International Journal of Pediatric Otorhinolaryngology 89 (2016) 28–32

Contents lists available at ScienceDirect

International Journal of Pediatric Otorhinolaryngology

journal homepage: http://www.ijporlonline.com/

The suppressive effects of metformin on inflammatory response of otitis media model in human middle ear epithelial cells

Jae Gu Cho, Jae Jun Song, June Choi, Gi Jung Im, Hak Hyun Jung, Sung Won Chae*

Department of Otolaryngology-Head and Neck Surgery, Korea University College of Medicine, Seoul, South Korea

ARTICLE INFO

Article history:
Received 9 March 2016
Received in revised form
19 July 2016
Accepted 21 July 2016
Available online 25 July 2016

Keywords:
Metformin
Otitis media
LPS
Human middle ear epitheilial cells

ABSTRACT

Objective: Metformin is a well-known anti-diabetic agent, but its mechanism is unclear. Recently, many reports have described the anti-inflammatory effects of metformin on various cell types, including human vascular smooth muscle cells and endothelial cells. This study was designed to investigate the anti-inflammatory effect of metformin on lipopolysaccharide (LPS) induced inflammation in human middle ear epithelial cell lines (HMEECs).

Methods: The effect of pretreatment by metformin (0, 1, 2, 4 mM) was evaluated by the inflammatory response in the HMEECs exposed to LPS (10 ng/ml). For verifying the suppression effect of metformin on the inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) was evaluated by real-time polymerase chain reaction, and COX-2 protein was assessed by western blotting. Intracellular reactive oxygen species (ROS) was measured using 2′, 7′-dichlorofluorescein diacetate (DCFHDA) fluorocytometer.

Results: Stimulation by LPS 10 ng/ml concentration showed 12.4 folds increase the expression of *TNF-α mRNA* compared to control on HMEECs. Pretreatment of metformin dose dependently suppressed the expression of *TNF-α mRNA* induced by LPS (2 mM, $p = 0.03$). The amount of COX-2 protein production was significantly decreased by metformin pretreatment (4 mM, $p = 0.01$). The production of ROS was decreased significantly by pretreatment of metformin ($p = 0.03$).

Conclusions: These findings suggest that the inflammatory response and oxidative stress induced by LPS could be suppressed by metformin in HMEECs. Therefore, metformin may have a therapeutic potential for the treatment of the otitis media.

1. Introduction

Otitis media (OM) is the most common inflammatory disease in middle ear cavity. Most children experiences at least one episode of OM, even though the majority heals spontaneously. In some cases of them, the disease evolves into a more chronic phase with sustained inflammation and effusion material entrapped in the middle ear [1]. In recent report, children with OM have a poor quality of life and suffer from sleep, loss of appetite, otalgia, and behavioral problems [2].

A major cause of OME seems to be middle ear infections. The microbial products such as lipopolysaccharide(LPS) activate the inflammation in the first place, and pro-inflammatory mediators including bacterial components, cytokines, and chemokines have established the vicious cycle after the initiating factors are eliminated [3]. These causes of inflammation increase vascular permeability, chemotaxis, and production of reactive oxygen species (ROS), and are responsible for middle ear inflammation [4]. Therefore, modulation of these inflammatory factors may influence middle ear inflammation.

The anti-inflammatory potential of metformin, a frontline drug for the treatment of type 2 diabetes mellitus, has been reported in many experimental models such as endothelial cells, human aortic smooth muscle cells, and activated macrophages [5–7]. In recent study, metformin inhibited the production of ROS from NADH in LPS-activated macrophage [8].

Aims of this study was to investigate the anti-inflammatory effect of metformin on LPS induced inflammation in HMEECs by measuring the expression of *TNF-α mRNA,* reactive oxygen species(ROS) suppression, and COX-2 protein production.

* Corresponding author. Department of Otorhinolaryngology-Head and Neck Surgery, Guro Hospital, Korea University College of Medicine, 80 Guro-dong, Guro-gu, Seoul 152-703, South Korea.
E-mail address: koreaschae@gmail.com (S.W. Chae).

http://dx.doi.org/10.1016/j.ijporl.2016.07.025
0165-5876/

2. Materials and methods

2.1. Cell culture

HMEECs was maintained in a mixture of DMEM and BEBM (1:1) supplemented with bovine pituitary extract (52 μg/ml), hydrocortisone (0.5 μg/ml), hEGF (0.5 ng/ml), epinephrine 0.5 (mg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), triiodothyronine (6.5 ng/ml), retinoic acid (0.1 ng/ml), gentamycin (50 μg/ml), and amphotericin-B (50 ng/ml). All cells were grown in a humidified atmosphere at 37 °C containing of 5% CO2 and 95% air. Growth media was changed every 3rd day.

A bacterial endotoxin LPS (10 ng/ml, Sigma, St Louis, MO, USA) from *Pseudomonas aeruginosa* was used to induce inflammatory response and metformin (0, 1, 2, and 4 mM, Sigma) was used to inhibit inflammatory response. Horseradish peroxidase-labeled anti-rabbit antibody was used as secondary antibody (Amersham, Buckinghamshire, UK).

2.2. Real-time polymerase chain reaction (PCR) analysis

Total RNA from HMEECs was extracted using RNeasy® Mini Kits (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions (RNeasy handbook, June 2001). Total cellular RNA (15 μg) was reverse-transcribed for 60 min at 50 °C in a 60 μl reaction mixture containing; 100U of Superscript-III reverse transcriptase (Invitrogen), 10 mM dithiothreitol, 1 mM of each of dATP, dTTP, dCTP, and dGTP, and 100 μg of Oligo-dT primer (Amersham Biosiences, Piscataway, NJ, USA) per milliliter. Reactions were stopped by heat inactivation for 10 min at 70 °C.

