Lipopolysaccharide (LPS) was performed to induce the inflammatory responses in human middle ear epithelial cells (HMEECs). The effects of glucocorticoids on the inflammatory mediators (tumor necrosis factor (TNF)-α, interleukin (IL)-6 and cyclooxygenase-2 (COX-2)), cell viability and cell apoptosis were investigated, as well as the underlying mechanisms and potential signaling pathway.

Materials and methods

Cell culture and treatment

HMEECs immortalized with the E6/E7 genes of human papilloma virus type were used in the study [16]. These cells were maintained in 1:1 mixture of Bronchial Epithelial Cell Basal Medium (BEBM; Lonza Walkersville, Inc.; Walkersville, MD, USA) and Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) supplemented with bronchial epithelial growth medium (BEGM; Lonza Walkersville, Inc.; Walkersville, MD, USA) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air. For cell treatment, the cells (5 × 10⁴) were inoculated into 96-well plates. When the cells were grown to 70-90% confluency, LPS was added to each well at a final concentration of 10 mg/ml and allowed to incubate for 6 h. Thereafter, the cells were maintained with or without glucocorticoids (5 μM) and phorbol 12-myristate 13-acetate (PMA) (5 nM; Sigma, St Louis, MO) for 12 h following treatment with LPS.

Cell viability

The cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, the cells (1 × 10⁵) were seeded in 96-well plates and treated with or without LPS and glucocorticoids. The cells were incubated for 24 h, 48 h and 72 h and then the cells were incubated with 10 μl MTT at a final concentration of 5 mg/ml (Sigma Chemical Co., USA) for 4 h. Thereafter, 100 μl dimethylsulfoxide (DMSO) was added to dissolve formazan crystals. Absorbance at 570 nm was detected using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, USA).

Apoptosis assay

The cell apoptosis was determined by Annexin-V/Propidium Iodide (PI) staining by flow cytometry according to the manufacturer’s protocol. Briefly, the cells (1 × 10⁶) were seeded in 96-well plates and treated with or without LPS and glucocorticoids. Then the cells were collected, washed three times with phosphate-buffered saline (PBS), trypsinized, and incubated with Annexin V-FLUOS labeling reagent (Roche Diagnostics Corporation, Indianapolis, IN) for 15 min in the dark at room temperature. The rate of cell apoptosis (%) was then analyzed by flow cytometry analysis.

Quantitative real-time RT-PCR (qRT-PCR)

After treatment with or without LPS, glucocorticoids and PMA, total RNAs were extracted from the cells using Trizol Reagent (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized by use of a random cDNA synthesis system (Roche Applied Science) according to the instructions of the supplier. Then the double-stranded cDNA was amplified with primers using a SYBR Green PCR kit (Qiagen, Hilden, Germany) in an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA). GAPDH was used as a loading control for mRNA expression. All primers were synthesized by Sangon Biotech. Co., Ltd. (Shanghai, China).

Western blot

After treatment with or without LPS, glucocorticoids and PMA, total protein was extracted from the cells using RIPA buffer (Santa Cruz Technology, CA, USA). Protein contents were
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measured by a standard Pierce Bicinchoninic Acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were resolved in 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were then blocked with 5% nonfat milk in Tris Buffered Saline with Tween (TBST) for 2 h at room temperature. Thereafter, the membranes were probed overnight at 4°C with the corresponding antibodies: anti-COX-2 antibody (Abcam, Cambridge, MA), anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-B-cell lymphoma (Bcl)-2 antibody (Santa), anti-inhibitory protein I-kappa-B-alpha (IκB-α) antibody (Cell Signaling Technology Inc., MA), or anti-phosphorylation-nuclear factor kappa B (p-NF-κB) antibody (Cell Signaling). After incubation with appropriate horse-radish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature, the bands were developed by using enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology).

