EGFR-INDUCED ERK1/2 AND P38 ACTIVATION OF THE MAPK SIGNALING PATHWAY CAUSES TNF-A PRODUCTION AND RELEASE AND PROMOTES ACUTE LUNG INJURY

MING YE[1#], ZHI WANG[2#], JIAN CHENG[3*]
[1]Department of emergency medicine, Central theater General Hospital, WuHan, HuBei, 430070, China - [2]Depart ment of emergency medicine,Taikang Tongji (Wuhan) Hospital, WuHan, HuBei 430050, China - [3]Department of emergency, Hefei BOE hospital Co, Ltd, Anhui, Hefei 230011, China
[#]These authors contributed equally to this work as co-first authors

ABSTRACT

Objective: *To analyze the activation of ERK1/2 and P38 induced by epidermal growth factor receptor (EGFR) in the MAPK signaling pathway to induce the generation and release of tumor necrosis factor α (TNF-α) and promote the occurrence of acute lung injury.*

Methods: *42 male SPF grade mice were selected, and 10 mice were divided into a normal group and a model group. The model group used lipopolysaccharide (2 mg/kg) to establish the ALI model. A western blot detected EGFR protein expression in the lung tissues of the two groups. The remaining 32 mice were randomly divided into a normal group, a positive control group (EGFR inhibitor erlotinib 100 mg/kg gavage), a model group (lipopolysaccharide 2 mg/kg modeling), and an EGFR inhibitor group (lipopolysaccharide + EGFR inhibitor erlotinib). The HE staining method was used to detect changes in lung tissue structure of ALI mice in each group, and the ELISA method was used to detect serum TNF-α levels in each group, and a western blot was used to detect ERK1/2 and P38 phosphorylation levels in each group.*

Results: *EGFR was expressed in the normal mice and the ALI model mice. The expression of p-EGFR protein in the model group was significantly higher than in the control group, and the p-EGFR/EGFR ratio was significantly higher than the that of the control group. The difference was statistically significant (P<0.05). In the model group, the lung tissue was seriously damaged, and the alveolar structure was destroyed, accompanied by a large amount of inflammatory cell infiltration and erythrocyte exudation. The lung tissue damage in the EGFR inhibitor group was significantly lower, and the normal alveolar structure was visible. The serum TNF-α expressions in the model group and the EGFR inhibitor group were significantly higher than those of the normal group and the positive control group (P<0.05). The expression of serum TNF-α in the EGFR inhibitor group was significantly lower than that in the model group, and the difference was statistically significant (P<0.05). The phosphorylation levels of ERK1/2 and P38 in the model group and the EGFR inhibitor group were significantly higher than those in the normal group (P<0.05). In addition, the phosphorylation levels of ERK1/2 and P38 in the EGFR inhibitor group were significantly lower than that in the model group, and the difference was statistically significant (P<0.05).*

Conclusion: *EGFR participates in the production and release of TNF-α induced by ALI. This mechanism may occur through the activation of ERK1/2 and P38 levels in the MAPK signaling pathway. Thus, the phosphorylation of ERK1/2 and P38 may promote the occurrence of ALI.*

Keywords: *EGFR, MAPK signaling pathway, ERK1/2, P38, TNF-α, acute lung injury.*

DOI: 10.19193/0393-6384_2021_5_452

Received *March 15, 2020;* ***Accepted*** *October 20, 2020*

Introduction

Acute lung injury (ALI) is the manifestation of systemic inflammatory response syndrome in the lungs. It is an acute and progressive exacerbation of respiratory failure caused by various direct or indirect factors. The main pathophysiological features are reduced lung volume, decreased lung compliance, and ventilation/blood flow imbalance. Among these, acute respiratory distress syndrome (ARDS) is the most severe stage of acute lung injury patients[1, 2]. The pathogenesis of ALI is complex and has not yet been fully elucidated. In recent years, relevant data have shown that ALI/ARDS is a different stage in the process of uncontrollable systemic immune and inflammatory response caused by severe injury[3].

Inflammatory response is the most basic self-protection response of the body, but an excessive response or a continuous spatiotemporal state can cause tissue and organ damage. The tumor necrosis factor α(TNF-α) is the earliest inflammatory factor in the process of the ALI inflammatory response, and it can bind to a TNF-α receptor in the lung tissue to damage lysosomes and cause enzyme leakage. In addition, this factor can exert a toxic effect by stimulating endothelial cells and neutrophils to release a large amount of proteases, oxygen free radicals, and other media[4].

