Construction of a cell model of β₂-adrenoceptor downregulation

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ABSTRACT

Objective: To establish a cell model of β₂-adrenergic receptor (β₂AR) downregulation of murine airway smooth muscle induced by salbutamol to elucidate the molecular and biological mechanisms of β₂AR downregulation.

Methods: Airway smooth muscle cells (ASMCs) derived from Balb/c mice were primary cultured. Passage 2-5 cells were characterized by cell morphology and indirect immunofluorescence. More than 95% pure cells at passage 3 or 4 were randomly divided into two groups: control and salbutamol-treated groups. β₂AR mRNA and protein expression levels were then detected by RT-PCR and western blot analyses.

Results: Primary cultured cells demonstrated a typical “peak and valley”-like growth characteristic. Smooth muscle α-actin filaments paralleled the cell longitudinal axis in the cytoplasm. The origin of the ASMCs was validated and consistent with their morphology and biological characteristics. β₂AR mRNA expression in the salbutamol-treated group was lower than that in the control group (P<0.05), and β₂AR protein expression was also markedly lower than that in the control group (P<0.05).

Conclusions: We successfully established a cell model of β₂AR downregulation in ASMCs, which may provide the foundation for further study of the mechanism of β₂AR downregulation in asthmatic patients.

KEY WORDS

Airway smooth muscle cells (ASMCs); β₂-adrenergic receptor (β₂AR); salbutamol; cell culture

Introduction

The mechanisms of β₂-adrenergic receptor (β₂AR) downregulation is an intensely researched area for the prevention and treatment of bronchial asthma (1). These studies mainly depend on animal models that reveal the pathogenesis as a whole, while the molecular levels are less detailed (2). Balb/c mice have been widely accepted as an animal model of bronchial asthma (3). However, primary cultured airway smooth muscle cells (ASMCs) may be established as a cell model that can reveal the molecular mechanisms of β₂AR downregulation. Nevertheless, primary culture of ASMCs is challenging, and the method for stimulation and dosage of stimuli are difficult to determine. Therefore, no classic cell model of β₂AR downregulation has been developed so far (4). In this study, we tried to construct a β₂AR downregulation cell model based on the previous studies and others’ experiences (2,5,6), which may allow further study of β₂AR downregulation.

Materials and methods

Experimental animals

Six to eight-week-old female Balb/c mice [SCXKL (Shanghai) 2011-0003] of a specific pathogen-free grade were purchased from the Shanghai Animal Center of Chinese Academy of Sciences. Mice were given free access to food and were allowed to adapt to their new surroundings for at least 1 week before experiment. This study was approved by the Animal Ethics Committee of Nantong University.

Equipment and reagents

Purification of mouse ASMCs

ASMCs were purified by differential adhesion. At 15 minutes after the first passage, only some of the cells were adherent. Unattached cells were transferred to a second bottle. After 15 minutes, unattached cells were transferred to a third bottle. Cells in the third bottle were pure ASMCs.

Identification of mouse ASMCs

ASMC morphology was observed by the size, shape, arrangement, and growth of the cells under an inverted phase contrast microscope. For immunocytochemical staining, passage 3-5 ASMCs were grown on glass slides in a 6-well plate. At 80% confluence, the cells were fixed with 4% paraformaldehyde for 20 minutes. Then, the cells were washed with distilled water three times for 3 minutes each time. Endogenous peroxidase activity was blocked by incubation with 0.6% hydrogen peroxide for 30 minutes, and then the cells were washed with 0.01 mol/L PBS three times for 2 minutes each time. Non-specific antibody binding was blocked by incubation with normal goat serum at room temperature for 20 minutes without washing. Cells were incubated with the mouse anti-a-actin monoclonal antibody at room temperature for 20 minutes, and then washed with 0.01 mol/L PBS three times for 2 minutes each time. Next, the cells were incubated with a biotinylated goat anti-rabbit IgG at room temperature for 20 minutes, and then washed as described above. Finally, the cells were incubated with SABC-FITC for 20 minutes, and then washed with 0.01 mol/L PBS three times for 5 minutes each time. After staining, the cells were observed and photographed under a laser scanning microscope.

