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Azithromycin inhibits double-stranded RNA-induced thymic stromal lymphopoietin release from human airway epithelial cells

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Received March 1, 2013, accepted March 29, 2013

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Pharmazie 68: 899–903 (2013) *doi: 10.1691/ph.2013.3588*

Thymic stromal lymphopoietin (TSLP) is elevated in asthma and triggers dendritic cell-mediated activation of T_H2 inflammatory responses. Viral stimuli, a major cause of asthma exacerbations, have been shown to induce overexpression of TSLP in asthmatic epithelium. Azithromycin has various anti-microbial and anti-inflammatory effects. However, the effect of azithromycin on the production of TSLP has not been studied. Here we explored the effects of azithromycin on viral surrogate (dsRNA)-induced TSLP in normal human bronchial epithelial (NHBE) cells. NHBE were stimulated with poly (I:C) in the presence azithromycin. The effects of azithromycin on dsRNA-induced inflammatory responses in NHBE cells were analyzed. We demonstrated that azithromycin inhibited the production and mRNA expression of TSLP in NHBE cells. Azithromycin also inhibited the nuclear factor-κB luciferase activity induced by poly (I:C), and it prevented dsRNA-induced loss of the NF-κB repressor protein IκBα. These results suggest that azithromycin can be useful to treat asthma exacerbations due to the inhibition of TSLP.

1. Introduction

Exacerbations of asthma are the main cause of asthma morbidity and mortality and account for significant healthcare costs (Holgate 2011). It is now evident that asthma deterioration and exacerbations in children and adults are frequently associated with viral infections, and the bronchial epithelium is the major target of infection (Gavala et al. 2011; Jackson et al. 2011) The viral infection of airway epithelial cells induces the production of proinflammatory cytokines and antiviral INF-β (Dougherty and Fahy 2009).

Recent findings indicate that thymic stromal lymphopoietin (TSLP) plays an important role in allergic inflammation (He and Geha 2010). TSLP is highly expressed by airway epithelial cells of patients with asthma. The pathophysiology of human TSLP involves innate allergic immune responses with direct effects of TSLP on mast cells and on dendritic cells (DCs). TSLP-activated DCs initiate adaptive allergic immune responses by triggering differentiation of naive T cells into inflammatory T_H2 cells that produce several traditional T_H2 cytokines and also large amounts of TNF-α. Thus, TSLP represents a critical factor linking responses at interfaces between the body and environment to allergic T_H2 immune reactions (Kato et al. 2007; Rochman and Leonard 2008).

Double-stranded RNA (dsRNA) is produced during vial replication and induces innate immune responses including epithelial generation of interferons and inflammatory cytokines (Takayama et al. 2011). Recently, it was demonstrated that polyinosinic-polycytidylic acid (poly I:C), a synthetic double-stranded RNA (dsRNA) recognized by dsRNA sensors including Toll-like receptor 3 (TLR3), was a strong trigger for TSLP production in human bronchial epithelial cells, and the TSLP release can be synergistically enhanced with an atopic cytokine milieu (Kinoshita et al. 2009). These findings imply that a respiratory viral infection, through epithelial TLR3 stimulation, may amplify T_H2 inflammation *via* the induction of TSLP in the asthmatic airway.

The use of macrolides has been proposed both in chronic and acute asthmatic inflammation (Friedlander and Albert 2010; Good et al. 2012). Besides direct anti-microbial activity against gram-positive cocci and atypical pathogens, C14 macrolides such as azithromycin also have immune modifying effects (Emmez et al. 2011; Iwamoto et al. 2011). However, their direct action on TSLP production in human bronchial epithelial cells is not well understood. Therefore, the objective of this study was to evaluate the effect of azithromycin on the dsRNA-induced TSLP expression in human bronchial epithelial cells.

2. Investigations and results

2.1. Effect of azithromycin on dsRNA-induced expression of TSLP in NHBE cells

To assess the inhibitory effect of azithromycin on the production of TSLP, we pretreated normal human bronchial epithelial (NHBE) cells with increasing concentrations of azithromycin for 4 h and subsequently stimulated the cells with poly (I:C) plus T_H2 cytokine milieu (IL-4 and IL-13) for 48 h in the presence of azithromycin. As shown in Fig. 1A, the stimulation with poly (I:C) increased TSLP production from NHBE cells. The levels of TSLP that increased due to poly (I:C) were significantly decreased by azithromycin treatment in a dose-dependent way.

