EGCG induces β-defensin 3 against influenza A virus H1N1 by the MAPK signaling pathway

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Abstract. Epigallocatechin gallate (EGCG) is the main component of green tea, which has been proven to inhibit a variety of viruses, including influenza A virus. However, the mechanism of EGCG against influenza virus remains to be further explored. The mechanism of EGCG against influenza virus was studied. The results showed that EGCG significantly increased the levels of HBD3 mRNA and protein, while the levels of phosphorylation of (p)-p38 MAPK, ERK and JNK after EGCG treatment were significantly up-regulated. p38 MAPK, ERK and JNK inhibitors significantly inhibited the expression of HBD3 induced by EGCG. On the other hand, EGCG significantly inhibited the expression of HA and NP proteins in influenza A virus H1N1, but attenuated the anti-influenza A virus effect of EGCG after silencing HBD3. Thus, the anti-influenza virus effect of EGCG is related to the induction of HBD3 expression. In addition, the expression of EGCG-induced HBD3 is related to the p38 MAPK, ERK and JNK signaling pathways. The research data show that EGCG can induce HBD3 expression through p38 MAPK, ERK and JNK signaling pathway to inhibit the replication of influenza A virus H1N1, providing a new and effective candidate drug for influenza virus.

Introduction

Influenza A virus (IAV) is a single negative chain RNA virus belonging to the positive myxovirus family, which is the main pathogen of seasonal influenza. Seasonal influenza kills 290,000 to 650,000 patients every year (1-3). Influenza vaccine and antiviral drugs are the main strategies for the prevention and treatment of influenza virus. However, at present, the influenza virus vaccine is only aimed at the corresponding subtypes of strains, and the anti-influenza virus drugs amantadine and neuraminidase inhibitors have serious drug resistance (4,5). Therefore, it is urgent to find a long-term and effective new strategy against influenza virus.

β-defensin 3 is a class of antibacterial peptides produced by epithelial cells (6,7). It has been found that β-defensin 3 enhances the resistance ability against influenza virus (8). MDCK cells transfected with β-defensin 3 plasmid also showed resistance to influenza virus (9). Respiratory epithelial cells are the first line of defense against influenza virus, so inducing β-defensin 3 in respiratory epithelial cells may become a new strategy against influenza virus. MAPKs are a key family of proteins involved in regulating gene expression in response to extracellular stimuli and activating a variety of cellular responses, including cell proliferation, differentiation and apoptosis (10). According to the literature, the expression and regulation of human β-defensin 3 in human lung epithelial cells are dependent on MAPKs ERK1/2 and JNK (11). Epigallocatechin gallate (EGCG) is the most abundant catechin in green tea, which has a variety of biological functions, including anti-tumor, anti-inflammatory and immunomodulatory effects (12). Recently, it has been found that EGCG also has anti-influenza virus effect. Previous studies have shown that EGCG mainly prevents the virus from entering the cell by destroying the viral envelope (13). EGCG can also play an antiviral role by inducing the expression of antiviral protein (14). However, it has not been clarified at this stage whether EGCG can play an antiviral role by inducing the expression of β-defensin 3 in respiratory epithelial cells and the mechanism of inducing the expression of β-defensin 3.

Therefore, in the present study, bronchial epithelial cells (BEAS-2B) were used as a model to explore the mechanism of β-defensin 3 expression induced by EGCG and the relationship between β-defensin 3 expression and anti-influenza A virus. The results confirmed that EGCG pretreatment against influenza A virus is related to the induction of the expression of β-defensin 3. EGCG induces the expression of β-defense 3...
through the p38 MAPK, ERK, JNK signaling pathway to inhibit the replication of influenza A virus H1N1 in BEAS-2B cells. We elucidated part of the mechanism of EGCG against influenza A virus.

Materials and methods

Cells, virus and reagents. BEAS-2B cells were purchased from Kunming Cell Bank of the Chinese Academy of Sciences (Kunming, China) and cultured in KCB medium at 37°C with 5% CO₂. Madin-Darby canine kidney (MDCK) cells were purchased from Kunming Cell Library of the Chinese Academy of Sciences (Kunming, China), and cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% v/v fetal bovine serum both from (Gibco; Thermo Fisher Scientific, Inc.). Penicillin (100 U/ml), and 100 μg/ml streptomycin both from (HyClone; GE Healthcare Life Sciences) at 37°C in 5% CO₂. Influenza A virus (A/PR/8/34 H1N1, PR8) in this experiment was cultured in the allantoic cavity of chicken embryo at 9 days of age for 48 h, and the Reed-Muench method was used to determine the TCID₅₀ of the virus in MDCK cells. The study was approved by the Ethics Committee of The Affiliated Hospital of Guizhou Medical university (Guiyang, China).