Stimulation by salbutamol

Passage 3 or 4 ASMCs in the exponential growth phase were seeded in a 24-well plate at 1×10^5 cells per well. Cells were randomly divided into two groups: control and salbutamol-treated groups. The salbutamol-treated group was cultured with medium containing 250 ng/mL salbutamol for eight days. The control group was cultured with normal medium.

RT-PCR analysis of β2AR mRNA expression in mouse ASMCs

The sequences of mouse α-tubulin and β2AR genes were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). Primers were designed using Primer Premier 5, Dnastar analysis software and on-line BLAST analysis, and synthesized by Shanghai Shenggong Biological Engineering Company as follows: a-tubulin: forward, TTGAGCCAGCCAACCAG; reverse, CACCCTCCACAGAATCCA (458 bp); β2AR: forward, ATCTCCTGAAGGTGCTGT; reverse: GATCCGATCCGCTTAT (321 bp). Total RNA was extracted using Trizol reagent and reverse transcribed to cDNA with a TaKaRa Reverse Transcription Kit according to the manufacturer's instructions. For PCR, 5 µL cDNA template was used for each amplification. The thermal cycling conditions were initial denaturation for 5 minutes at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 40 seconds and extension at 72 °C for 60 seconds, and then a final extension at 72 °C for 10 minutes. PCR products were subjected to 1% agarose gel electrophoresis, observed under an ultraviolet lamp, and photographed with a gel analysis system. Electrophoretic bands were analyzed with grayscale scanning software.

Western blot detection of β2AR protein expression in mouse ASMCs

Cells were collected and lysed in RIPA buffer containing a protease inhibitor on ice for 30 minutes with intermittent agitation. The
supernatant was collected, mixed with loading buffer, and boiled for 3-5 minutes. The protein samples were then stored at −20 °C until use. Protein concentration was determined by a BCA Protein Quantify Kit (SNBC) using an Eppendorf Biophotometer.

Protein samples (100 µL) were electrophoresed, and then transferred to a polyvinyl difluoride membrane. After blocking with 5% skim milk powder in 1× TBST at 37 °C for 1 hour, the membrane was washed with TBST, and then incubated with primary antibodies (1:1,000) for 1 hour. Then, the membrane was thoroughly washed with TBST, followed by incubation with the second antibody (1:6,000) for 1 hour. The membrane was washed, and then visualized by ECL reagent. α-tubulin was used as an internal control.

Statistical analysis

Statistical analyses were performed using SPSS software version 16.0. Measurement data are described as ±s. Data with a normal distribution were analyzed using a t-test. A P-value of less than 0.05 was considered to be significant.

Results

Culture and identification of ASMCs

After the tissues were incubated for 3-6 days, a small number of round cells began to migrate and erupt from the outer edges as observed under an inverted phase contrast microscope. In general, 10-15 days was needed for the cells to reach confluency for subculture (Figure 1). During subculture, some tissues separated from the container and would adhere again, followed by outgrowth of cells. The subcultured cells reached confluency in 8-10 days (Figure 2). The growth characteristics of the subcultured cells were similar to those of the primary cells.

ASMC morphology

The sizes of primary cultured cells appeared to be different under the inverted phase contrast microscope. Prior to confluency, ASMCs were fusiform, round or polygonal, and had one or multiple nuclei with one or several processes extending toward areas of low cell density. At confluency, ASMCs typically grew as a long fusiform with a ranked fascicular. The cells alternated and overlapped mutually. This typical peak and valley growth is characteristic of smooth muscle cells (Figure 3).

