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HIF-1α promotes inflammatory response of chronic obstructive pulmonary disease by activating EGFR/PI3K/AKT pathway

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Abstract. – OBJECTIVE: Chronic obstructive pulmonary disease (COPD) is an incomplete, reversible disease with progressive inflammation obstruction in airways. This study aims to explore the regulatory mechanism of hypoxia-inducible factor-1α (HIF-1α) in inflammatory response and progression of COPD.

PATIENTS AND METHODS: 71 bronchoalveolar lavage fluid (BALF) samples were collected, including 59 samples from COPD patients (COPD group) and 12 from patients with normal pulmonary function (control group). The mRNA and protein levels of HIF-1α and epidermal growth factor receptor (EGFR) in BALF were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. Serum levels of interleukin-13 (IL-13), IL-9, IL-1, and tumor necrosis factor-α (TNF-α) were detected by enzyme-linked immunosorbent assay (ELISA). Hypoxia cell model was constructed by COCl2 induction in human embryonic lung cells. Expression levels of HIF-1α, EGFR and p-AKT in NCI-H1563 cells treated with 740Y-P, the phosphoinositide 3-kinase (PI3K) agonist were detected. Finally, we detected proliferation and apoptosis in NCI-H1563 cells with HIF-1α overexpression by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively.

RESULTS: The mRNA and protein levels of HIF-1α and EGFR were higher in COPD groups compared with those of control group. Serum levels of IL-13, IL-9, IL-1, and TNF-α in COPD patients were elevated. CoCl2 induction in NCI-H1563 cells led to upregulated levels of IL-13, IL-9, IL-1, and TNF-α. 740Y-P treatment remarkably activated EGFR/PI3K/AKT pathway. Overexpressed HIF-1α inhibited proliferation but induced apoptosis of NCI-H1563 cells.

CONCLUSIONS: HIF-1α was overexpressed in COPD, which upregulated expressions of inflammatory factors via activating the EGFR/PI3K/AKT pathway. The activated EGFR/PI3K/AKT pathway induced by pulmonary inflammation further upregulated HIF-1α expression in a feedback loop, thus aggravating COPD pathological changes.

Key Words:
HIF-1α, EGFR, COPD, Inflammation, PI3K/AKT pathway.

Introduction

Chronic obstructive pulmonary disease (COPD) is a common chronic respiratory disease with high morbidity and mortality. COPD is defined as a preventable, treatable disease characterized by persistent, incompletely reversible and progressive airflow limitation. COPD is related to the chronic inflammatory reaction of airway and lung caused by harmful gases or particles[1-3]. Clinical studies[4-6] have shown that the main clinical symptoms of COPD are repeated cough, expectoration, dyspnea, which is often progressed from chronic bronchitis or emphysema. Evident pathological alterations in bronchi and lung tissues, as well as disordered pulmonary function of COPD patients severely affect their life quality.

Song et al[7] found that lipopolysaccharide (LPS) stimulates the secretion of inflammatory factors *via* epidermal growth factor receptor (EGFR)/PI3K (phosphoinositide 3-kinase) pathway in NCI-H292 cells[8]. The EGFR/PI3K pathway exerts a vital role in the upregulation of secretory proteins in airway epithelial cells induced by LPS or neutrophil elastase. Yang et al[9] found that EGFR/PI3K pathway is activated in the transcription process of 16HBE cells treated with neutrophil elastase. Lee et al[10]

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have demonstrated that EGFR/PI3K pathway is closely related to high secretion of mucus in the airway epithelium.

HIF-1 (hypoxia-inducible factor-1) is a vital transcription factor that regulates hypoxic responses. Current researches found that there are three proteins in the HIF-1 family, namely HIF-1, HIF-2, and HIF-3. HIF-1 plays a major role in the adaptive response under hypoxic state[11]. HIF-1 is widely expressed in many mammalian and human tissue cells, which is composed of α and β subunit. Among them, HIF-1α contains a PAS (Per/ARNT/AbR/Sim) domain in amino-terminal that binds to DNA. The independent transactivation domains and an oxygen-dependent degradation domain in carboxyl-terminal determine the HIF-1 activity. Li et al reported[12] that HIF-1α regulates the oxygen balance, hypoxia adaptation, inflammatory response, and tumor development. In recent years, the specific role of HIF-1α in allergic inflammation has been well recognized[13-15].