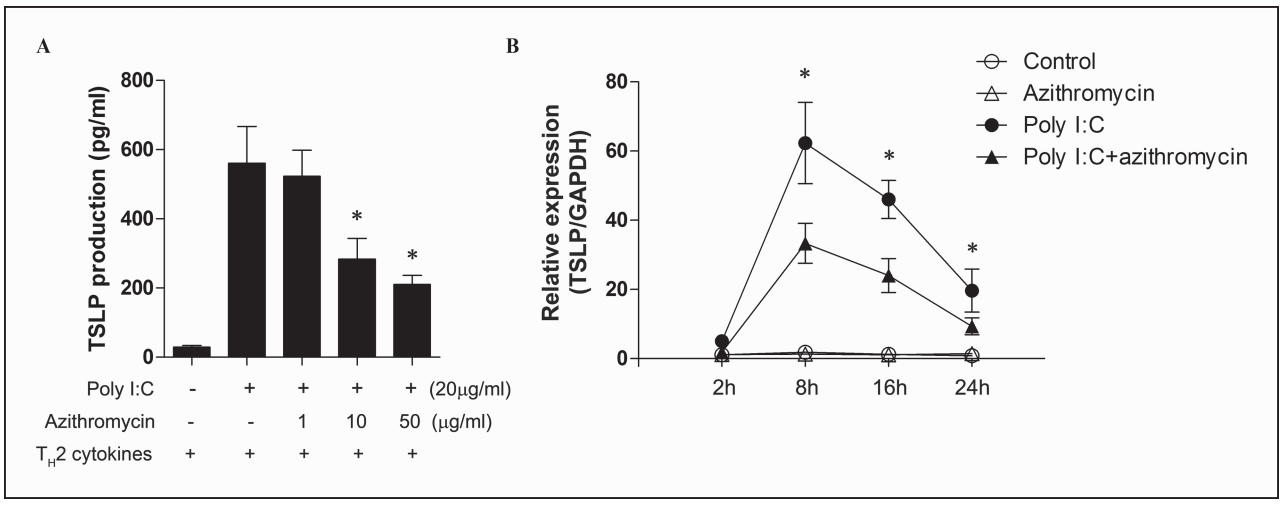

Fig. 1: Azithromycin decreases dsRNA-induced expression of TSLP in NHBE cells. (A) NHBE cells were pretreated with increasing concentrations of azithromycin (0, 1, 10 and 50 μg/ml) for 4 h and subsequently were stimulated with poly (I:C) (20 μg/ml) plus T_H2 cytokine milieu (IL-4 and IL-13) in the presence of azithromycin. Concentrations of TSLP in the culture supernatant collected 48 h after stimulation were measured. Data shown are the means for two wells and are representative of three independent experiments (*$p < 0.05$). (B) NHBE cells were pretreated with azithromycin (50 μg/ml) for 4 h, and then the cell were stimulated with poly (I:C) (20 μg/ml) for 2, 8, 16 and 24 h in the absence of 100 ng/ml IL-4 and 100 ng/ml IL-13. TSLP gene expression was analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control at 2 h. The data are presented as the mean ± SD of a total of two independent experiments (*$p < 0.5$).

To determine whether azithromycin can modulate poly (I:C)-induced mRNA expression of TSLP, we pretreated the cells with azithromycin for 4 h before the poly(I:C) stimulation. We then stimulated the cells with poly (I:C) for 2, 8, 16 and 24 h in the absence of IL-4 and IL-13. The dsRNA induced gene expression of TSLP peaked at 8 h, and azithromycin showed an inhibitory effect at 8, 16 and 24 h (Fig. 1B)

2.2. Effect of azithromycin on dsRNA-induced gene expression of IL-8 and IFN-β in NHBE cells

To investigate whether azithromycin suppresses the dsRNA-induced innate inflammatory response, NHBE cells were pretreated with azithromycin in concentrations ranging from 1 to 50 μg/ml for 4 h before they were challenged by poly (I:C) 20 μg/ml for 12 h in the absence of T_H2 cytokines. Azithromycin displayed a concentration-dependent inhibitory effect on dsRNA-stimulated mRNA expression of IL-8, whereas the expression of IFN-β was not significantly modified (Fig. 2).