Effect of EGCG on BEAS-2B cell activity. Different concentrations of EGCG (Solarbio Life Science; SE8120) (6.25, 12.5, 25, 50, 100 μg/ml) were added to the monolayer BEAS-2B cells in 96-well plates. Five wells were repeated for each concentration and cultured for 48 h. Cell viability was determined by CCK-8 kit (Dojindo Molecular Technologies Inc.) and absorbance was detected at 450 nm by BioTek Instruments, Inc.

Intervention of EGCG on BEAS-2B cells. Different concentrations of EGCG (0, 6.25, 12.5, 25 and 50 μg/ml) were added to the single-layer BEAS-2B cells in the 6-well plate and cultured for 24 h. EGCG of 50 μg/ml was added to the single-layer BEAS-2B cells in the 6-well plate and cultured for 2, 4, 12, 24 and 48 h. One hour before adding 50 μg/ml EGCG, 20 μM SB203580, 20 μM SP600125 dissolved in dimethyl sulfoxide was added to inhibit the MAPK signaling pathway.

Transfection of silenced HBD3 in BEAS-2B cells. When 50% of the bottom area of the confocal dish was covered by cells, the culture solution was discarded. After rinsing 3 times with 1X PBS, serum-free DMEM basal medium was added at 2 ml/well. After culture for 12 h, 120 μl rife FECTCP buffer, 5 μl, 20 μM siRNA and 12 μl ribo FECTCP reagent was taken. HBD3, sequence 5'-TGTGGAAATGCCTTCTTAA-3' and control siRNA (NC), article number: N Control_05815, transfection reagent and primer design were provided by Guangzhou RiboBio Co., Ltd. The mixture was placed at room temperature for 15 min to form a transfection complex, then mixed with 1,863 μl of serum-free DMEM medium (confocal dish 1 well system). Then cells were added and incubated at 37°C for 24 h.

Virus infection of BEAS-2B cells. At 24 h after transfection, the original culture solution was removed and rinsed 3 times with 1X PBS. The experimental groups were control, IAV, EGCG + IAV, EGCG + siNC and EGCG + IAV + siHBD3 group. In IAV, EGCG + IAV and EGCG + IAV + siHBD3 group, 100X TCID₅₀ H1N1 collected after chicken embryo culture was added, and 50 μg/ml EGCG was added to EGCG + IAV group, EGCG + siNC group and EGCG + IAV + siHBD3 group for 12 h culture. After washing twice with PBS, the medium was changed to KCB and cultured for 12 h.

RNA extraction and real-time fluorescence quantitative PCR. Total RNA was extracted using total RNA extraction kit (Tiangen Biotech Co., Ltd.) and tested on NanoDrop 2000 (Thermo Fisher Scientific, Inc.) UV spectrophotometer for RNA purity and concentration. cDNA was synthesized using PrimeScript™ RT kit with gDNA Eraser (Takara Bio, Inc.). Quantitative PCR was performed on CFX96 real-time fluorescent quantitative PCR instrument (Bio-Rad Laboratories, Inc.). Primer sequence-β-defensin 3 forward: 5'-TTATTTGCAGAGT CAGAGCGCG-3', and reverse: 5'-CGACACCTTGGCGAT CTGT-3'. GAPDH forward, 5'-AGAAAGGCTGGGCTCAT TTG-3' and reverse 5'-AGGGGCCATACGAGTCTTC-3'. The expression of β-defensin 3 mRNA was calculated using the 2⁻ΔΔCq method.