To study the interaction of HIF-1α and EGFR in the pathogenesis of COPD, we first detected expressions of HIF-1α and EGFR in COPD patients. Subsequently, human embryonic lung epithelial cells were utilized to investigate the potential mechanism of HIF-1α in the occurrence and progression of COPD.

Patients and Methods

Patients

59 COPD patients treated in our hospital from April 2015 to June 2016 were selected in the COPD group. Among the 59 COPD patients (including patients with acute exacerbation of chronic obstructive pulmonary disease and stable of chronic obstructive pulmonary disease), 37 were male and 22 were female. Enrolled patients met the criteria of 2007 GOLD COPD classification and grouping guidelines. This study was approved by the Ethics Committee of Beijing Luhe Hospital Affiliated to Capital Medical University. The signed written informed consents were obtained from all participants before the study. Exclusion criteria were: 1. Severe diseases of the immune system or immunosuppressive agents administration for the past 7-8 weeks; 2. Combination of interstitial lung disease, pneumothorax, and other restrictive ventilation dysfunction; 3. Combination of severe cardiovascular, renal, hepatic, and hematopoietic system diseases that cannot undergo bronchoalveolar lavage; 4. Combination of severe lung diseases such as lung cancer, tuberculosis, and allergic pneumonia; 5. Combination of malignancies; 6. Subjects who could not be actively cooperated. Meanwhile, 12 inpatients with normal pulmonary function in the same period were selected in the control group, including 6 males and 6 females.

Collection and Treatment of Bronchoalveolar Lavage Fluid (BALF)

Each patient received a detailed explanation of the major processes of bronchoalveolar lavage (BAL), highlighting the risks and matters needing attention. All patients gave signed the informed consent. BAL was performed by the fiberoptic bronchoscope, with reference to Technical Specification of Cytology Test on Bronchoalveolar Lavage published by Chinese Medical Association in 2002. The detailed procedure was as followings: (1) Medical history of each patient was inquired. Cardiopulmonary function and other physical examination were routinely performed. The results of routine laboratory tests were studied. (2) Patient could not eat or drink 4 hours before surgery. (3) Intravenous pathway was established, sedated if necessary. Meanwhile, 0.5% of tetracaine spray was used for anesthesia. (4) Fiberoptic bronchoscope through the nostrils was introduced in the lungs to detect the situation of the tracheal and bronchial mucosa. (5) The middle of the right lung was routinely used for the lavage site. 1-2 ml of 2% lidocaine was administered for local anesthesia. 37°C sterile saline was injected in the lavage site for multiple times, with the total amount of 100-250 ml and a single injection of about 25-30 ml. 50-100 mmHg negative pressure was used to aspirate lavage fluid (40-60% of the recovery rate). If each recovery rate was lower than 5%, the operation should be immediately stopped and treated based on the specific situations. 20-30 ml of the collected BALF was centrifuged at 3500 rpm for 5 min at 4°C, and the shed alveolar epithelial cells in the BALF were collected.

$CoCl_2$ Induction of Hypoxia in Human Embryonic Lung Cells

1×10^5/mL NCI-H1563 cells were seeded in a 6-cm dish for overnight culture. Medium containing 1% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) was then replaced and cells were further cultured for 12 h. Subsequently,

NCI-H1563 cells were treated with 125 μM $CoCl_2$ or isodose phosphate-buffered saline (PBS), respectively, for 48 h.

740Y-P Treatment in Human Embryonic Lung Cells

NCI-H1563 cells were seeded in 6-well plates. After cell adherence, cells were washed with PBS twice and incubated with PI3K agonist 740Y-P for 24 h. Cells in the control group were treated with isodose DMSO (dimethyl sulfoxide) for 24 h.

Western Blot

Total protein was extracted, separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) under denaturing conditions and transferred to PVDF (polyvinylidene difluoride) membranes (Roche, Basel, Switzerland). Membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

RT-PCR (Real-Time Polymerase Chain Reaction)

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract total RNA for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. Primers used in RT-PCR were listed as follows: HIF-1a: F: CGTTCCTTCGATCAGTTGTC, R: TCAGTGGTGGCAGTGGTAGT; EGFR: F: TCTCAGCAACATGTCGATGG, R: TCGCACTTCTTACACTTGCG; GAPDH: F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Extraction of Peripheral Blood Mononuclear Cells (PBMCs)

2 mL of peripheral blood was collected and preserved in EDTA-Na (ethylene diamine tetraacetic acid-Na) anticoagulant tube. After gently mixed and centrifuged at a gradient density, the remaining portion of the tube was diluted with PBS. The mixture was then added in erythrocyte lysate, followed by dissolution and precipitation. Cells were resuspended in 100 μL of PBS that were PBMCs.

