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E-prostanoid receptor distribution in airway smooth muscle cells of a rat model of asthma

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Abstract: Airway remodeling is a main pathological characteristic of asthma, and strongly associated with migration and proliferation of airway smooth muscle cells. E-prostanoid (EP) receptor can regulate airway remodeling. This study established a rat model of asthma and evaluated EP changes in airway smooth muscle cells under the asthmatic state so as to provide theoretical evidence for developing EP drugs to treat airway remodeling in asthma. A total of 20 clean Sprague-Dawley rats were randomly assigned to asthma group and control group. 28 days later, they were sacrificed for histological examination. Airway smooth muscle cells were isolated, cultured and measured using quantitative fluorescent PCR. Histopathological examination revealed that rat models of asthma were in accordance with the manifestations of asthmatic airway remodeling. After reverse transcription, real-time quantitative fluorescent PCR was performed using miRNA Q-PCR diagnostic kit. GAPDH was considered the internal reference. Relative expressions of E-prostanoid 1–4 (EP1–4) ($2^{-\Delta\Delta Ct}$) in the control and asthma groups were respectively as follows: EP1: 4.35±0.18, 6.55±1.21; EP2: 3.64±0.12, 1.35±1.06; EP3: 4.59±1.14, 5.89±1.74; EP4: 2.89±1.85, 1.69±0.44. EP2/4 significantly decreased, but EP1 significantly increased in the asthma group ($P < 0.01$). These results suggested that the reduced EP2/4 and increased EP1 expressions in airway smooth muscle cells of rat models of asthma were probably important factors for asthmatic airway remodeling.

Keywords: Asthma, Airway Smooth Muscle Cells, Prostaglandin E Receptor

1. Introduction

Huber and Koessler have verified that thickening of the epithelium and smooth muscle layer was observed in fatal asthmatic airways [1]. Bronchial asthma is a common chronic allergic airway disease, but so far, pathogenesis of asthma remains unclear. Of them, airway remodeling is considered a key pathological characteristic of bronchial asthma, and a main reason for failure to cure asthma in the clinic [2]. Airway remodeling is strongly associated with hyperplasia and hypertrophy of airway smooth muscle cells. The reasons for these changes were associated with some extracellular signaling molecules-mediated various pathophysiological effects. Of them, prostaglandin E_2 (PGE_2) is one of essential extracellular signaling molecules[3]. PGE_2 has already been applied in the clinic, and has been shown to lessen asthma by controlling airway smooth muscle contraction and relaxation. Braking or "brake" mechanism of PGE_2 has been considered as an important factor in sensitization-, other stimulus- and NSAID-induced asthma [4]. However, it remains poorly understood whether these phenomena were associated with the alterations in PGE_2 receptor in airway smooth muscle cells. This study sought to determine EP in airway smooth muscle cells of rat models of asthma using quantitative fluorescent PCR and to identify the distribution of EP receptor in airway smooth muscle cells during asthma.

2. Experimental Methods

2.1. Animal Model Replication

A total of 20 male clean Sprague-Dawley rats aged 4–6 weeks and weighing 140–200 g were provided by the Zhejiang Provincial Experimental Animal Center, China. All rats were randomly and equally divided into asthma group and control group. Ovalbumin was used in the asthma group. Control group took aerosol inhalation of physiological saline. The rats were intraperitoneally injected with freshly prepared ovalbumin aluminum hydroxide solution 1 ml, containing 10 mg ovalbumin and 200 mg algeldrate, at 1 and 8 days. At 15–24 days, aerosol inhalation of 1% ovalbumin dissolved

physiological saline was conducted to induce asthma, once a day. Control group used physiological saline, instead of medicine. At 18–24 hours after medication (at 28 days), the rats were sacrificed by anesthesia with 20% urethane solution, and samples were collected.

Primary culture and identification of airway smooth muscle cells

After sacrificing, trachea and lung tissue were sterilely rapidly isolated and placed on a super clean bench. Various tissues except trachea and bronchi were removed. After longitudinal dissection, adventitia and intimal tissue were carefully erased until transparence. The trachea was cut into 1 mm or smaller blocks using an iris scissors. Cells were cultured in accordance with previous methods [5]. Airway smooth muscle cells were identified using Streptavidin-Peroxidase method (α-actin), showing positive staining [6]. Passage 3–5 of airway smooth muscle cells were seeded in a 96-well plate at 1×10^4 cells/mL.

