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Original Article

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

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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% CO_2/95% air. For cell treatment, the cells (5×10^4) 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 (1×10^5) 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 manufcturer's protocol. Briefly, the cells (1×10^6) 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

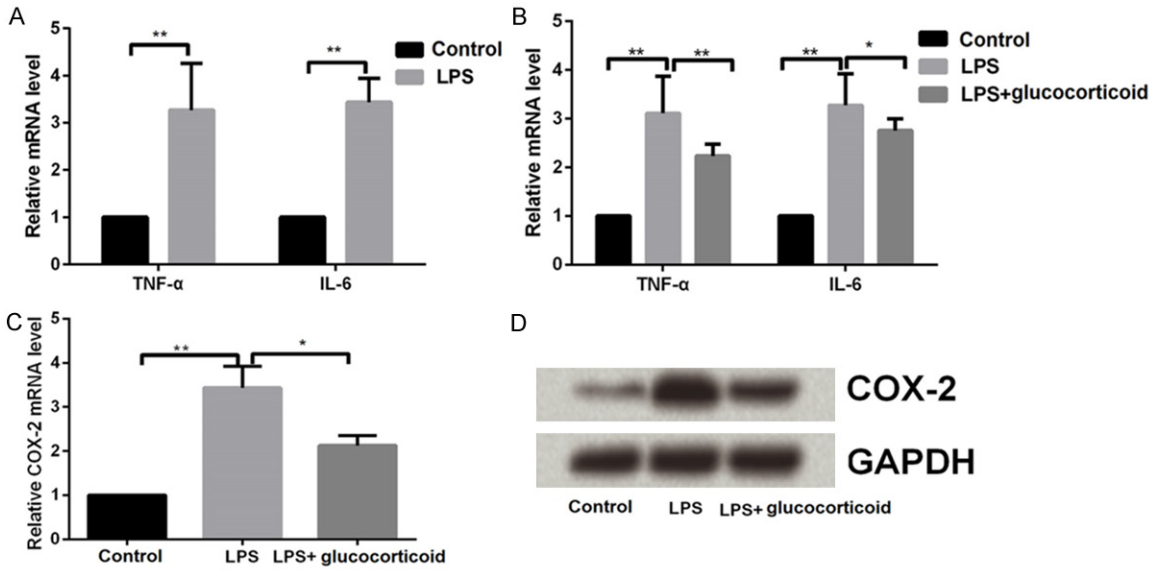

Figure 1. Glucocorticoids inhibit LPS-induced inflammatory reaction in HMEECs. A: The expression of TNF-α and IL-6 was significantly increased by LPS; B: The expression of TNF-α and IL-6 was statistically decreased by administration of glucocorticoids. Glucocorticoids suppress LPS-induced expression of COX-2 in HMEECs. C: The relative mRNA expression of COX-2 was markedly increased by LPS but decreased by glucocorticoids; D: The protein expression of COX-2 was distinctly elevated by LPS but reduced by glucocorticoids. (TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; HMEECs, human middle ear epithelial cells; COX, cyclooxygenase; LPS, lipopolysaccharide; HMEECs, human middle ear epithelial cells. $^{*}P < 0.05$; $^{**}P < 0.01$).

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 horseradish 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

