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Epoxyeicosatrienoic acids attenuate cigarette smoke extract-induced interleukin-8 production in bronchial epithelial cells

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ABSTRACT

In response to endothelial cell activation, arachidonic acid can be converted by cytochrome P450 (CYP) epoxygenases to epoxyeicosatrienoic acids (EETs), which have potent vasodilator and anti-inflammatory properties. In this study, we investigated the effects of exogenous EETs on cigarette smoke extract (CSE)-induced inflammation in human bronchial epithelial cells (NCI-H292). We found that CSE inhibited the expression of CYP2C8 and mildly stimulated the expression of epoxide hydrolase 2 (EPHX2) but did not change the expression of CYP2J2. Treatment with 11,12-EET or 14,15-EET attenuated the CSE-induced release of interleukin (IL)-8 by inhibiting the phosphorylation of p38 mitogen-activated protein kinases (MAPKs). Our results demonstrated that CSE may reduce the anti-inflammatory ability of epithelial cells themselves by lowering the EET level. EETs from pulmonary epithelial cells may play a critical protective role on epithelial cell injury.

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1. Introduction

Chronic obstructive pulmonary disease (COPD) remains a major cause of chronic morbidity and mortality around the world. The prevalence, morbidity, and mortality of COPD vary across countries and across different groups within countries. However, in general, these are directly related to the prevalence of tobacco smoking [1]. Airway epithelial cells are the first innate defense system in the airways for cigarette smoke (CS) and are able to respond to CS with the increased production of proinflammatory cytokines [2]. IL-8 is as proinflammatory cytokine that has been suggested to be the most important mediator in airway inflammatory and innate immunity [3,4]. In particular, it is a potent neutrophil chemoattractant, and its level is elevated in the induced sputum of patients with COPD [5].

It is well established that arachidonic acid (AA) can be converted to eicosanoid mediators by the cyclooxygenase (COX), lipoxygenase, and cytochrome P450 (CYP) monooxygenase pathways [6]. Unlike COX and lipoxygenase products, the role of CYP450 pathways in the immune response is poorly understood. EETs are synthesized predominantly by the epoxygenases of the CYP2 family, including the 2C and 2J classes [7]. CYP2C8 is expressed mainly in the endothelium, CYP2C9 is expressed mainly in the kidney, and CYP2J2 is expressed mainly in the endothelium and myocardiocytes [8]. Recent research has also found that 2J2 is expressed in human platelets [9]. These CYP epoxygenases add an epoxide group to one of the four double bonds of AA and form four regioisomeric EETs: 5,6-, 8,9-, 11,12-, and 14,15-EETs [10]. An accumulating body of evidence indicates that EETs exhibit important, but diverse physiological and pathophysiological roles. EETs can protect the cardiovascular system and directly contribute to vasodilation and reduction in blood pressure under hypertension [8]. Recent studies suggest that the forced expression of CYP2J2 and elevated levels of EETs promote tumor malignancy, including proliferation and metastasis in vivo and in vitro, whereas the selective inhibition of CYP2J2 attenuated these effects [11]. Moreover, previous studies have also found that 14,15-EET can

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affect the uterine contractile function and inhibit sodium-dependent phosphate transport in opossum kidney cells [12,13]. In addition, the roles of CYP epoxygenases and EETs in the regulation of inflammatory processes are being increasingly studied.

Previous studies have demonstrated that CYP epoxygenases and EETs significantly attenuate the inflammatory responses in various cardiovascular pathological inflammation model systems [14–16]. Soluble epoxide hydrolase (sEH) converts EETs to dihydroxyeicosatrienoic acids (DHETs). sEH inhibition can increase the biological activity of EETs [17]. The administration of AUDA-nBE (a selective sEH inhibitor) causes a marked reduction in tobacco smoke-induced inflammation in rats [18]. Similarly, treatment with a sEH inhibitor increases fatty acid epoxides and indirectly reduces the production of Th1 cytokines and proinflammatory lipid mediators, as well as minimizes airway obstruction and reduces weight loss in a rat model of COPD [19]. In addition, 14,15-EET displays an anti-inflammatory effect on TNF-α-treated human bronchi that is related to the inactivation of NF-κB [20]. These findings suggest that sEHs and EETs can relieve airway inflammation. However, the function of CYP epoxygenases in pulmonary epithelial cells has not yet been investigated.

In this study, we detected the epoxygenases of the CYP2 family in pulmonary epithelial cells and subsequently explored the anti-inflammatory effects of EETs on cigarette smoke extract-exposed pulmonary epithelial cells and the underlying mechanisms.

2. Materials and methods

2.1. Cells culture

NCI-H292 cells, a human bronchial epithelial cell line, were purchased from the Cell Bank of the Chinese Academy of Sciences. The cells were grown in RPMI 1640 (Hyclone) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin (Hyclone) and were maintained in a humidified incubator at 37 °C with 5% CO_2. Fresh media were replenished every 2–3 days. For each experiment, the cells (2×10^5 cells/ml) were plated in six-well culture plates.