Flow Cytometry

PBMCs were resuspended and incubated with $AnnexinV^+/7\text{-}AAD^-$ (eBioscience, San Diego, CA, USA) for 15 min in the dark. Subsequently, PBMCs were washed, centrifuged, and resuspended for determination using a flow cytometer (Partec AG, Arlesheim, Switzerland).

ELISA (Enzyme-Linked Immunosorbent Assay)

Culture medium was collected for detecting cytokine dose according to the instructions of ELISA detection kit (Bio Legend, San Diego, CA, USA). The optical density at the wavelength of 562 nm and 450 nm was detected using a microplate reader.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in 6-well plates after corresponding treatments. 10 μL of CCK-8 reagent (Dojindo, Kumamoto, Japan) was added in each well. 2 hours later, the optical density of each sample was detected at the wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

We used GraphPad Prism (v6.0) for all statistical analysis (La Jolla, CA, USA). The quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was used for comparing differences between the two groups. Bonferroni analysis was introduced for detecting the significance among each group. $p < 0.05$ was considered statistically significant.

Results

Pulmonary Function Decline in COPD Patients

Although COPD is highly heterogeneous, current diagnosis is only based on pulmonary function detection. The basic clinical characteristics of enrolled patients were analyzed (Table I). We found that FEV_1/FVC (forced expiratory volume in one second/forced vital capacity), FEV_1% pred (FEV_1 predicted) and RV/TLC (residual volume/total lung volume) were remarkably lower in COPD patients compared with those of controls. No significant differences in other basic indicators were found between the two groups, except for BMI.

Table I. Clinical data of patients in COPD and control groups (mean ± standard deviation).

Groups	COPD group (n = 59)	Control group (n = 12)	T (χ^2)	p
Sex (male/female)	37/22	6/6	0.663	0.521
Age (year)	58.32 ± 7.89	56.44 ± 9.31	1.229	0.412
Smoke (yes/no)	37/22	4/8	3.504	0.106
BMI (kg/m^2)	23.41 ± 2.56	24.22 ± 3.11	-1.581	0.004
FEV_1/FVC	57.77 ± 13.79	89.11 ± 7.92	-19.89	0.000
FEV_1 % pred	51.28 ± 13.79	119.08 ± 16.56	-13.68	0.000
RV/TLC	46.59 ± 6.98	42.11 ± 7.23	2.22	0.012

HIF-1α and EGFR Were Involved in the Occurrence and Progression of COPD

Both protein and mRNA levels of HIF-1α and EGFR were elevated in BALF of COPD patients than those of controls (Figure 1A and 1B). Meanwhile, expression levels of HIF-1α and EGFR were also upregulated in PBMCs of COPD patients (Figure 1C). Subsequently, the relationship between HIF-1α and EGFR was analyzed by Spearman correlation. The data indicated that the mRNA level of HIF-1α was positively correlated to EGFR level (rs = 0.73).

HIF-1α Promoted Inflammation in COPD

It is reported that IL-13, IL-9, IL-1, TNF-α, and EGFR are important cytokines in regulating pulmonary inflammation. We found that serum levels of IL-13, IL-9, IL-1, and TNF-α were remarkably higher in the COPD group than those of the control group ($p < 0.001$, Figure 2).

HIF-1α Promoted Inflammation in COPD Via EGFR/PI3K/AKT Pathway

Hypoxia cell model was conducted by 125 μM $CoCl_2$ treatment in NCI-H1563 cells. Up-regulated mRNA levels of HIF-1α and EGFR were found in hypoxic cells compared with those of controls ($p < 0.001$, Figure 3A). 740Y-P treatment obtained the similar results. Besides, elevated serum levels of IL-13, IL-9, IL-1, and TNF-α were observed after 740Y-P treatment in hypoxic cells than those of controls ($p < 0.001$, Figure 3B). Subsequently, we detected the protein expressions of HIF-1α, EGFR, and p-AKT by Western blot. Both $CoCl_2$ treatment and 740Y-P treatment remarkably upregulated protein expressions of HIF-1α, EGFR and p-AKT in NCI-H1563 cells (Figure 3C).