Western blot analysis. Cells were washed with PBS at 37°C and RIPA lystate buffer was added into the cells and centrifuged at 4°C for 5 min at 14,800 x g to obtain complete cell lysates. Protein samples (20 μg) were electrophoresed using SDS polyacrylamide gel (Bio-Rad Laboratories, Inc.) and the target protein molecules were transferred to a polyvinylidene fluoride membrane. Polyvinylidene fluoride membrane was sealed for 2 h. Then the corresponding resistance p-ERK (cat. no. 4370; 1:1,000), and ERK (cat. no. 4695; 1:1,000) (both from Cell Signaling Technology, Inc.), p-JNK (Abcam; 1:1,000), JNK (Abcam; cat. no. ab179461; 1:1,000), p-p38 lightning MAPK (Cell Signaling Technology, Inc.; cat. no. 9211; 1:1,000), p38 lightning MAPK (Cell Signaling Technology, Inc.; cat. no. 9212; 1:1,000), NP (BIOS; cat. no. bs-4976R; 1:1,000) and GAPDH (Boster Biological Technology; cat. no. M00227-5; 1:700) were added and incubated overnight at 4°C. After washing, horseradish peroxidase-labeled

Figure 1. Toxicity test of EGCG in BEAS-2B cells. BEAS-2B were treated with EGCG at various concentrations (0, 6.25, 12.5, 25 and 100 μg/ml) for 48 h. Cell viability was analyzed by CCK-8 assay. Data are shown as means ± SD in five parallel experiments. *p<0.05, when compared with the control without EGCG. The data are representative of three independent experiments. EGCG, Epigallocatechin gallate; SD, standard deviation.
secondary antibodies (Boster Biological Technology; cat. no. BA1054; 1:5,000) were added for incubation and exposure in the gel imaging system.

Cytokine detection. The supernatant of cell culture was taken and the content of β-defensin 3 was detected by human β-defensin 3 ELISA kit (Wuhan Gene-beauty Organism).

Immunofluorescence detection of influenza virus replication in BEAS-2B cells. When the BEAS-2B cells were fused to 80%, the cells were washed with PBS twice, fixed with 4% paraformaldehyde for 30 min, and then treated with 0.2% triton for 2 min. After blocking with 5% BSA, rabbit anti-NP antibody (Boster Biological Technology) and rabbit anti-HA antibody (Cell Signaling Technology, Inc.) were added at 4˚C overnight. After washing with PBS, fluorescence labeled secondary antibody (Boster Biological Technology) was added avoiding light for 30 min and photographed under a confocal microscope.

Statistical analysis. Data are expressed as the mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA), with Bonferroni multiple comparison post hoc test, or Student's t-test. Statistical calculations were carried out using the GraphPad InStat3 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of EGCG on cell activity. Different concentrations of EGCG (0, 6.25, 12.5, 25, 50, 100 μg/ml) were applied to BEAS-2B cells for 48 h, and CCK8 kit was used to detect cell activity. The results showed that the activity of BEAS-2B cells was significantly inhibited only by 50 μg/ml EGCG compared with the control group, and the remaining concentrations were not significantly different from those in the control group (Fig. 1). Therefore, EGCG of up to 50 μg/ml was selected for the next experiment.

Expression of β-defensin 3 in BEAS-2B cells induced by EGCG. After confirming that EGCG at concentrations of up to 50 μg/ml had no effect on cell activity, the time-dose relationship was studied between EGCG-induced expression of β-defensin 3 in BEAS-2B cells. After the intervention of different concentrations of EGCG in BEAS-2B cells, the expression level of β-defensin 3 mRNA was detected by real-time fluorescence quantitative PCR and the protein expression level of β-defensin 3 was detected by ELISA. The results showed that the expression of β-defensin 3 in BEAS-2B cells was up-regulated in a dose-dependent manner, with the highest concentration of 50 μg/ml (Fig. 2A and B). When EGCG was applied to BEAS-2B cells at a concentration of 50 μg/ml, the expression of β-defense 3 mRNA and protein in BEAS-2B cells at different time periods was up-regulated.
in a time-dependent manner and reached its peak at 24 h (Fig. 2C and D).

Expression of phosphorylation protein in MAPK signaling pathway induced by EGCG in BEAS-2B cells. It was confirmed that β-defensin 3 was most strongly induced by 50 μg/ml EGCG, so the signal pathway of β-defensin 3 expression in BEAS-2B cells was determined by 50 μg/ml EGCG. BEAS-2B cells were treated with EGCG for 0, 2, 4, 8, 12 and 24 h. The effects of EGCG on the activation of p38 MAPK, ERK and JNK in BEAS-2B cells were determined. The results showed that EGCG did not affect the levels of total p38 MAPK, ERK and JNK protein over time, but significantly increased the levels of phosphorylated p38 MAPK, p-ERK and p-JNK (Fig. 3).