Epidermal growth factor receptor (EGFR) is mainly involved in cell proliferation, migration, and differentiation, and it can participate in the pathogenesis of various immune inflammatory reactive diseases under pathological conditions. EGFR chiefly activates downstream pathways by binding to corresponding ligands and causing corresponding changes in cells. Beyond this, the MAPK signaling pathway plays an important role in this process[5]. Some scholars have found that EGFR can affect the production of TNF-α by activating downstream MAPK signaling pathways[6]. Therefore, this study aimed to analyze the mechanism of EGFR-induced activation of ERK1/2 and P38 in the MAPK signaling pathway to cause TNF-α production and release to promote acute lung injury.

Materials and methods

Experimental reagents and instruments

Isopropyl alcohol and chloroform were purchased from Tianjin Beifang Tianyi Chemical Reagent Factory. Glycine and sodium lauryl sulfate were purchased from Sigma Corporation. RIPA lysate and a protein quantitative kit were purchased from Shanghai Biyuntian Company. A BCA protein concentration determination kit was purchased from Thermo Fisher Scientific Co., Ltd. Rabbit anti-mouse ERK1/2, P38 antibodies were purchased from Abcam. Rabbit anti-mouse β-actin antibody was purchased from Background Biosen Biotech Co., Ltd. The centrifuge and pipette were purchased from Eppendorf, Germany.

The electrophoresis apparatus and electrophoresis tank were purchased from Bio-Rad Corporation, USA. A low-temperature refrigerator was purchased from Qingdao Haier Company, and the vortex oscillator was purchased from Beijing Liuyi Biotechnology Co., LTD. Pressure steam sterilizer was purchased from Shanghai ShenAn Medical instrument Factory. PVDF membrane was purchased from Millipore, Inc., USA. The tissue grinder was purchased from IKA, Germany. The microplate reader was purchased from Thermo Fisher Scientific Co., Ltd.

Experimental animal models and grouping

For this study, 42 male SPF grade mice, 6-8 weeks old and weighing 19-22 g, were purchased from the Animal Experimental Center of Huazhong University of Science and Technology. They were subsequently raised in our hospital with an ambient temperature of 22-24 °C, a humidity of 50-60%, and a day–night light rhythm of 12 h: 12 h, drink water and eat freely.

In addition, 10 mice were randomly divided into a normal group and a model group. The model group was anesthetized by intraperitoneal injection of pentobarbital sodium 60 mg/kg, and intratracheal instillation of lipopolysaccharide (2 mg/kg) was used to establish a mouse ALI model. The remaining 32 mice were randomly divided into a normal group, a positive control group, a model group, and an EGFR inhibitor group, with 8 mice in each group. The normal group was given no treatment, and the positive control group was given the EGFR inhibitor erlotinib (100 mg/kg) gavage. The model group was given lipopolysaccharide (2 mg/kg) for modeling, and the EGFR inhibitor group was given lipopolysaccharide (2 mg/kg) for modeling + EGFR inhibitor erlotinib (100 mg/kg) for gavage.

Observation indicators

• Western blot detection of EGFR protein expression in lung tissue of ALI mice involved the following: We collected the lung tissues of mice in each group, checked the protein concentrations with reference to the instructions of the BCA Protein Assay kit, and analyzed the protein concentrations of the resulting samples. After this, we used the SDS-PAGE gel configuration kit to configure a 10% separation gel and a 5% concentration gel, to perform gel electrophoresis, and to transfer to the PVDF membrane. After the electrophoresis was completed, we immersed the PVDF membrane in a 5% skimmed milk powder blocking solution and shook it at room temperature for 1 h at room temperature. After blocking, we added rabbit anti-mouse β-actin antibody (1:1000), rabbit anti-mouse EGFR (1:1000), and rabbit anti-mouse p-EGFR (1:1000) antibody to incubate the PVDF membrane. We incubated it at 4 °C refrigerator overnight, TBST

rinsed the PVDF membrane 3 times, added the corresponding secondary antibody, and incubated at room temperature for 1 h, exposed and developed with ECL chemiluminescence reagent. The latter was stored after development.