3. RNA Extraction

After centrifugation, RNA was extracted in accordance with the Trizol kit (Invitrogen life technologies). RNA concentration and purity were measured using NanoDrop® ND-1000. Absorbance values at 260 nm (A_{260}) were determined using ultraviolet spectrophotometer. 1 at 260 nm represents 40 ng RNA/ul. RNA concentration was calculated by A_{260} × 40 ng/ul. Briefly, RNA was dissolved in 20 μl diethyl pyrocarbonate. 1 μl sample was detected, and it is concluded that A_{260} = 65.003, RNA concentration = 65.003 × 40 ng/ul = 2,600.12 ng/μl. Sample purity was detected using A_{260}/A_{280} ratio, and the ratio ranged from 1.8 to 2.1. 3 μg of RNA was utilized in agarose gel electrophoresis under denaturing conditions. Samples were observed and photographed with an ultraviolet transmittance analyzer. The bands of 28S and 18S ribosomal RNAs were very bright and dark. EB （Ethidium bromide） diffuse staining substances could be detected between 18S and 28S ribosomal bands. These substances were possibly composed of mRNA and other types of RNA (Figure 1).

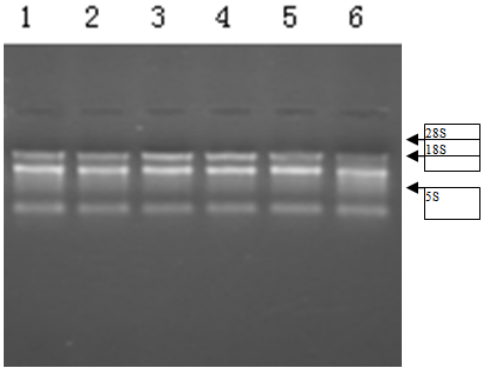

Figure 1. *electrophoresis for RNA extraction and denaturation*

1–6: samples grouped by A260/A280 ratio :in 2.01, 1.97, 2.01, 2.02, 2.04 and 2.01

4. Primer Design

Gene sequences were searched in miRBase database (http://www.mirbase.org/) and GeneBank. Primers were designed using Primer 5 software, and synthesized by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China. The primer sequences of above-mentioned four kinds of genes are listed in Table 1. Four target mRNAs: EP1 (http://www.ncbi.nlm.nih.gov/gene/25637), EP2 (http://www.ncbi.nlm.nih.gov/gene/81752), EP3 (http://www.ncbi.nlm.nih.gov/gene/24929), EP4 (http://www.ncbi.nlm.nih.gov/gene/84023).

Table 1. *EP primers*

Gene	Bidirectional primer sequence	Annealing temperature (°C)	Product length (bp)
EP1	F: 5′CACTTCAACCACAGCTGCCC3′ R: 5′CAGGATCTGGTTCCACGACG3′	59	232
EP2	F: 5′CTTCAGCTGTACGCCACGGT3′ R: 5′GGCCAGGAGAATGAGGTGGT3′	58	210
EP3	F: 5′CAACCTGGCGACCATCAAAG3′ R: 5′TCCCATCTGCGTCTTGCATT3′	58	217
EP4	F: 5′CTCACGCTCTTCGCCGTCTA3′ R: 5′AGCACATTGCAGAGCACGGT3′	59	208

EP: E-prostanoid.

4.1. Reverse Transcription of Mirna and Real-Time Quantitative Fluorescent PCR

A poly (A) tail was added to RNA. OligodT adaptor primer was applied in reverse transcription. miRNA-specific primer served as forward primer, conjunct and adaptor-paired universal primer as reverse primer, and GAPDH as internal reference. Real-time quantitative fluorescent PCR amplification was performed in accordance with the instruction of miRNA Q-PCR diagnostic kit (Fulen Gene, Guangzhou, China). Reverse transcription: 25 μl of reaction system: 2 μg of total RNA, 0.5 μg/μl Oligo $(dT)_{18}$ (Sangon Biotech (Shanghai) Co., Ltd.) 1 μl, 10 × RT buffer 2 μl, 2.5 mM dNTP mixed liquor 4 μl, RNase inhibitor (Promega) 1 μl, MMLV reverse transcriptase (Promega) 1 μl. 10 μl RT reaction solution (Epicentre) was added to 10 μl annealed mixture in a water bath at 37 °C for 60 minutes, which was heated to 95 °C for 5 minutes. The products were diluted five times and stored at –20°C for further use.

Synthesized cDNA used in real-time quantitative PCR (20 μl reaction system): $MgCl_2$ (TaKaRa) 2.4 μl, 10 × buffer

(TaKaRa) 2 μl, SYBR (Invitrogen) 0.5 μl, dNTP (10 mM/each) (Promega) 0.4 μl, Primer 1 (50 pM/μl) 0.2 μl, Primer 2 (50 pM/μl) 0.2 μl, Taq enzyme (Promega) (5 U/μl) 0.3 μl (1.5 U), first-strand cDNA 1 μl obtained in reverse transcription, and double distilled water 13 μl. Reaction condition: pre-denaturation at 95 °C for 2 minutes, denaturation at 95 °C for 10 seconds, at 60 °C for 10 seconds, 72 °C for 40 seconds, totally 40 cycles. The experiments were conducted in ABI PRISM7500 system (Applied Biosystems). PCR was conducted five times in each sample.