Inhibition of MAPK signaling pathway induces expression of β-defensin 3 mRNA and protein in BEAS-2B cells induced by EGCG. The expression of β-defensin 3 induced by p38 MAPK, ERK and JNK signaling pathway in EGCG was further detected using 20 μM SB203580, 20 μM U0126 or 10 μM SP600125 in the pretreatment of the BEAS-2B cells for 1 h and then 50 μg/ml EGCG was added to the cells. Pretreatment with 20 μM SB203580, 20 μM U0126 and 10 μM SP600125 significantly inhibited the expression of β-defensin 3 mRNA and protein induced by EGCG (Fig. 4).

Expression of HBD3 mRNA and protein in BEAS-2B cells after silent transfection qPCR and ELISA were used to detect the expression of HBD3 mRNA and protein in BEAS-2B cells of siNC and siHBD3. The results showed that compared with the siNC group, the expression of HBD3 mRNA and protein in the siHBD3 group was significantly reduced (P<0.01) (Fig. 5). This suggested the successful silencing of the HBD3 gene of BEAS-2B cells.

EGCG induces BEAS-2B cells against influenza A virus H1N1. The above study confirmed that EGCG can induce the expression of β-defensin 3 in BEAS-2B cells. Whether EGCG anti-influenza virus H1N1 was related to the induction of β-defensin 3 expression was explored. Immunofluorescence was used to detect the expression of HA and NP proteins in BEAS-2B cells. The results showed that compared with the IAV group (Fig. 6B), the expression of HA and NP in the EGCG + IAV group (Fig. 6C) was significantly inhibited, but the inhibition of the expression of HA and NP in the EGCG + IAV + siHBD3 group (Fig. 6E) was significantly lower than that in the EGCG + IAV group (Fig. 6C), indicating that silencing HBD3 inhibited the antiviral effect of EGCG (Fig. 6).
Discussion

In this study, EGCG was used to intervene in BEAS-2B cells, and the results showed that EGCG significantly up-regulated the expression levels of β-defensin 3 mRNA and protein in BEAS-2B cells. Therefore, it was confirmed that EGCG can induce the production of HBD3 in BEAS-2B cells, and EGCG stimulates the up-regulation of HBD3 mRNA and protein expression in a concentration- and time-dependent manner. Gan et al (14) have shown that the expression levels of β-defensin 2 mRNA and protein in 16HBECS cells are in a concentration- and time-dependent relationship with paeoniflorin (PF) stimulation, which is similar to the trend in this study, but the concentration and the length of time were different, which related to the experimental subjects and the different types of drugs and defensins. β-defensin 3 has strong antiviral (9) and antibacterial activity (15). HBD3 can be induced by viruses, bacteria, microbial products, toll-like receptors, epidermal cell growth factors, and pro-inflammatory factors (16). Although the exact mechanism of action is not yet clear, it is currently believed that HBD3 works through ion channels (17).

The mechanism of EGCG-induced β-defensin 3 expression in BEAS-2B cells was explored. MAPKs in mammals include c-Jun NH2 terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK). These enzymes are serine-threonine protein kinases that can regulate various cellular activities, including proliferation, differentiation, apoptosis, survival, inflammation and innate immunity (18-20). It has been confirmed that EGCG can regulate the activation of p38 MAPK, ERK, and JNK signaling pathways in colon cancer cells (21). ERK and JNK-dependent HBD3 expression has been confirmed in Helicobacter pylori infected gastric epithelial cells (22). In this study, it was found that EGCG can significantly increase the level of phosphorylated (p)-p38 MAPK, ERK and JNK, and the results show that EGCG can

Figure 4. Effects of EGCG on the expression of HBD3 in BEAS-2B cells. (A) Real-time RT-PCR assay for the upregulated expression of HBD3 mRNA treated with inhibitors for intracellular pathways. The cells were treated with inhibitors for intracellular pathways in a series of experiments. BEAS-2B cells were pre-incubated with SB203580 (an inhibitor of p38 MAPK), U1026 (an inhibitor of ERK), and SP600125 (an inhibitor of JNK) for 1 h and then treated with 50 µg/ml EGCG for 12 h. SB203580 (an inhibitor of p38 MAPK), U1026 (an inhibitor of ERK), and SP600125 (an inhibitor of JNK) completely inhibited the upregulated expression of HBD3 by EGCG (*P<0.05, **P<0.01, compared with the control group; #P<0.05, ##P<0.01 compared with the EGCG group). The data are representative of three independent experiments. (B) Competitive ELISA for the quantification of HBD3 secretion by cultured BEAS-2B cells after EGCG and inhibitor treatments. The cell were preincubated with SB203580, U1026, and SP600125 for 1 h and then treated with 50 µg/ml EGCG for 12 h. Serial dilutions of synthetic mature HBD3 peptide were used to generate a standard curve, and the HBD3 peptide concentration of each experimental group was considered. In accordance with the RT-PCR result, SB203580, U1026, and SP600125 completely inhibited the secretion of HBD3 by EGCG (*P<0.05, **P<0.01, compared with the control group; #P<0.01, compared with the EGCG group). The data are representative of three independent experiments. EGCG, Epigallocatechin gallate.