**Statistical analysis**

All data are expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to determine statistically significant differences between groups. Results were analyzed using GraphPad Prism V6.0 (Graphpad Software San Diego, CA). A statistical significance was defined when $P < 0.05$.

**Results**

**Glucocorticoids inhibit LPS-induced inflammatory reaction in HMEECs**

To investigate the effects of glucocorticoids on OM, we first used LPS to induce the inflammatory reaction in HMEECs and then analyzed the expression of tumor TNF-α and IL-6. TNF-α and IL-6 are important inflammatory mediators that contribute to the development of OM. As shown in **Figure 1A**, both the mRNA expression of TNF-α and IL-6 was significantly elevated after exposure to LPS for 6 h compared to the control group (both $P < 0.01$). Next, glucocorticoids were added to each well after exposure to LPS, and then the expression of TNF-α and IL-6 was analyzed again. We observed that both the mRNA expression of TNF-α and IL-6 was remarkably reduced by application of glucocorticoids compared to the LPS group ($P < 0.05$ or $P < 0.01$) (**Figure 1B**). The results suggested...
that glucocorticoids could inhibit LPS-induced inflammatory reaction in HMEECs.

**Glucocorticoids suppress LPS-induced expression of COX-2 in HMEECs**

We then analyzed the expression of COX-2 after exposure to LPS and administration of glucocorticoids. As shown in Figure 1C, the relative mRNA expression of COX-2 was prominently increased by LPS compared to the control group ($P < 0.01$). However, the expression of COX-2 was statistically decreased by administration of glucocorticoids compared to the LPS group ($P < 0.05$). The protein expression of COX-2 was similar to the mRNA expression (Figure 1D). The protein level of COX-2 was significantly upregulated by LPS, and while downregulated by glucocorticoids, indicating that glucocorticoids could suppress LPS-induced expression of COX-2 in HMEECs.

**Glucocorticoids promote cell viability and reduce cell apoptosis of HMEECs**

The cell viability and the rate of cell apoptosis were determined after LPS or glucocorticoids treatment. As demonstrated in Figure 2A, the results showed that the cell viability at 24, 48, and 72 h was all significantly decreased by LPS exposure compared to the control group (all $P < 0.01$). However, the cell viability at 24, 48, and 72 h was all statistically increased by application of glucocorticoids compared to the LPS group (all $P < 0.05$). On the contrary, the rate of cell apoptosis was strikingly increased by LPS but decreased by glucocorticoids (both $P < 0.05$) (Figure 2B). To further explore the underlying mechanism of apoptosis, the apoptosis-related factors, Bax and Bcl-2, were analyzed by qRT-PCR and Western blot. We found that both the mRNA expression of Bax was significantly increased by LPS but was statistically decreased by glucocorticoids (both $P < 0.01$). However, the mRNA expression of Bcl-2 was significantly decreased by LPS but was statistically increased by glucocorticoids (both $P < 0.01$) (Figure 2C). The protein expression of Bax and Bcl-2 showed similar results as mRNA results (Figure 2D).

**Glucocorticoids inactivate NF-κB pathway in HMEECs**

Previous studies have proved that the activation of NF-κB pathway plays significant roles in inflammatory reaction of HMEECs. To investigate whether the inhibitory effect of glucocorticoids on the inflammatory reaction in HMEECs was related to the NF-κB pathway, we deter-
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mined the expression of IkB-α and NF-κB after exposure to LPS and glucocorticoids by qRT-PCR and Western blot. As indicated in Figure 3A-C, the results showed that the expression levels of IkB-α were significantly decreased, and while levels of NF-κB (for mRNA) p-NF-κB (for protein) were dramatically elevated by LPS (both \( P < 0.01 \)), demonstrating that LPS induced the degradation of IkB-α and activated the NF-κB pathway. However, we observed that glucocorticoids significantly suppressed the LPS-induced degradation of IkB-α (\( P < 0.001 \)) and decreased the levels of p-NF-κB (\( P < 0.05 \)). The results indicated that glucocorticoids could inactivate the NF-κB pathway in HMEECs.