4.2. Statistical Treatment

The data were analyzed using SPSS 16.0 software. CT values of each group were expressed as mean ± SD. U6 served as internal reference for correction. EP miRNA expression in airway smooth muscle cells was calculated by $2^{-\Delta\Delta CT}$ method. $\Delta Ct = Ct\ (miRNA) - Ct(U6)$, $\Delta\Delta Ct = (Ct_{target} - Ct_{internal\ reference})$ in the experimental group $- (Ct_{target} - Ct_{internal\ reference})$ in the control group. The difference was analyzed using independent samples *t*-test. A value of $P \leq 0.05$ was considered statistically significant.

5. Results

The rat model of asthma was accorded with asthma standard as detected by histopathology. These structural changes include epithelial detachment, subepithelial fibrosis, increased airway smooth muscle mass, mucous gland and goblet cell hyperplasia.

6. RNA Quality and Purity

A_{260}/A_{280} ratio of RNA samples was between 1.8 and 2.0.

7. Amplification Curve and Solubility Curve (Figure 2)

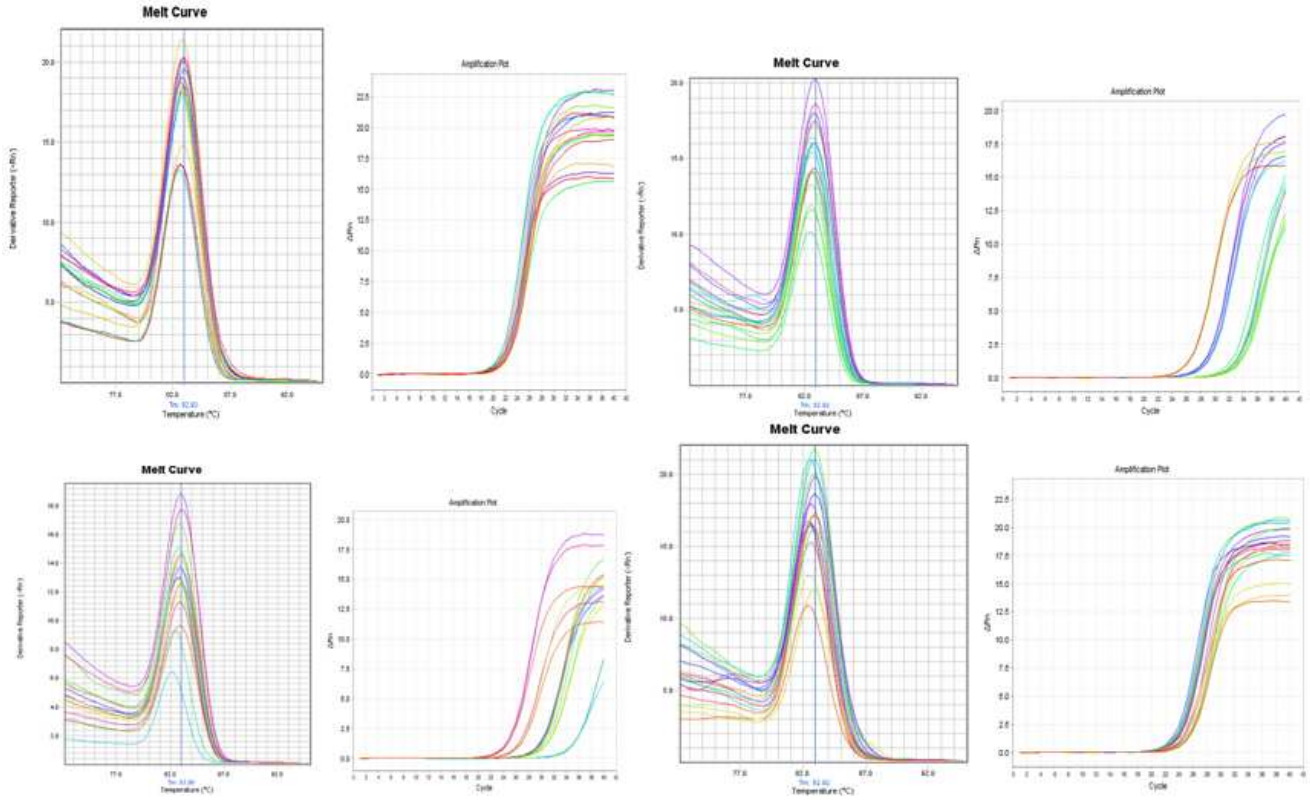

Figure 2. *Solubility curve and amplification plot of E-prostanoid 1–4 (EP1–4) as detected by real-time quantitative fluorescent PCR.*

8. Results of Quantitative Fluorescent PCR

Relative expression ($2^{-\Delta\Delta Ct}$) of EP IS? altered in airway smooth muscle cells of rat models of asthma, showing that EP1 levels significantly increased. EP2 and EP4 levels significantly decreased. EP3 levels did not apparently changed (Table 2).

