ANTI-INFLAMMATORY EFFECTS OF SALBUTAMOL ON HUMAN BRONCHIAL EPITHELIAL CELLS STIMULATED BY CIGARETTE SMOKE EXTRACT

EFEITOS ANTI-INFLAMATÓRIOS DO SALBUTAMOL EM CÉLULAS EPITELIAIS BRÔNQUICAS HUMANAS ESTIMULADAS POR EXTRATO DE FUMO DE CIGARRO

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ABSTRACT

Cigarette smoking, the major cause of chronic obstructive pulmonary disease (COPD), induces activation of pro-inflammatory pathways in the airway epithelium. Salbutamol, a selective and short-acting β2-adrenoceptor agonist, is used for bronchospasm relief in patients with asthma and COPD. In addition, salbutamol also presents anti-inflammatory effects. Here, we evaluated whether salbutamol (10^{-5}-10^{-7} M) can reduce on bronchial epithelial cells (BEAS-2B cells) the inflammatory parameters induced by cigarette smoke extract (CSE; 1%). After 24h, Salbutamol reduced the IL-1β production induced by CSE in a concentration-response manner. Salbutamol (10^{-6} M) reduced the IL-8 production and increased the IL-10 production on CSE-stimulated cells. Also, salbutamol (10^{-6} M) decreased the ICAM-1 expression and the reactive oxygen species production. These anti-inflammatory effects could be associated with the down regulation of activation of NF-κB. Salbutamol may be a potential alternative treatment to airway inflammation caused by cigarette smoking such as in COPD patients.

KEYWORDS: Cigarette smoke extract, bronchial epithelial cells, smoking.

RESUMO

O tabagismo, principal causa da doença pulmonar obstrutiva crônica (DPOC), induz a ativação de vias pró-inflamatórias no epitélio das vias aéreas. O salbutamol, um agonista seletivo e de curta duração do β2-adrenoceptor, é utilizado para o alívio do broncoespasmo em doentes com asma e DPOC. Além disso, o salbutamol também apresenta efeitos anti-inflamatórios. Aqui, avaliamos se o salbutamol (10-5-10-7 M) pode reduzir nas células epiteliais brônquicas (células BEAS-2B) os parâmetros inflamatórios induzidos pelo extrato de fumo de cigarro (CSE; 1%). Após 24 horas, o salbutamol reduziu a produção de IL-1β induzida pelo CSE de uma forma correspondente à concentração. O salbutamol (10-6 M) reduziu a produção de IL-8 e aumentou a produção de IL-10 em células
estimuladas por CSE. Além disso, o salbutamol (10-6 M) diminuiu a expressão de ICAM-1 e a produção de espécies reativas de oxigênio. Estes efeitos anti-inflamatórios podem estar associados à regulação negativa da ativação do NF-κB. O salbutamol pode ser um potencial tratamento alternativo para a inflamação das vias respiratórias causada pelo consumo de cigarros, como nos doentes com DPOC. 

PALAVRAS-CHAVE: Extrato de fumo de cigarro, células epiteliais brônquicas, tabagismo.

INTRODUCTION

Smoking is recognized as a serious preventable public health problem, contributing to the increase in morbidity and mortality rates. Several diseases are associated with smoking, such as chronic obstructive pulmonary disease (COPD), which is characterized by a progressive restriction of airflow and mainly by an abnormal inflammatory response of the lungs\(^1\).

Bronchial epithelium acts as a defensive barrier once it is in the interface between the external environments. In consequence of this, it is able to initiate and orchestrate immune and inflammatory responses by releasing chemokines and cytokines. Cigarette smoke can activate the airway epithelial barrier function, increasing the oxidative stress and the release of pro-inflammatory mediators favoring the exacerbation of airway inflammation\(^2\).

Salbutamol, a selective and short acting β2 agonist, is a bronchodilator used in treatment of bronchial asthma and COPD diseases to improve the forced expiratory flow\(^3,4\). In addition, salbutamol can also demonstrate anti-inflammatory activities such as the reduction of IL-1β, IL-6, and IL-8 both in vitro and in vivo models\(^5,6\).