2.3. Effect of azithromycin on the expression of RNA-recognizing receptors in NHBE cells

Several intracellular receptors have been described to bind to dsRNA, such as Toll-like receptor 3 (TLR3), PKR, RIG-I and MDA5 (Kalali et al. 2008). To reveal the inhibitory mechanisms of azithromycin, we examined the mRNA expression of these RNA-recognizing receptors. As shown in Fig. 3, stimulation of NHBE cells with poly (I:C) led to increases in the different receptors to a variable degree, whereas azithromycin treatment did not affect the mRNA level of these genes.

2.4. Suppressive effects of azithromycin on dsRNA-stimulated TSLP production via NF-κB signaling pathway

NF-κB is one of the key transcription factors regulating the expression of proinflammatory genes and is known to be involved in mediating TSLP release from NHBE cells stimulated with dsRNA (Vu et al. 2011). Next, we examined whether azithromycin could regulate the luciferase expression specifically *via* NF-κB activation. As shown in Fig. 3, the poly (I:C) stimulation increased the reporter gene activity. However, this

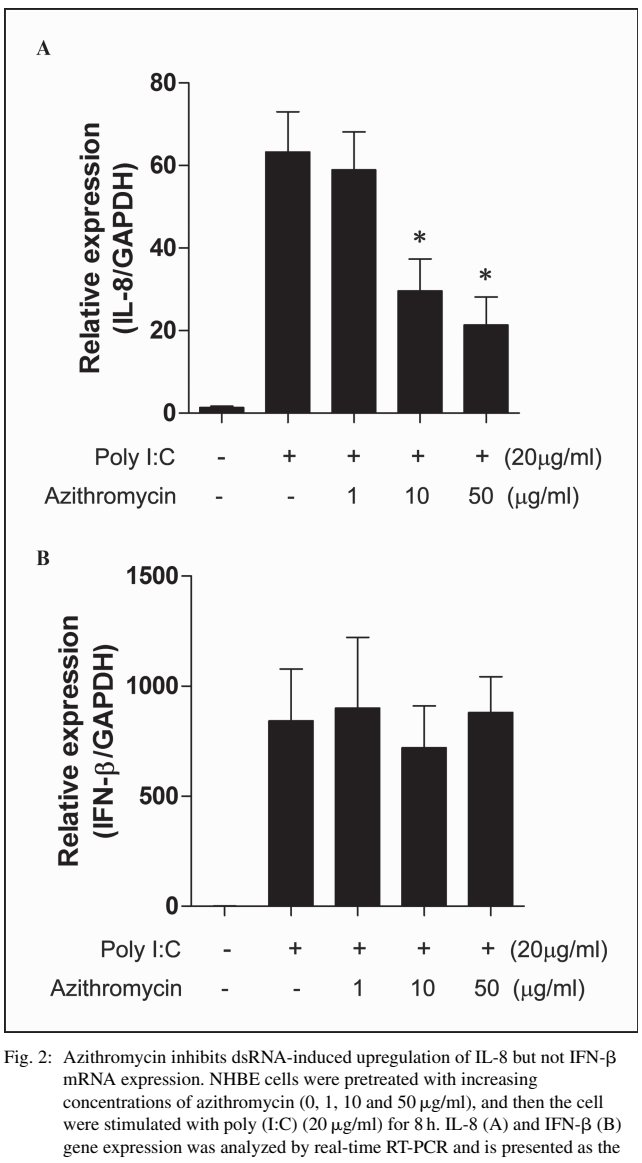

Fig. 2: Azithromycin inhibits dsRNA-induced upregulation of IL-8 but not IFN-β mRNA expression. NHBE cells were pretreated with increasing concentrations of azithromycin (0, 1, 10 and 50 μg/ml), and then the cell were stimulated with poly (I:C) (20 μg/ml) for 8 h. IL-8 (A) and IFN-β (B) gene expression was analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control. The data are presented as the mean ± SD of a total of two independent experiments (*$p < 0.5$).