HIF-1α Promoted Proliferation and Apoptosis of NCI-H1563 Cells

NCI-H1563 cells were treated with different doses of 740Y-P (0.125, 0.25, 0.5, 1, 2, 4, 6, and 8 μmol/L) for 24 h, respectively. The CCK-8 assay showed that the proliferative capacity was increased in a dose-dependent manner (Figure 4A, r = -0.8311, $p < 0.01$). A significant difference in cell viability was found between the two groups after treatment of 1, 2 and 4 μmol/L 740Y-P, respectively ($p < 0.05$, Figure 4B).

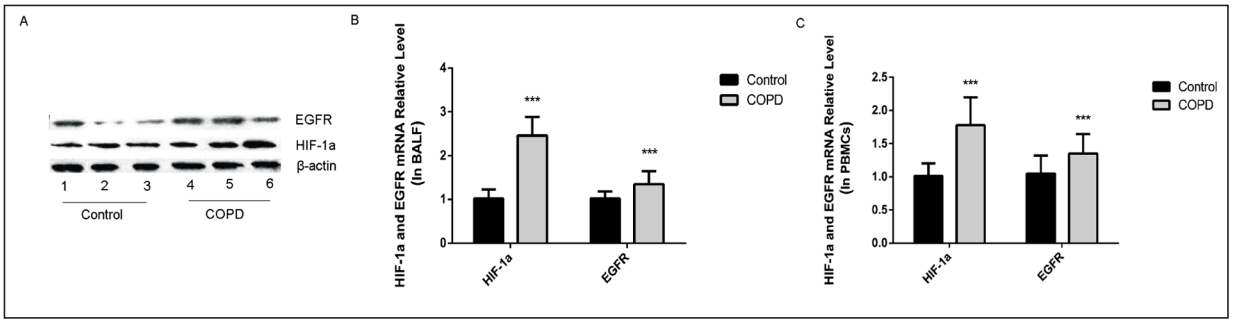

Figure 1. HIF-1α and EGFR were involved in the occurrence and progression of COPD. ***A, B,*** Both protein (***A***) and mRNA (***B***) levels of HIF-1α and EGFR were elevated in BALF of COPD patients than those of controls. ***C,*** Expression levels of HIF-1α and EGFR were upregulated in PBMCs of COPD patients.

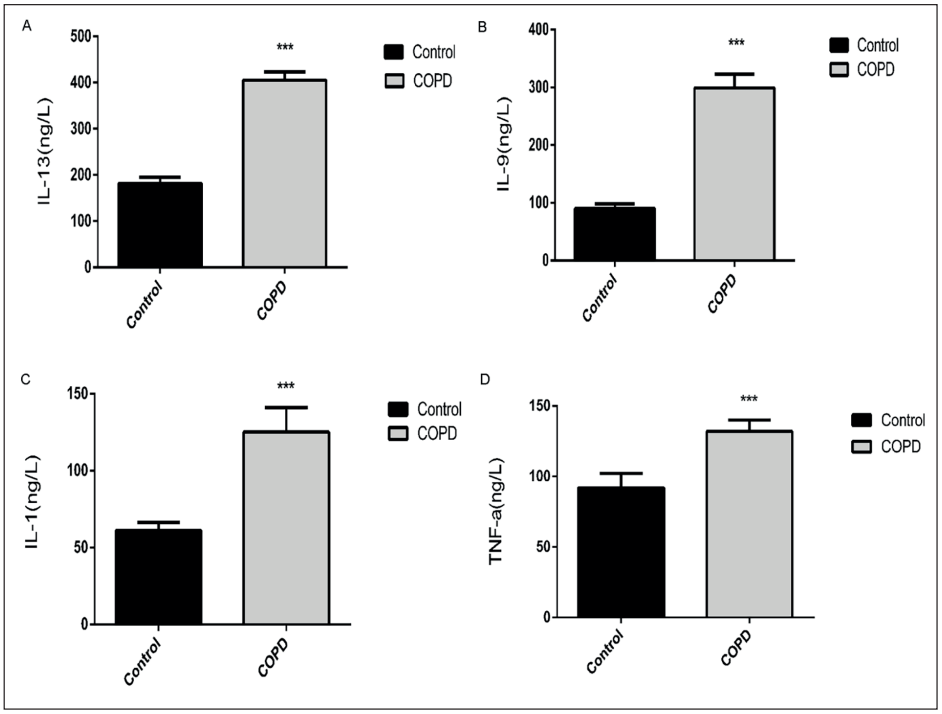

Figure 2. HIF-1α promoted inflammation in COPD. Serum level of IL-13, IL-9, IL-1, and TNF-α was remarkably higher in the COPD group than that of the control group detected by ELISA, respectively.