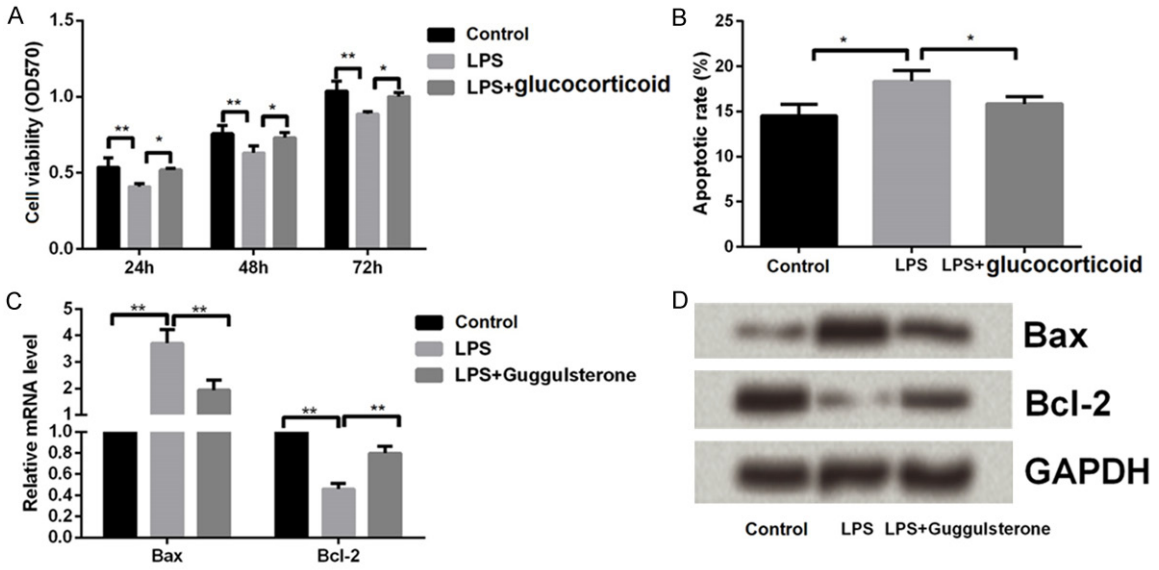

Figure 2. Glucocorticoids promote cell viability and reduce cell apoptosis of HMEECs. A: The cell viability was significantly decreased by LPS exposure but increased by glucocorticoids at different time points (24, 48, and 72 h); B: The rate of cell apoptosis was strikingly increased by LPS but decreased by glucocorticoids; C and D: LPS statistically increased the mRNA and protein expression of Bax but decreased Bcl-2, and glucocorticoids reversed the results. LPS, lipopolysaccharide; HMEECs, human middle ear epithelial cells; Bcl, B-cell lymphoma. $^{*}P < 0.05$; $^{**}P < 0.01$.

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-

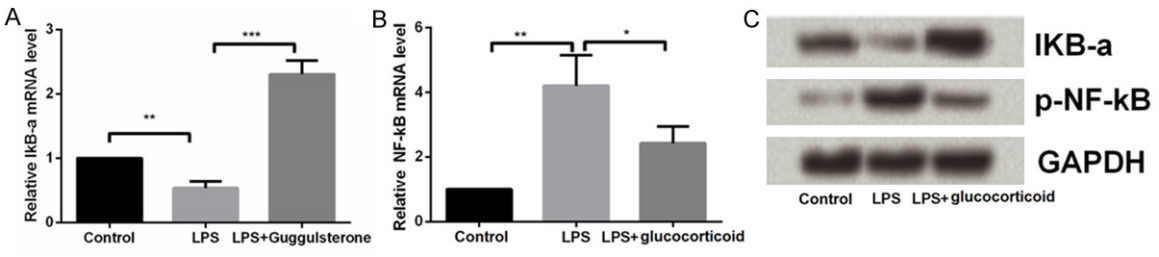

Figure 3. Glucocorticoids inactivate NF-κB pathway in HMEECs. A: The relative mRNA expression of IκB-α was dramatically decreased by LPS but upregulated by glucocorticoids; B: The relative mRNA expression of NF-κB was significantly upregulated by LPS but downregulated by glucocorticoids; C: The protein expression of IκB-α and p-NF-κB induced by glucocorticoids. LPS, lipopolysaccharide; HMEECs, human middle ear epithelial cells; IκB-α, I-kappa-B-alpha; p-NF-κB, phosphorylation-nuclear factor kappa B. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