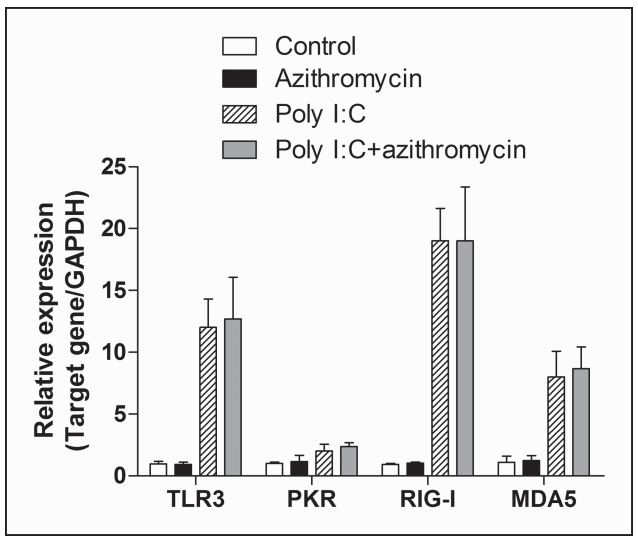

Fig. 3: Azithromycin have no effects on the expression of RNA-recognizing receptors in NHBE cells. NHBE cells were treated or not with azithromycin (50 μg/ml) in the presence or absence of poly (I:C) stimulation (20 μg/ml) for 8 h, and then TLR3, PKR, RIG-I and MDA5 mRNA expression were analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control. The data are presented as the mean ± SD of a total of two independent experiments.

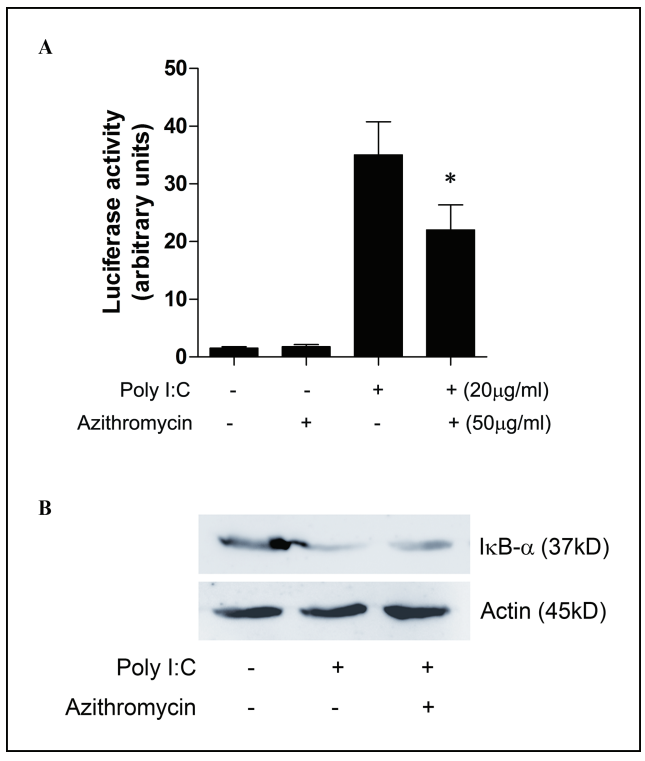

Fig. 4: Azithromycin inhibits dsRNA-induced NF-κB activation in NHBE cells. (A) NHBE cells (1×10^7) were transiently transfected pGL4.32 and treated with or without azithromycin (50 μg/ml) for 2 h and then stimulated with or without poly (I:C) (20 μg/ml) for 48 h. The NF-κB activity was assessed with a luciferase assay. The data are presented as the mean ± SD of a total of two independent experiments (*$p < 0.5$). (B) NHBE cells were incubated with or without poly (I:C) (20 μg/ml) in the presence or absence of azithromycin (50 μg/ml) for 18 h, and degradation of IκBα in cytoplasmic extract from NHBE cells was assessed by western blot analysis. Data shown are representative of two independent experiments.

increased NF-κB luciferase activity was significantly decreased by azithromycin treatment (Fig. 4A).

As activity of NF-κB is controlled by IκB, we examined by means of western blot analysis whether azithromycin influenced the degradation of IκBα. In this study, treatment of NHBE cells with poly (I:C) showed increased degradation of IκBα in the cytoplasm extracts measured by immunoblotting. Azithromycin treatment (50 μg/ml) partially restored the level of IκBα in dsRNA-stimulated NHBE cells (Fig. 4B).