Figure 5. The levels of HBD3 mRNA and protein. Confirmation of suppression after transfection with siRNA-HBD3 in BEAS-2B cells using real-time RT-PCR and ELISA analysis. *P<0.01, compared with the siRNA-NC group. The data are representative of three independent experiments. (A) Expression level of HBD3 mRNA. (B) Expression level of HBD3 protein.
activate the p38 MAPK, ERK, or JNK signaling pathways. On the other hand, p38 MAPK, ERK, or JNK inhibitors were used to inhibit the signaling pathway. p38 MAPK, ERK, or JNK inhibitors significantly reduced the expression level of β-defensin 3 in BEAS-2B cells induced by EGCG. The results in this study were similar to other studies which suggested that the expression of β-defensin 3 is also regulated by p38 MAPK, ERK, and JNK signaling pathways (14, 23, 24). Therefore, it is suggested in this study that EGCG can induce β-defensin 3 expression in BEAS-2B cells through p38 MAPK, ERK, and JNK signaling pathways. In addition, the expression of β-defensin 3 mRNA and protein peaked at 12 or 24 h under EGCG stimulation, while the activation peak of MAPK pathway was 8 or 12 h, but the expression at 24 h was significantly reduced. The reason may be that since β-defensin 3 was downstream of MAPK pathway, when MAPKs start to be highly expressed at 8 h, the downstream β-defensin 3 was gradually activated to express. At 12 h, β-defensin 3 expression began to increase sharply, and although the expression of MAPKs was reduced at 24 h, the gradual reactions persisted. β-defensin 3 continued to maintain an expression peak, and the expression level was slightly higher than at 12 h.

EGCG has been proven to be resistant to influenza virus (13), but the role of EGCG in immune modulation against influenza virus remains to be further studied. Our experiments found that EGCG pretreatment can significantly inhibit the expression of HA and NP proteins of influenza virus in BEAS-2B cells. However, silencing HBD3 expression in cells will reduce the ability of EGCG to inhibit the expression of HA and NP of influenza virus. In the report of EGCG
against influenza A virus, Colpitts and Schang pointed out that EGCG competitively binds to the sialic acid receptor on the surface of the virus, thereby inhibiting influenza disease (25). Kesic et al found that EGCG can upregulate the expression of Nrf-2 gene, the most important transcriptional regulator in the second phase of the cellular antioxidant pathway, to inhibit influenza virus (26). In recent study, it was pointed out that EGCG can significantly down-regulate TLR4 and NF-κB protein levels and treat acute lung injury caused by swine influenza virus H9N2 (27). These findings indicate that EGCG can fight influenza virus through a variety of mechanisms, but there are few reports on EGCG inhibiting influenza virus by regulating HBD3 secretion. However, our results confirm that EGCG can induce HBD3 expression in BEAS-2B cells and inhibit the expression of HA and NP in influenza A virus. Because HA plays an important role in the process of virus introduction into host cells, NP is a key protein for viral transcription and replication, it is speculated that EGCG can regulate the concentration of HBD3 produced by cells to inhibit influenza virus adsorption, invasion and replication, and then produce anti-influenza virus effects.

In conclusion, it has been shown in this study that EGCG can induce the expression of β-defensin 3 through p38 MAPK, ERK and JNK signaling pathways in BEAS-2B cells to inhibit influenza A virus H1N1, which indicates that EGCG may be an effective drug against influenza A virus.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QM conceived the study and wrote the manuscript. YJ interpreted and analyzed the data. LZ and ZZ designed the study and performed the experiment. TR was responsible for the analysis and discussion of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Affiliated Hospital of Guizhou Medical university (Guiyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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