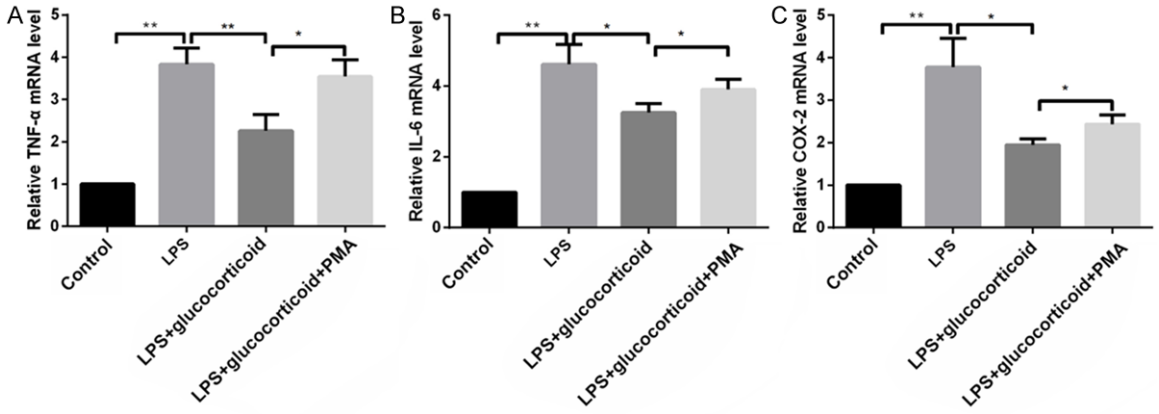

Figure 4. Effects of glucocorticoids on expression of inflammatory mediators are inhibited by PMA. A: The relative expression of TNF-α was significantly increased by administration of PMA; B: The relative expression of IL-6 was markedly upregulated by administration of PMA; C: The relative expression of COX-2 was remarkably raised by administration of PMA. LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; COX, cyclooxygenase; PMA, phorbol 12-myristate 13-acetate. $^{*}P < 0.05$; $^{**}P < 0.01$.

mined the expression of IκB-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 IκB-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 IκB-α and activated the NF-κB pathway. However, we observed that glucocorticoids significantly suppressed the LPS-induced degradation of IκB-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 relative 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-

tistically increased the cell viability but decreased the cell apoptosis. Besides, LPS-induced the production of apoptosis-related factor Bax and NF-κB pathway related proteins p-NF-κB was dramatically inhibited by administration of glucocorticoids. However, the levels of Bcl-2 and IκB-α were promoted by glucocorticoids. Furthermore, the effects of glucocorticoids on the expression of inflammatory mediators were remarkably alleviated by the activator of NF-κB, PMA. Thus, we concluded that the anti-inflammatory effect of glucocorticoids might be regulated by the inhibition of NF-κB activation.

OM is a very common bacterial infection in the middle ear [17]. The bacterial infection rapidly stimulates the host mucosal immune response and inflammatory reactions, leading to the production of cytokines and chemokines [18, 19]. A variety of cytokines have been discovered in middle ear effusions, e.g. TNF-α, IL-6 and COX-2 [20-23]. These cytokines are known to be important for the development of OM by increase of vascular permeability and secretion activity [19]. TNF-α is a significant and potent proinflammatory mediator that causes inflammation. It can be significantly induced by the stimulation of bacterial LPS and viruses [24, 25]. In addition, TNF-α has been considered to be responsible for the tissue damage, fibrosis, and bone resorption in OM [26]. Moreover, it has been found that the inhibitor of TNF-α shows a therapeutic effect on LPS-induced OM in rats [27, 28]. IL-6 is also regarded as a significant proinflammatory mediator in the inflammatory cascade in OM [29]. It has been reported that IL-6 is involved in the early stage of OM and contributes to the defensive reaction [22]. COX-2 is an inducible enzyme that promotes the production of prostaglandins (PGs), which plays a critical role in inflammation [30]. Previous studies proved that the expression of COX-2 was significantly elevated in animal models of LPS-induced OME and may be one important factor contributing to the development of OME [31, 32], and that administration of COX-2 inhibitor within 12 h might be considered effective because of the relief of inflammation [31]. In the present study, we observed that the expressions of TNF-α, IL-6 and COX-2 were all significantly increased by LPS, which was in line with the above studies. But the production was statistically inhibited by glucocorticoids.