In this study, we used human bronchial epithelial cells stimulated by cigarette smoking extract (CSE) in vitro and evaluated the anti-inflammatory effect of salbutamol.
MATERIALS AND METHODS

CELL CULTURE
The human bronchial epithelial cell line (BEAS-2B; ATCC) was kept in culture flasks containing DMEM/F-12 culture medium supplemented with fetal bovine serum (10%) and 1% penicillin + streptomycin (Gibco-Life Technologies) and maintained under appropriate conditions (5% CO₂ atmosphere at 37°C)⁷.

CIGARETTE SMOKE EXTRACT (CSE) PROCESS
In this study, we used commercial cigarettes (Marlboro®) to carry out the CSE extract¹¹. Briefly, the smoke from one or five cigarettes were bubbled into kitassato containing 25 mL of PBS using vacuum. The smoke extract was adjusted to reach a desirable pH of 7.4, considered as 100%, and then filtered using a 0.22 μm filter for further dilution for subsequent experiments. The absorbance of the dilution samples was measured by spectrophotometry at 320 nm to create a standard concentration of the extract in each independent experiment.

TREATMENT AND STIMULUS
Bronchial epithelial cells were plated 1 x 10⁵ cells / mL and incubated in 96-well plates, treated with salbutamol (10⁻⁵-10⁻⁷ M) (Devalia et al. 1992⁸) and stimulated 30 minutes later with CSE (1%) for a period of 24 hours.

QUANTIFICATION OF IL-1β, IL-8 and IL-10
The quantification of IL-1β, IL-8 and IL-10 present in the epithelial cell culture supernatant was performed using an ELISA enzyme immunoassay (BD Biosciences).

ICAM-1 EXPRESSION
The analysis of the expression of ICAM-1, also known as CD54 (Cluster of Differentiation 54), was carried out according to the methodology of Pereira et al. (2021)⁹. After stimulation with CSE, there was recovery of cells that were
subsequently washed twice in PBS and stained at 4°C for 30 min with anti-CD54 antibodies (BD Biosciences). In the study, the negative control was determined by the PE-conjugated goat IgG1 isotype. Cells were then washed 3 times with 0.5 ml of PBS and resuspended in 0.5 ml of 2% paraformaldehyde in PBS for fixation. Cells were washed, resuspended and analyzed. Flow cytometry (FACSCalibur; BD Biosciences Pharmingen) was used to analyze the expression of signaling molecules in 50,000 viable cells. All data obtained were analyzed using the FlowJo software.

NF-κB EXPRESSION

The NF-κB signaling pathways were assessed by cytometry according to de Oliveira et al. (10). After CSE stimulation, the cells were fixed with 4% paraformaldehyde for 10 min at 37°C and centrifugated. After, the cells were permeabilized in ice-cooled methanol for 30 minutes and then stained with Anti-phospho-NF-κB (BD Biosciences Pharmingen) or its corresponding isotype monoclonal antibody for 60 minutes, followed by incubation with the secondary antibody labeled with FITC for another 45 minutes in the dark. The cells were washed, resuspended, and subjected to analysis. The evaluation of the expression of populations of 50,000 viable cells corresponding to phosphorylated intracellular signaling molecules was performed using flow cytometry (FACSCalibur; BD Biosciences Pharmingen). All data obtained were analyzed using the FlowJo software.

ROS ASSAY

ROS quantification was performed after 24 h of stimulation by incubating cells with the diluted fluoroprobe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at 37°C with gentle agitation for 5 min. Serum-free culture medium was used for the washing procedure and later cell analysis was performed using fluorescence microscopy (exc: ~485nm/em: ~535nm).
STATISTICAL ANALYSIS

All experiments were performed in duplicate and the results were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by test of variance (ANOVA) followed by a Tukey post-test between means using GraphPad PRISM (Version 6.0; GraphPad Software Inc., San Diego, CA, USA) with a significance level of P values lower than 0.05.

RESULTS

CIGARETTE SMOKE MEDIUM-INDUCED IL-1β RELEASE SUPPRESSION

In the first set of experiments, we analyzed the effect of different concentrations of salbutamol on IL-1β production in BEAS-2B cell cultures that received CSE stimulation. The IL-1β production was increased in cells stimulated by CSE when compared to the control group (Fig 1). Salbutamol (10⁻⁵ and 10⁻⁶ M), but not at 10⁻⁷ M, reduced the concentration of IL-1β when compared to CSE-stimulated cells. IL-1β production was decreased by 56.03% and 53.64% from 777.19 ± 85.41 (CSE) to 341.76 ± 75.59 (salbutamol at 10⁻⁵M) and to 360.30 ± 67.51 (salbutamol at 10⁻⁶M) (mean ± SEM), respectively.