3. Discussion

The current treatment of asthma comprises corticosteroids and/or β_2-agonists and only partially prevents asthma exacerbations (Alavaikko et al. 2011). A vital need exists for new treatment regimes for this form of asthma. For more than 20 years macrolide antibiotics have been used to treat chronic inflammatory airway diseases (Hernando-Sastre 2010). Clinical studies have shown that macrolides have beneficial effects in chronic inflammatory lung diseases including diffuse panbronchiolitis, bronchiectasis, bronchiolitis obliterans syndrome, and cystic fibrosis (Friedlander and Albert 2010; Cai et al. 2011; Fisher 2011; Serisier and Martin 2011). Macrolides exert their anti-inflammatory actions through multiple mechanisms. Macrolide antibiotics down-regulate damaging prolonged inflammation as well as increase mucus clearance. Macrolide therapy appears to suppress the secretion of proinflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8 and TNF-α from bronchial epithelial cells, lung fibroblasts, macrophages and leukocytes (López-Boado and Rubin 2008; Morinaga et al. 2009; Matsumura et al. 2011). In the present study, we showed that azithromycin inhibited dsRNA-induced TSLP from normal human bronchial epithelial cells by inhibiting NF-κB signaling, while expression of anti-virus cytokine IFN-β was not significantly influenced

The observation that viral products induce TSLP production by bronchial epithelium may be related to the well-documented aggravating role of infection in allergic as well as asthma (Proud 2011). During viral replication, single-stranded (ss) RNA viruses such as rhinovirus produce double-stranded (ds) RNA which is detected as a 'danger signal' by the innate immune system. Previous studies have shown that exposure to rhinovirus infection or dsRNA *in vitro* induces TSLP production in bronchial epithelial cells (Uller et al. 2010), suggesting that this TSLP may link the innate antiviral response and the T_H2 adaptive immune response. TSLP was originally reported to exert its T_H2-promoting properties through a dendritic cell-mediated pathway in human beings that involved induction of the OX40 ligand on DCs (Wang and Liu 2009). There has therefore been great interest in targeting the pathways downstream of TSLP such as TSLP-TSLP receptor interaction and OX40L-OX40 interaction. In the present study, azithromycin inhibited the production and mRNA expression of TSLP. To our knowledge, this is the first study showing an inhibition of TSLP by azithromycin in bronchial epithelial cells.

Induction of immune responses by dsRNA and RNA viruses involves endosomal receptor TLR3 or present in the cytoplasm including the RNA helicases RIG-I and MDA5 and the serine-threonine kinase PKR (Kalali et al. 2008). Our data demonstrated that none of these cytoplasmic dsRNA-recognition receptors were modulated by azithromycin treatment, however the roles of MDA5, RIG-I or PKR could not be completely excluded. Activation of TLR3 by dsRNA RNA viruses transduces its signals to NF-κB and IRF-3 and induces proinflammatory genes and type I IFNs including IFN-β (Vu et al. 2011). However, the effects of macrolide antibiotics on TLR3/IRF3 signaling and IFN-β production have shown contradictory results. Previous data showed that erythromycin treatment down-regulated TLR3/IRF3 signaling and IFN-β production, while in another report, azithromycin significantly

increased rhinovirus-induced interferons (Aghai et al. 2007; Gielen et al. 2010). Here, we showed that secretion of IFN-β, which may have a protective role in asthma exacerbations, was not compromised by azithromycin treatment, suggesting a positive role of macrolides in the antiviral ability, which is independent of TSLP production.

The transcription factor NF-κB mediates cytokine gene activation downstream of TLR3 signaling. Drugs that inhibit NF-κB by stabilizing its binding to IκB have also been shown to be effective inhibitors of TSLP generation in dsRNA-stimulated bronchial epithelial cells (Lee et al. 2008). Additionally, such drugs have produced a less desirable inhibition of the antiviral IFN-β (Le et al. 2010). The present findings suggest possibilities of separating anti-TSLP and anti-IFN-β effects since azithromycin reduced expression of TSLP without inhibiting the IFN-β production.

In conclusion, we demonstrated that azithromycin suppresses the dsRNA-induced release of TSLP from normal human bronchial epithelial cells. TSLP is considered the master switch for T_H2 responses. Therefore, it is a promising strategy to shut down the release of TSLP in the bronchial epithelial cells of asthma patients. Azithromycin could therefore be effective in the treatment of asthma exacerbation through the suppression of TSLP release from bronchial epithelial cells, when exogenous dsRNA is involved in the pathogenesis or exacerbation.