2.2. Preparation of cigarette smoke extract

Research-grade cigarettes (3R4F) were obtained from the Kentucky Tobacco Research Council (University of Kentucky, Lexington, KY, USA). The composition of 3R4F research-grade cigarette was the following: total particulate matter – 10.9 mg/cigarette, tar – 9.4 mg/cigarette, nicotine – 0.726 mg/cigarette. The cigarette smoke extract (CSE) was prepared by bubbling smoke from three cigarettes into 30 ml of PBS according to a previously published method [21].

2.3. RT-PCR and Q-PCR

Confluent NCI-H292 cells in a six-well plate were pre-treated with 11,12-EET (1 μM; Sigma, St. Louis, MO, USA) or 14,15-EET (1 μM; Sigma, St. Louis, MO, USA) for 30 min, which were stored in indirect light and under an inert atmosphere with the bulk kept below 20 °C. Subsequently, the cells were incubated with CSE for different times. Total RNA was extracted with TRIzol reagent (Takara, Japan) according to the manufacturer's instructions. The PCR primers were purchased from Shanghai Bioengineering Ltd. (Shanghai, China). After PCR reaction, the products were run on a 1.5% agarose gel electrophoresis and stained with ethidium bromide. All primers were checked against BLAST for selectivity. Real-time PCR amplification was performed using an Applied Biosystems 7500 sequence detection system. Thermal cycler conditions were 10 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C for 40 cycles. GAPDH was amplified as an internal control. mRNA levels were calculated by using the comparative parameter threshold cycle (Ct) and normalized to GAPDH. Primer sequences were as follows: human IL-8: 5′-AGATATTGCACGGGGAA-3′ (sense) and 5′-GAAATAAAGGAGAAACCA-3′ (antisense); GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′′ (sense) and 5′-TCCACCACCCTG TTGCTG TA-3′ (antisense); CYP2C8: 5′-CACCCAGAGGTCACAGCTAAAGT-3′ (sense) and 5′-CATGTGGCTCCTATCCTGCAT-3′(antisense); CYP2J2: 5′-GGACTCTCCTACTGGGCACT-3′ (sense) and 5′-CTCCGAAGGTGATGGAGCAA-3′(antisense); EPHX2: 5′-ACTGCCATCCTCACCAACAC-3′ (sense) and 5′-GGTTCAGGTTTGACCATTCC-3′(antisense).

2.4. Measurement of IL-8 by ELISA

NCI-H292 cells were plated in a 24-well plate. A subconfluent monolayer of the cells was exposed to a different concentration of CSE for 24 h. The supernatant was collected and stored at −80 °C until assayed for IL-8 using an ELISA kit (eBioscience, USA) following the manufacturer's instruction.

2.5. Western blot analysis

The cells were washed three times with ice-cold PBS and lysed in 100 μL of RIPA with 1 mM PMSF (Beyotime, China). The protein concentration was measured using a BCA protein assay kit (Cwbiotech, China). A sample of protein (30 μg) from the cell lysates was separated by SDS-PAGE in 8% polyacrylamide gel and transferred to nitrocellulose membranes (PALL, USA), which were further blocked in 5% nonfat dry milk dissolved in Tris-buffered saline with Tween (TBS–T; pH 7.4) for 1 h at room temperature (RT). Incubation with rabbit monoclonal primary antibodies (1:1000) was conducted overnight at 4 °C, and incubation with goat anti-rabbit 800 antibodies (1:5000, Invitrogen, CA, USA) was conducted at RT for 2 h. The immunoreactive bands were visualized by a two-color infrared imaging system (Odyssey, USA). The primary antibodies for CYP2C8 and β-actin were obtained from Abcam (Cambridge, MA, USA), and the p38, p-p38, p-Erk and Erk antibodies were purchased from Cell Signaling (Danvers, MA, USA).

2.6. Statistical analysis

The GraphPad prism V5.0 software was used for the statistical analyses. The data are presented as the mean ± S.E.M. The statistical tests were performed using the SPSS software (version 16.0; SPSS, Chicago, IL, USA). Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) or post-hoc tests. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. CSE decreases the production of CYP2C8 and increases EPHX2 mRNA expression in bronchial epithelial cells

EETs are synthesized predominantly by the epoxygenases of the CYP2 family, including the 2C and 2J classes [7]. We first investigated whether human pulmonary epithelial cells express the 2C and 2J classes. RT-PCR was performed using the total RNA prepared from NCI-H292 cells. As shown in Fig. 1A, NCI-H292 cells mainly expressed CYP2C8 and marginally expressed CYP2J2. The treatment of NCI-H292 cells with CSE (2.5%) for 24 h decreased the expression of CYP2C8 but did not affect the CYP2J2 mRNA level (Fig. 1A). Moreover, 24-h exposure to CSE decreased CYP2C8 mRNA

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