**Effects of glucocorticoids on expression of inflammatory mediators are inhibited by PMA**

Based on the results, to further confirm glucocorticoids could inactivate the NF-κB pathway in HMEECs, we applied the activator of NF-κB, PMA, to the cells. Then we analyzed the expression of TNF-α, IL-6 and COX-2 by qRT-PCR. As demonstrated in Figure 4A-C, the results showed that glucocorticoids significantly lowered the expression of TNF-α, IL-6 and COX-2 (\( P < 0.05 \) or \( P < 0.01 \)). Nevertheless, these effects were reversed by PMA. That means, the relative expression of TNF-α, IL-6 and COX-2 were statistically elevated by PMA (\( P < 0.05 \)), indicating that PMA was able to inhibit the effects of glucocorticoids on anti-inflammatory actions in HMEECs.

**Discussion**

In the present study, we demonstrated that glucocorticoids significantly inhibited the expression of inflammatory mediators (TNF-α, IL-6 and COX-2) induced by LPS in HMEECs, and sta-
Glucocorticoids are a family of steroid hormones that have a wide range of physiological and biological properties, particularly immunoinflammatory responses. It has been well established that glucocorticoids are widely used in clinical practice as anti-inflammatory therapeutics to inhibit a large variety of inflammatory and immune responses. Although the use of glucocorticoids in treatment for OM is controversial, there is increasing support in the literature for using these drugs in patients with OM [33]. In our study, we had not only found that glucocorticoids could statistically decrease the expression of inflammatory mediators but also observed that glucocorticoids could significantly increase the cell viability but decreased the cell apoptosis. The possible molecular mechanism of cell apoptosis was investigated by determining the expression of apoptosis-related protein. The results demonstrated that glucocorticoids dramatically decreased the expression of apoptosis-promoting gene (Bax) but increased the expression of anti-apoptotic gene (Bcl-2), leading to anti-apoptosis.

NF-κB is a key transcription factor that participates in various regulation involved in inflammatory responses, cell proliferation, and cell apoptosis. Inhibition of NF-κB has been shown to downregulate the expression of many proinflammatory cytokines [34]. NF-κB is held in an inactive state in the cytoplasm, but is activated by the phosphorylation and degradation of IκB-α following inflammatory stimulation, resulting in nuclear translocation of NF-κB [35]. Hence, inhibition of IκB-α degradation might be regarded as a potential manner to inhibit inflammatory responses. To further explore whether glucocorticoids exhibits its anti-inflammatory responses through NF-κB signaling pathway, we analyzed the expression of IκB-α and p-NF-κB. Our data demonstrated that LPS-treated HMEECs had lower levels of IκB-α than non-treated control cells, and that administration of glucocorticoids inhibited IκB-α degradation. Our results were consistent with previous studies, in which glucocorticoids showed its immunosupression by inhibition of NF-κB activity through induction of IκB-α synthesis [36, 37]. In addition, we applied PMA, the activator of NF-κB, to prove the above results. As expected, the results showed that after application of PMA, the expression levels of TNF-α, IL-6 and COX-2 were significantly reversed, indicating...
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PMA inhibited the effects of glucocorticoids on inflammatory mediators.

In conclusion, our results demonstrate that glucocorticoids inhibit LPS-induced the production of TNF-α, IL-6 and COX-2 in HMEECs, promot cell viability and inhibit cell apoptosis. The effects might be by inhibition of NF-κB signaling pathway. Our study provides the evidence that glucocorticoids might be effective drugs to treat OM in cellular mechanisms.

Disclosure of conflict of interest

None.

Address correspondence to: An-Ting Xu, Department of Otorhinolaryngology, The Second Hospital of Shandong University, 247 Beiyouanda Street, Jinan 250033, Shandong Province, China. E-mail: xuanting4769@126.com

References

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