Figure 1. Anti-inflammatory effects of salbutamol on the IL-1β production on BEAS-2B cells stimulated by cigarette smoke extract (CSE). BEAS-2B cells were treated for 30 min with salbutamol (10⁻⁵-10⁻⁷ M) prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n= 6. * P < 0.05 versus the control group, # P < 0.05 versus the CSE group.
No alteration on IL-1β production was observed in cells only treated with salbutamol at higher concentration (10^{-5}M) separately when compared to control (data not shown).

We have chosen the minimal effective concentration of salbutamol (10^{-6}M) on IL-1β production to experiments that followed.

EFFECTS OF SALBUTAMOL IN THE IL-10 AND IL-8 IN BEAS-2B CELLS STIMULATED BY CSE

Next, we evaluated the effect of salbutamol in IL-10 and IL-8. CSE increased the IL-10 and IL-8 productions when compared to the control group (Fig 2A and 2B, respectively). Salbutamol increased the IL-10 production (Fig 2A) and reduced the IL-8 production (Fig 2B) when compared to the CSE group. IL-10 production was increased by ~1.3 folds from 858.31 ± 12.24 (CSE) to 1,038.09 ± 147.90 (salbutamol + CSE) (mean ± SEM). IL-8 production was decreased by ~36% from 1,419.70 ± 51.01 (CSE) to 910,343 ± 284,80 (salbutamol + CSE) (mean ± SEM). No significant effect was observed in ROS production by treatments when compared to the CSE group (Fig 5C).

Figure 2. Effect of salbutamol on the IL-10 (A) and IL-8 (B) productions on BEAS-2B cells stimulated by cigarette smoke extract. BEAS-2B cells were treated for 30 min with salbutamol (10^{-6} M) prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n= 5. * P < 0.05 versus the control group, # P < 0.05 versus the CSE group.
EFFECTS OF SALBUTAMOL IN THE ICAM-1 EXPRESSION AND REACTIVE OXYGEN SPECIES (ROS) PRODUCTION IN BEAS-2B CELLS STIMULATED BY CSE

CSE increased the ICAM-1 expression (Fig 3A) and ROS production (Fig 3B) in BEAS-2B cells when compared to the control group. Salbutamol decreased the ICAM-1 expression and ROS production when compared to the CSE group.

Figure 3. Effect of salbutamol on the ICAM-1 (A) expression and ROS (B) production on BEAS-2B cells stimulated by cigarette smoke extract. BEAS-2B cells were treated for 30 min with salbutamol (10^-6 M) prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n= 5. * P < 0.05 versus the control group, # P < 0.05 versus the CSE group.

EFFECTS OF SALBUTAMOL IN THE NF-κB ACTIVATION IN BEAS-2B CELLS STIMULATED BY CSE

CSE increased the activation of NF-κB in bronchial epithelial cells when compared to the control group (Fig 4). Salbutamol decreased the phosphorylation of NF-κB when compared to the CSE group.

Figure 4. Effect of salbutamol on the activation of NF-κB on BEAS-2B cells stimulated by cigarette smoke extract. BEAS-2B cells were treated for 30 min with salbutamol (10^-6 M) prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n= 5. * P < 0.05 versus the control group, # P < 0.05 versus the CSE group.
DISCUSSION

Airway epithelial cells play an important role in host defense during innate immune responses by producing pro-inflammatory chemokines and cytokines\(^\text{(10)}\). However, dysregulation of this epithelium can trigger the onset of chronic airway inflammation, such as COPD. Smoking causes an ongoing inflammatory process, airway remodeling, and pulmonary emphysema in COPD patients\(^\text{(11)}\).