Real-time PCR was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reaction mixtures contained 12.5 μl of 2× SYBR Green Master Mix (containing; 200 μM of each of dATP, dGTP, and dCTP, 400 μM dUTP, 2 mM $MgCl_2$, 0.125U uracil N-glycosylase, and 0.313U Ampliteq Gold DNA polymerase), 6.25 pmol of each sense and antisense primer, and 2 μl of cDNA in a final volume of 25 μl. Oligonucleotide primers were synthesized commercially at Bioneer Company (Daejon, Republic of Korea) as follows: sense primer; 5′-ATC TTC TCG AAC CCC GAG TGA -3′ and antisense primer; 5′- GGG TTT GCT ACA ACA TGG GC -3′ for *TNF- α*; and sense primer; 5′-CGC CCT GTT CGC TCT GGG -3′ and antisense primer; 5′- AGG AGG TCC GCA TGC TCA -3′ for *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. Reaction mixtures were incubated for 2 min at 55 °C followed by 10 min at 95 °C to activate the Ampliteq Gold DNA polymerase. The amplification profile used was; 40 cycles of 15sec of denaturation at 95 °C followed by 1 min of annealing at 60 °C for *TNF- α* and *GAPDH*. Data were analyzed using Sequence Detection System software (Applied Biosystems). Results were obtained in 3 repeated experiments with triplicate samples.

2.3. Western blotting

Cells were stimulated with LPS for 4 h and were pretreated for 20 h with four different concentration of 0, 1, 2, and 4 mM metformin.

After the treatment of HMEECs with CSS, the medium was removed out and the cells were washed twice in PBS (10 mM, pH 7.4). The cells were incubated in 0.4 ml ice-cold lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris [pH 7.5], 1 mmol/L EDTA, 0.1% Triton X-100) containing 0.5% protease inhibitor cocktail III (Calbiochem., San Diego, CA, USA). The cells were then centrifuged at 13,000 *g* for 25 min at 4 °C, and the supernatant (total cell lysate) was collected, aliquoted and stored at −70 °C. The protein concentration was determined by RC DC Protein Assay kit using the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

Aliquots (25 μg protein) were mixed with sample buffer (Bio-Rad) containing 2% mercaptoethanol, boiled for 5 min, and electrophoresed on 12% tris-HCl gels. After transfer to nitrocellulose membranes (Hybond ECL; Amersham Biosciences Corp.), membranes were blocked with PBS, 0.1% Tween-20 containing 5% (w/v) dry milk and 1% bovine serum albumin for 1 h at room temperature. Membranes were probed with COX-2 mouse anti-human antibody (clone 45 M1, 1:200; Neomarkers, Fermont, CA, USA), α-tubulin (Merck Biosciences, San Diego, CA, USA) or control goat IgG, followed by donkey antigoat IgG coupled to horseradish peroxidase (1:10,000 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), developed using an ECL detection kit (Pierce, Rockford, IL, USA) and exposed to X-ray film (XAR5, Kodak, Rochester, NY, USA). Densitometric analysis was performed using a Scion imager (Scion, Frederick, MD, USA). Relative protein expressions were calculated by determining the ratio of protein to α-tubulin. Results were obtained in 2 repeated experiments.

2.4. Measurement of ROS

Cells plated in 100 mm dishes subjected to measurement of intracellular ROS using 2′,7′-dichlorofluorescein diacetate (DCFHDA). HMEECs were seeded in 100 mm plates (1×10^6 cells/dish) and incubated with growth media for 24hr. In separate experiments, cells were pretreated by metformin for 20 h. After 4 h LPS treated, cells were rinsed with PBS (Biosolution, BP007, Korea), loaded with 50 μM DCFHDA, and incubated for 30 min at 37 °C. After which DCF fluorescence was measured by Cytomics FC500 (Beckman Coolter).

2.5. Statistics

All data are expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to determine statistically significant differences between groups. Scheffé's F-test was used to correct for multiple comparisons when statistical significances were identified in the ANOVA. $P < 0.05$ for the null hypothesis was accepted as indicating a statistically significant difference.

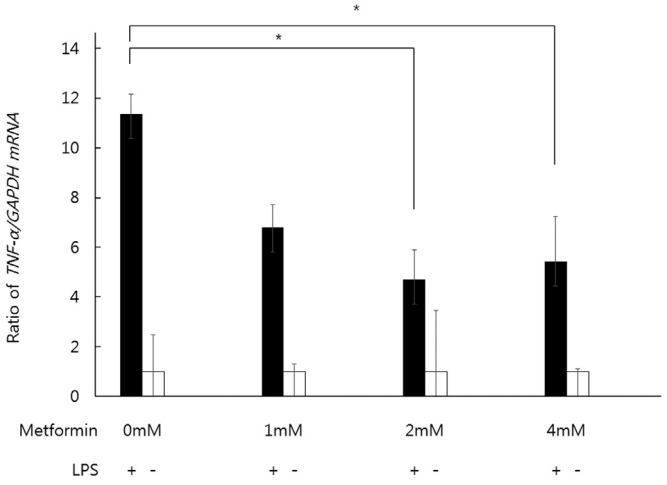

Fig. 1. Inhibitory effect of metformin on LPS-induced *TNF-α mRNA* expression. Pretreatment with metformin dose-dependently suppressed *TNF-α mRNA* expression. In the 2 mM and 4 mM concentration of metformin pretreatment, there were statistically significant suppressive effect on expression of *TNF-α mRNA*. But there were no different effect between in the 2 and 4 mM concentration of metformin pretreatment. LPS: lipopolysaccharide (*: $p < 0.05$).