Immunocytochemical staining of α-actin, which is specific for smooth muscle cells, was performed with a mouse anti-α-actin monoclonal antibody. More than 95% of cells were strongly positive for α-actin in the cytoplasm under a laser scanning microscope. α-actin fluorescence was parallel to the longitudinal axis of the cells, indicating smooth muscle α-actin. Some cells were multinucleated (Figures 4,5).
β2AR mRNA expression in mouse ASMCs

β2AR mRNA expression in the salbutamol-treated group was lower than that in the control group as shown by RT-PCR (P<0.05) (Figure 6).

β2AR protein expression in mouse ASMCs

β2AR protein expression in the salbutamol-treated group was lower than that in the control as group as shown by western blotting (P<0.05) (Figure 7).

Discussion

Cell models have become important for the study of various diseases (7). Although there is no uniform standard, a cell model should conform to the following criteria (8): (I) Primary culture and expansion of cells; (II) There should be an appropriate treatment or modification based on the pathogenesis or characteristics of the specific model; (III) A corresponding index or experimental method is needed (an acknowledged gold standard is optimal) to test whether establishment of the cell model is successful or needs improving.
$\beta_2$AR downregulation is an important aspect of $\beta_2$AR desensitization (9) that involves numerous factors, and has an uncertain observational indicator and unstable means of testing (10). Therefore, in this study, we constructed a cell model of $\beta_2$AR downregulation as the observational indicator. As mentioned previously, the present studies of a cell model of $\beta_2$AR downregulation have been deficient. Vayttaden (4) et al. overexpressed and silenced wild-type $\beta_2$AR in HEK293 cells by genetic engineering methods to study the molecular mechanisms of $\beta_2$AR downregulation. However, their study did not use a stimulation factor or airway smooth muscle cells. Thus, it is not a qualified model from the perspective of cell models.

$\beta_2$AR agonists are effective drugs for clinical treatment of asthma. However, their application is limited because of $\beta_2$AR downregulation (11). To study the molecular mechanisms of $\beta_2$AR downregulation, models should include airway smooth muscle from patients or animal models of asthma and adopt a stimulation method similar to the clinical setting during establishment (12). Therefore, construction of a cell model of $\beta_2$AR downregulation by stimulating the airway smooth muscle of an animal model with salbutamol or another $\beta_2$AR agonist may reflect the molecular mechanisms of $\beta_2$AR downregulation. Balb/c mice have been widely accepted as an animal model of bronchial asthma (3). Construction of cell models of $\beta_2$AR downregulation with airway smooth muscle is direct and objective. Salbutamol is one of the most commonly used short-acting $\beta_2$AR agonist because of minor systemic side effects, low-cost, and a rapid effect of relieving acute dyspnea (13). Our study adopted salbutamol as the stimulus to construct the cell model, so that it was more similar to the pathological status of clinical patients who are medicated with salbutamol. Thus, our study is more accurate and reliable. We constructed the cell model of $\beta_2$AR downregulation by adding salbutamol to the medium and stimulating ASMCs over a long-term. The concentration of salbutamol used in this study was determined according to the concentration applied to normal human ASMCs by Luo et al. (14). Their study showed that salbutamol induces apoptosis of human ASMCs in a time- and concentration-dependent manner. Therefore, the stimulation time and concentration of salbutamol needs further exploration during construction of the cell model of $\beta_2$AR downregulation.

In our study, Balb/c mouse ASMCs were primarily cultured and stimulated by salbutamol. $\beta_2$AR expression in the control and salbutamol-treated groups was evaluated by RT-PCR and western blot analyses, and together with observation of cytomorphology and identification by immunocytochemistry. We successfully achieved preliminary construction of a cell model of $\beta_2$AR downregulation. We are currently in the exploration stage, and this cell model requires further optimization such as special conditions other than the short-acting $\beta_2$AR agonist as well as its optimal concentration. Furthermore, we should explore whether it is feasible to obtain a cell model by primary culture of ASMCs derived from animal models of $\beta_2$AR downregulation and whether it would be equivalent to the cell model constructed in this study.

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