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

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.

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References

[1] Pichichero ME. Otitis media. Pediatr Clin North Am 2013; 60: 391-407.
[2] Qureishi A, Lee Y, Belfield K, Birchall JP and Daniel M. Update on otitis media-prevention and treatment. Infect Drug Resist 2014; 2014: 15-24.
[3] Önerci TM. Complications of Otitis Media. Diagnosis in Otorhinolaryngology 2010; 43-44.
[4] Daniero JJ, Clary MS, O'Reilly RC. Complications of otitis media. Infect Disord Drug Targets 2012; 12: 267-270.
[5] Acuin J and Organization WH. Chronic suppurative otitis media: burden of illness and management options. 2004.
[6] Charmandari E, Tsigos C and Chrousos G. Endocrinology of the stress response 1. Annu Rev Physiol 2005; 67: 259-284.
[7] Reul JM, Collins A, Saliba RS, Mifsud KR, Carter SD, Gutierrez-Mecinas M, Qian X and Linthorst AC. Glucocorticoids, epigenetic c and stress resilience. Neurobiol Stress 2014; 1: 44-59.
[8] Benghuzzi HA, Tucci MA, Farah I, Hamadain E, Cameron JA. The Effect of Glucocorticoids and LPS on the Functional Activity of RAW Cell Line, 2016 32nd Southern Biomedical Engineering Conference (SBEC). IEEE 2016; 27-28.
[9] Bijlsma J, Jacobs J and Buttgereit F. Glucocorticoids in the treatment of rheumatoid arthritis. Clin Exp Rheumatol 2015; 33: 34.
[10] Wood AJ and Barnes PJ. Inhaled glucocorticoids for asthma. N Engl J Med 1995; 332: 868-875.
[11] Keatings VM, Jatakanon A, Worsdell YM and Barnes PJ. Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. Am J Respir Crit Care Med 1997; 155: 542-548.
[12] Annane D. Glucocorticoids in the treatment of severe sepsis and septic shock. Curr Opin Crit Care 2005; 11: 449.
[13] Baggett HC, Prazma J, Rose AS, Lane AP and Pillsbury HC. The role of glucocorticoids in endotoxin-mediated otitis media with effusion. Arch Otolaryngol Head Neck Surg 1997; 123: 41-46.
[14] Butler CC and van der Voort JH. Steroids for otitis media with effusion: a systematic review. Arch Pediatr Adolesc Med 2001; 155: 641-647.
[15] MacArthur CJ, Kempton JB, DeGagne J and Trune DR. Control of chronic otitis media and sensorineural hearing loss in C3H/HeJ mice: glucocorticoids vs mineralocorticoids. Otolaryngol Head Neck Surg 2008; 139: 646-653.
[16] Moon SK, Brackmann DE, Lee HY, Rhim JS, Webster P and Lim DJ. Immortalization of normal adult human middle ear epithelial cells using a retrovirus containing the E6/E7 genes of human papillomavirus type 16. Ann Otol Rhinol Laryngol 2002; 111: 507-517.
[17] Trune DR and Zheng QY. Mouse models for human otitis media. Brain Res 2009; 1277: 90-103.
[18] Song JJ, Cho JG, Hwang SJ, Cho CG, Park SW and Chae SW. Inhibitory effect of caffeic acid phenethyl ester (CAPE) on LPS-induced inflammation of human middle ear epithelial cells. Acta Oto-laryngol 2008; 128: 1303-1307.
[19] Leibovitz E. The challenge of recalcitrant acute otitis media: pathogens, resistance, and treatment strategy. Pediatr Infect Dis J 2007; 26: S8-S11.
[20] Yellon RF, Leonard G, Marucha PT, Craven R, Carpenter RJ, Lehmann WB, Burleson JA and Kreutzer DL. Characterization of cytokines present in middle ear effusions. Laryngoscope 1991; 101: 165-169.
[21] Maxwell KS, Leonard G, Carpenter R, Kreutzer DL, Fitzgerald JE and Burleson JA. Interleukin-8 expression in otitis media. Laryngoscope 1994; 104: 989-995.
[22] Sato K, Kawana M, Nonomura N and Nakano Y. Course of IL-1β, IL-6, IL-8, and TNF-α in the middle ear fluid of the guinea pig otitis media model induced by nonviable Haemophilus influenzae. Ann Otol Rhinol Laryngol 1999; 108: 559-563.
[23] Smirnova MG, Kiselev SL, Gnuchev NV, Birchall JP and Pearson JP. Role of the pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin-1 beta, interleukin-6 and interleukin-8