Discussion

The respiratory tract has inherent immunity. Mucus secreted by the airway epithelium adhere foreign bodies and pathogens, and then gradually cleared away with the cilia swing. Under normal circumstances, the mucosal surface of the airway system is covered by a liquid barrier, which is formed by mucus secreted by the airway[16,17]. A small amount of mucous secretion could protect the airway and inherent immunity. However, long-term secretion of mucus results in impaired mucociliary clearance function, further leading to repeated respiratory infections[18-20].

HIF-1α is an essential transcription factor that mediates the hypoxic response of the body. COPD leads to cellular hypoxia, thus upregulating HIF-1α expression[21]. Current investigations have already confirmed the role of HIF-1α in tumorigenesis. However, the underlying mech-

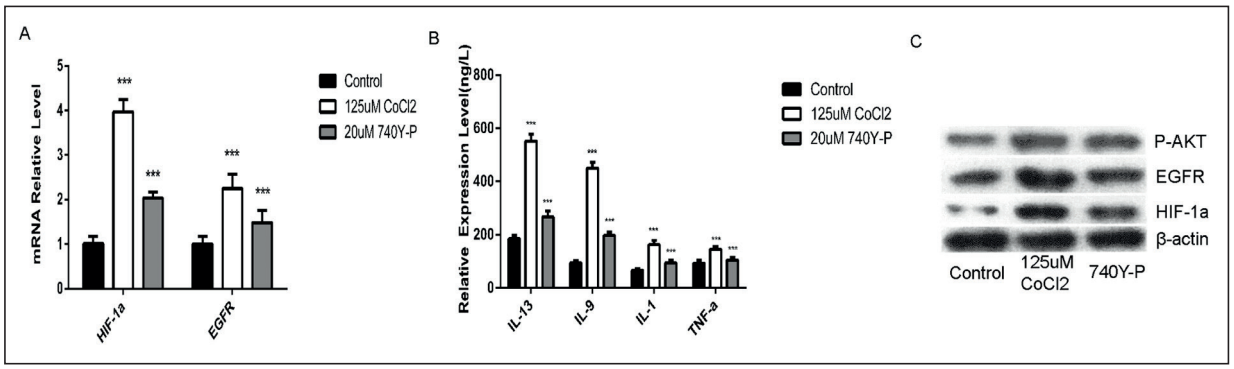

Figure 3. HIF-1α promoted inflammation in COPD *via* EGFR/PI3K/AKT pathway. ***A,*** Upregulated mRNA levels of HIF-1α and EGFR were found in hypoxic cells compared with those of controls. 740Y-P treatment obtained the similar results. ***B,*** Elevated serum levels of IL-13, IL-9, IL-1, and TNF-α were observed after 740Y-P treatment in hypoxic cells than those of controls. ***C,*** Both $CoCl_2$ treatment or 740Y-P treatment remarkably upregulated protein expressions of HIF-1α, EGFR and p-AKT in NCI-H1563 cells.