4. Experimental

4.1. Reagents and cell culture

Poly (I:C) was purchased from InvivoGen (San Diego, CA, USA). 100 ng/ml IL-4 and 100 ng/ml IL-13 were purchased from R&D Systems (Minneapolis, MN, USA). Azithromycin was obtained from Sigma (St Louis, MO, USA). Antibodies against IκBα and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Normal human bronchial epithelial (NHBE) cells (Lonza, Cleveland, USA) were maintained in serum-free bronchial epithelial cell growth medium (Cambrex, East Rutherford, NJ, USA). Cells were seeded in flat-bottomed 96-well microculture plates and cultured until they reached 100% confluence, and then the medium was changed to fresh medium. After further cultivation for 24 h, cells were stimulated with poly (I:C) and T_H2 cytokines (100 ng/ml IL-4 and 100 ng/ml IL-13) in the absence or presence of azithromycin.

4.2. ELISA

Concentration of TSLP was quantified by ELISA using commercially available paired antibodies and standards, following the manufacturer's instructions (R&D Systems). The sensitivity of each assay was 10 pg/ml.

4.3. RNA isolation and real-time RT-PCR

RNA was isolated and purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). For real-time RT-PCR analyses, 1 μg of DNase-treated total RNA was reverse transcribed. The amplification of the cDNA was accomplished using the ABI Prism 7900HT sequence detection system in the presence of the commercially available SYBR Green PCR Master Mix (Toneker, Shanghai, China) in a 40-cycle PCR. The primer sequences were as follows: GAPDH (Forward, 5'-GGT CGG AGT CAA CGG ATT TG-3'; Reverse, 5'-ATG AGC CCC AGC CTT CTC CAT-3'); TSLP (Forward, 5'-TAT GAG TGG GAC CAA AAG TAC CG-3'; Reverse, 5'-GGG ATT GAA GGT TAG GCT CTG G-3'); IL-8 (Forward, 5'-AAG CTG GCC GTG GCT CTC TTG-3'; Reverse, 5'-AGC CCT CTT CAA AAA CTT CTC-3'); IFN-β (Forward, 5'-CCA ACA AGT GTC TCC TCC AAA T-3'; Reverse, 5'-AAT CTC CTC AGG GAT GTC AAA G-3'); TLR3 (Forward, 5'-AAA TTG GGC AAG AAC TCA CAG G-3'; Reverse, 5'-GTG TTT CCA GAG CCG TGC TAA-3'); PKR (Forward, 5'-AAA GCG AAC AAG GAG TAA G-3'; Reverse, 5'-TTC AGA AGG GCT CTA ACA-3'); RIG-I (Forward, 5'-TAT TCT GAT TGC CAC CTC-3'; Reverse, 5'-TAA ATA CTG CTT CGT CCC-3'); MDA5 (Forward, 5'-GTT ATC CGT TAT GGT CTC G-3'; Reverse, 5'-GGA AAG TTA TTA GTG ATG GGT T-3'). The denaturing, annealing, and extension conditions of each PCR cycle were 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 34 s, respectively. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The mRNA levels of each target gene were normalized to the levels of GAPDH and were represented as fold induction.

4.4. Transient transfection and luciferase assay

For transfection, we seeded NBHE cells (1×10^7) in a 100 mm culture dish. We then used Lipofectamine™2000 purchased from Invitrogen (Carlsbad, CA, USA) to transiently transfect pGL4.32 reporter gene constructs into NBHE cells. To measure the luciferase activity, we used a luminometer Gene-Light 55 (Microtech Nichion, Chiba, Japan) in accordance with the manufacturer's protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of firefly luciferase activity to renilla luciferase activity.

4.5. Western blotting

NHBE cell lysates were separated by SDS-PAGE (10% acrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membranes, following detailed protocols described previously (Aghai et al. 2007). The blots were probed with IκBα, and β-actin antibodies and then proteins were visualized by the ECL Western Blotting System (Pierce, Rockford, IL, USA).

4.6. Statistical analysis

Results are expressed as the mean ± SD. A Student's t-test was used to determine significance among the groups. A value of $p < 0.05$ was considered significant. Analyses and graphical representation were performed using Graph-Pad Prism 5.01 software (Graphpad).

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