Table 2. *Quantitative fluorescent PCR results ($2^{-\Delta\Delta Ct}$)*

Receptor	Relative expression (2-ΔΔCt)		Statistical results
	Control group	Asthma group	
EP1	4.35±0.18	6.55±1.21	P<0.01
EP2	3.64±0.12	1.35±1.06	P<0.01
EP3	4.59±1.14	5.89±1.74	
EP4	4.89±1.85	1.69±0.44	P<0.01

9. Discussion

Histological examination showed that rat models of asthma in this study were in accordance with the manifestations of airway remodeling, with the presence of evident airway smooth muscle cell proliferation and airway structural changes. These phenomena were possibly correlated with regulatory effect of PGE_2 secretion on airway smooth muscle cells. It is known that four kinds of EP were observed on airway smooth muscle cells, including EP1–4. EP as a transmembrane protein belongs to G-protein-coupled receptor superfamily, containing four subtypes EP_1, EP_2, EP_3 and EP_4[7]. Airway smooth muscle cells involves in airway remodeling. The abilities of PGE_2 synthesis and secretion are associated with EP function and surface expression[8]. In airway smooth muscle cells, activated EP_2/EP_4 mediated cyclic adenosine monophosphate increase, induced smooth muscle loose, so it is called relaxation receptor. Activated EP_3 mediated cyclic adenosine monophosphate decrease and inhibited smooth muscle contraction, so it is called inhibitory receptor. Activated EP_1 mediated Ca^{2+} influx and caused smooth muscle contraction[7].

A few present studies addressed EP distribution in airway smooth muscle cells. Säfholm J et al.[9] detected EP in guinea pig airways using real-time PCR and mRNA expression of four EP receptors in airway smooth muscle. PGE_2 exhibited bell-shaped concentration-response curves. EP1 receptor antagonist ONO-8130 suppressed the initial contraction. EP2 receptor antagonist PF-04418948 induced smooth muscle relaxation. EP3 (ONO-AE5-599) and EP4 (ONO-AE3-208) selective receptor antagonists did not affect smooth muscle. ONO-8130 and specific PGE_2 antibody eliminated spontaneous airway tone, and EP2 antagonist PF-04418948 caused tone increase. It is believed that endogenous PGE_2 was synthesized mainly by COX-2. The balance of contractile EP1 receptor and diastolic EP2 receptor maintains spontaneous airway tone. Results of the present study suggested that EP2/4 reduced in airway smooth muscle cells of rats with asthma, which possibly led to the reduction of relaxation ability of airway smooth muscle cells and produced EP1 receptor-mediated effects. That is, PGE_2 did not lead to protective airway relaxation of airway smooth muscle at the asthmatic status, but turn on EP1-mediated contraction. Tilley et al.[10] verified that PGE_2 binding to EP_1 receptor in airway smooth muscle cells could enhance airway responsiveness. Fortner et al. [11] confirmed that PGE_2 caused relaxation of airway smooth muscle cells through EP2 receptor mediation by the deletion of the EP2 receptor (–/–). This conclusion was identical to the results from this study.

Different EP subtypes have different affinity on PGE_2 (the smaller the value in parentheses, the bigger the affinity was): EP_3 (1) > EP_4 (2) > EP_2 (12) > EP_1 (20) [7], which suggested that different PGE_2 levels probably depended on the major subtype of EP. Simultaneously, the alterations in EP subtype expression changed the affinity of PGE_2. PGE_2 inhibited airway smooth muscle cell migration via EP_2 signaling transduction pathway. The decrease in EP2/4 expression levels reduced the inhibitory effects of EP2/4. Simultaneously, the increased EP1/3 expression increased the migration and proliferation of airway smooth muscle cells, resulted in airway structural changes and airway remodeling. Aso H et al.[12] confirmed that EP2 and EP4 receptor agonists suppressed the migration of airway smooth muscle cells induced by platelet-derived growth factor-BB. However, the use of antagonist could lead to cell migration. EP3 displayed opposite results. Quantitative detection of EP in this study further strengthened above-mentioned conclusions. These indicated the regulatory effects of EP on contraction, relaxation and migration of airway smooth muscle cells.

In conclusion, airway remodeling in rat models of asthma is possibly associated with EP2/4 decrease and EP1 expression increase in airway smooth muscle cells. PGE_2 possibly has protective effects on normal airway smooth muscle cells, and enhances cell migration and proliferation. This might be a key factor for airway remodeling in asthma.

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