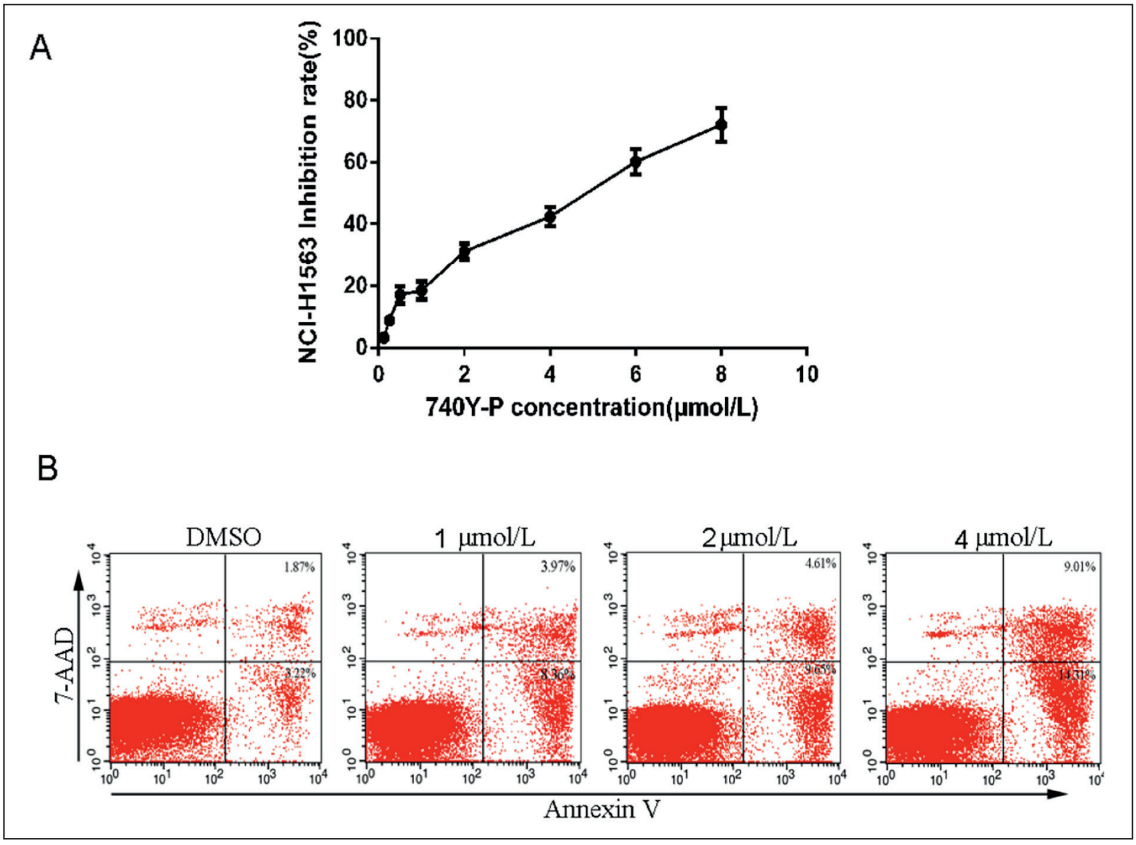

Figure 4. HIF-1α promoted proliferation and apoptosis of NCI-H1563 cells. ***A,*** CCK-8 assay showed that the proliferative capacity was increased in a dose-dependent manner. ***B,*** A significant difference in cell viability was found between the two groups after treatment of 1, 2 and 4 μmol/L 740Y-P, respectively.

anism of HIF-1α in COPD progression has not been well elucidated. In our study, we found that HIF-1α was highly expressed in COPD patients, which may be explained by the pulmonary function decline during the long-term hypoxic response. Currently, IL-13, IL-9, IL-1, TNF-α, and EGFR are considered to be related to mucus hypersecretion. IL-13 and IL-9 are considered as two major factors that promote mucus secretion in asthma[22].

In the present work, hypoxia model in human embryonic lung cells was conducted by $CoCl_2$ induction based on the previous research[23]. Our data showed that HIF-1α remarkably upregulated EGFR in hypoxia cell model. Besides, expression levels of IL-13, IL-9, IL-1, and TNF-α were increased, which were similar to pathological changes in COPD patients.

PI3K exerts a crucial role in basic cell metabolism, including vesicle transport, cell degranulation, cell migration, and glucose transport[24]. 740Y-P is an effective PI3K agonist, which reduces cell death by binding to p85 *via* PI3K-dependent AKT phosphorylation[24-26]. Our data demonstrated that 740Y-P remarkably upregulated HIF-1α and inflammatory factors. Rabe et al[27] showed that treatment of 20 μM 740Y-P for 24 h in human melanoma MNT-1 cells significantly reduced the amount of sucrose-induced M6PR-positive vacuoles, which is based on PI3K-dependent AKT phosphorylation. PI3K activation further stimulates MAPK signaling pathway, thereafter regulating HIF-1α expression.

To sum up, our findings suggested that HIF-1α upregulation resulted by hypoxia in COPD patients activates EGFR/PI3K/AKT pathway, which further aggravates inflammatory response.

Conclusions

We found that HIF-1α was overexpressed in COPD, which upregulated expressions of inflammatory factors *via* activating the EGFR/PI3K/

AKT pathway. The activated EGFR/PI3K/AKT pathway induced by pulmonary inflammation further upregulated HIF-1α expression in a feedback loop, thus aggravating COPD pathological changes in a vicious circle.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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