in the pathogenesis of the otitis media with effusion. Eur Cytokine Netw 2001; 13: 161-172.
[24] Schwabe RF and Brenner DA. Mechanisms of liver injury. I. TNF-α-induced liver injury: role of IKK, JNK, and ROS pathways. Am J Physiol Gastrointest Liver Physiol 2006; 290: G583-G589.
[25] DeMaria TF and Murwin DM. Tumor Necrosis Factor During Experimental Lipopolysaccharide-Induced Otitis Media. Laryngoscope 1997; 107: 369-372.
[26] Ophir D, Hahn T, Schattner A, Wallach D and Aviel A. Tumor necrosis factor in middle ear effusions. Arch Otolaryngol Head Neck Surg 1988; 114: 1256-1258.
[27] Jeun EJ, Park YS, Yeo SW, Choi YC and Jung TT. Effect of inhibitor of tumor necrosis factor-alpha on experimental otitis media with effusion. Ann Otol Rhinol Laryngol 2001; 110: 917-921.
[28] Kim DH, Park YS, Jeon EJ, Yeo SW, Chang KH and Lee SK. Effects of tumor necrosis factor alpha antagonist, platelet activating factor antagonist, and nitric oxide synthase inhibitor on experimental otitis media with effusion. Ann Otol Rhinol Laryngol 2006; 115: 617-623.
[29] Barzilai A, Dekel B, Dagan R and Leibovit E. Middle ear effusion IL-6 concentration in bacterial and non-bacterial acute otitis media. Acta Paediatr 2000; 89: 1068-1071.
[30] Crofford L. COX-1 and COX-2 tissue expression: implications and predictions. J Rheumatol Suppl 1997; 49: 15-19.
[31] Cho JG, Lee ES, Woo JS, Lee HM, Jung HH, Hwang SJ and Chae SW. Expressions of cyclooxygenase 1 and 2 in endotoxin-induced otitis media with effusion in the rat. Int J Pediatr Otorhinolaryngol 2007; 71: 101-106.
[32] Kakiuchi M, Tsujigiwa H, Orita Y, Nagatsuka H, Yoshinobu J, Kariya S, Haginomori SI, Orita S, Fukushima K and Okano M. Cyclooxygenase 2 expression in otitis media with effusion. Am J Otolaryngol 2006; 27: 81-85.
[33] Waldron CA, Thomas-Jones E, Cannings-John R, Hood K, Powell C, Roberts A, Tomkinson A, Fitzsimmons D, Gal M and Harris D. Oral steroids for the resolution of otitis media with effusion (OME) in children (OSTRICH): study protocol for a randomised controlled trial. Trials 2016; 17: 1.
[34] Gasparini C and Feldmann M. NF-κB as a target for modulating inflammatory responses. Curr Pharm Des 2012; 18: 5735-5745.
[35] Karin M, Delhase M. The IκB kinase (IKK) and NF-κB: key elements of proinflammatory signalling, Seminars in immunology. Elsevier 2000; 12: 85-98.
[36] Auphan N, DiDonato JA, Rosette C, Helmberg A and Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappaB activity through induction of IkappaB synthesis. Science 1995; 270: 286.
[37] Scheinman RI, Cogswell PC, Lofquist AK and Baldwin AS. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science 1995; 270: 283-286.