Beta-2 adrenergic agonists are administered to COPD patients as a reliever therapy, primarily for their bronchodilatory effects, relaxation of smooth muscle cells, thereby providing critical symptomatic relief\(^\text{(12)}\). In addition, they may also exert potential anti-inflammatory effects on all cell types including structural cells (epithelial cells and fibroblasts) by several distinct mechanisms\(^\text{(13)}\). These effects improve their efficacy in the management of airway inflammatory diseases.

IL-1\(\beta\) is a pro-inflammatory mediator that affects a broad spectrum of immunological and inflammatory responses and plays a significant role in smoking-induced airway diseases\(^\text{(11)}\). Salbutamol reduced the IL-1\(\beta\) production in an air pouch model of inflammation in rats \textit{in vivo}\(^\text{(23)}\). In our study, salbutamol also reduced the IL-1\(\beta\) production induced by CSE on BEAS-2B cells. These results demonstrated the anti-inflammatory effects of salbutamol.

IL-8 is a potent pro-inflammatory chemokine that induces trafficking of neutrophils to the lungs and contributes to the pathogenesis of COPD\(^\text{(15)}\). Salbutamol increased the IL-8 production in human transformed bronchial epithelial cells 16HBE cells\(^\text{(16)}\). In another study, no alteration in the IL-8 production was observed by salbutamol treatment in human bronchial epithelial cells from control and asthmatic patients stimulated by TNF-\(\alpha\)\(^\text{(17)}\). However, the salbutamol reduced the IL-8 production in bronchial epithelial cells stimulated by aqueous extracts of organic dust derived from hog CAFOs\(^\text{(6)}\). In our study, salbutamol reduced the IL-8 production induced by CSE. These results reinforce the anti-inflammatory effects of salbutamol.
IL-10 is a cytokine with diverse anti-inflammatory and immunosuppressive phenotypic effects, limiting inflammation\(^{(18)}\). During the examination of the sputum of the patient with COPD and in smokers, a reduction in the concentration of IL-10 was observed\(^{(19)}\). In mice immunized with lipopolysaccharides and ovalbumin, salbutamol favored IL-10-producing CD4+ T cells\(^{(20)}\). However, in another study, salbutamol was not able to enhance the IL-10 production on CD4+ T cells stimulated by allergen (house dust mite, grass, or cat extract)\(^{(21)}\). In our study, salbutamol increased IL-10 concentration when compared to CSE-stimulated BEAS-2B cells. The increased IL-10 production could contribute to reduce airway inflammation induced by cigarettes.

ICAM-1 plays an important role in leukocyte (neutrophils and macrophages) trafficking mediating the interaction between these cells and bronchial epithelial cells\(^{(22)}\). ICAM-1 expression is upregulated in smokers and COPD patients\(^{(23)}\). Salbutamol reduced the ICAM-1 expression in epithelial cells (BEAS-2B) stimulated by aqueous extracts of organic dust derived from hog CAFOs\(^{(24)}\). Our results agree with these findings: salbutamol reduced the ICAM-1 expression induced by CSE in BEAS-2B cells. These results, in association with the earlier results, confirm the anti-inflammatory effect of salbutamol.

Oxidative stress plays an important role in the pathogenesis of COPD caused by cigarette smoke\(^{(25)}\). Salbutamol inhibited the ROS production in RAW264.7 macrophages stimulated by lipopolysaccharide\(^{(26)}\). In neutrophils stimulated by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), salbutamol reduced the generation of ROS\(^{(27)}\). Our results are in agreement with the previous studies: salbutamol reduced the ROS production when compared to CSE-stimulated BEAS-2B cells.

NF-κB signaling is upregulated in COPD patients and plays a significant role in the airway epithelium in regulating cigarette smoking-induced inflammation. Salbutamol inhibited the activation of NF-κB in bone marrow derived dendritic cells stimulated by lipopolysaccharide\(^{(28)}\). Our results demonstrated that the activation of NF-κB induced by CSE on BEAS-2B cells
was reduced by salbutamol. The downregulation observed during NF-κB stimulation by salbutamol, which plays a significant role in airway inflammatory mechanisms in the pathogenesis of COPD and is important during IL-1β and IL-8 synthesis and ICAM-1 expression may provide benefits for the treatment of COPD patients.

CONCLUSION

Salbutamol could be a potential alternative treatment and a candidate to treat airway inflammation caused by cigarette smoking in patients with COPD.

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