• The HE staining method was used to detect changes in the lung tissue structure of ALI mice in each group: Mice were sacrificed 24 hours after administration, the upper left lung was cut with ophthalmic scissors, and the lung tissue was fixed in 4% paraformaldehyde for dehydration, and transparency. Dip wax was used to embed the mice, making them into paraffin specimens. Lung tissue paraffin sections were soaked in xylene for 15 minutes, followed by 100%, 95%, 85%, and 75% alcohol hydration for 5 minutes each, washed with PBS buffer, and stained with hematoxylin. After this, they were stained again with PBS buffer rinse 3 times, and we then used 6% ammonia to return them to blue. Next, we observed whether the nuclear staining was appropriate under the microscope, whether the nuclear structure was clear, and whether there was residual hematoxylin in the cytoplasm. The sections were stained to be transparent with eosin, and a bit of neutral gum was dripped onto the sectioned tissues. Finally, the sections were dried and placed under an optical microscope for observation and image capture.

• The enzyme-linked immunosorbent assay (ELISA) method was used to detect the changes of serum TNF-α levels in each group of mice.

• Western blot was used to detect the ERK1/2 and P38 phosphorylation levels of MAPK signaling pathway in each group of mice. The method steps are the same as those in (1).

Statistical methods

The data in this study were analyzed using the SPSS21.0 software package, and the measurement data were expressed by ($\bar{x}\pm s$), using a t test to compare the data between the two groups, using variance analysis to compare the data between the groups. In addition, $P<0.05$ was regarded as statistically significant.

The western blot detection results showed that EGFR was expressed in normal mice and ALI model mice, in which the p-EGFR protein expression in the model group was significantly higher than that of the control group. In addition, the p-EGFR/EGFR ratio was significantly higher than that of the control group. The difference was statistically significant ($P<0.05$) (see Picture 1).

Results

EGFR expression in lung tissue of ALI mice

The western blot detection results showed that EGFR was expressed in normal mice and ALI model mice, in which the p-EGFR protein expression in the model group was significantly higher than that in the control group. Beyond this, the p-EGFR/EGFR ratio was significantly higher than that of the control group. The difference was statistically significant ($P<0.05$) (see Figure 1).

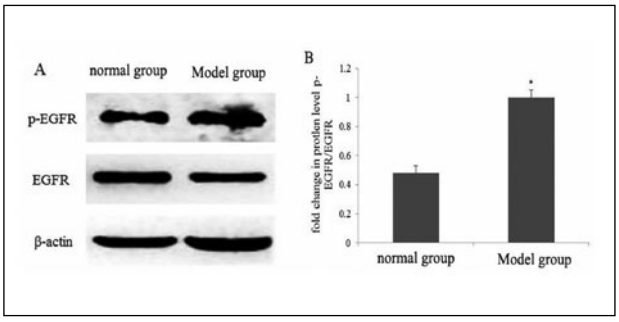

Figure 1: EGFR expression in lung tissue of ALI mice.
*Note: Compared with normal group *P<0.05.*

The effect of EGFR inhibitors on the lung tissue structure of ALI mice

The HE staining results showed that the lung tissue of the normal group of mice displayed a mesh structure, whereas the alveolar structure was normal. The positive control group of mice had normal alveolar structure and were not damaged. There was a large amount of inflammatory cell infiltration and erythrocyte exudation. Furthermore, the lung tissue damage of mice in the EGFR inhibitor group was significantly reduced, and normal alveolar structures were visible (see Figure 2).

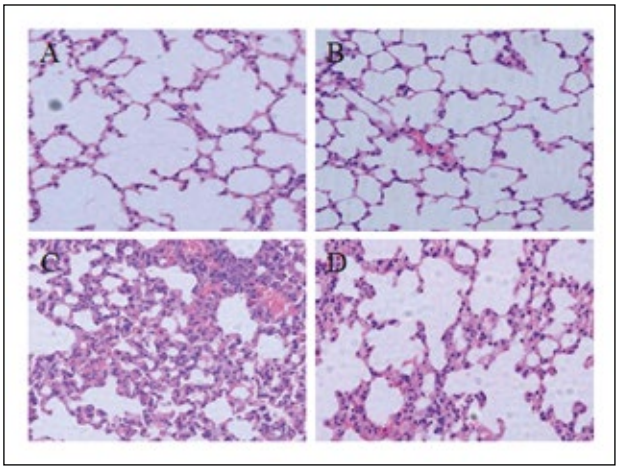

Figure 2: Effect of EGFR inhibitors on lung tissue structure of ALI mice.
A: Normal group; B: Positive control group; C: Model group; D: EGFR inhibitor.

The effect of EGFR inhibitors on serum TNF-α in ALI mice

The results of the ELISA test showed that the expressions of serum TNF-α in the model group and the EGFR inhibitor group were significantly higher than those in the normal group and the positive control group (P<0.05).

The expression of serum TNF-α in the EGFR inhibitor group was significantly lower than that of the model group, and the difference was statistically significant (P<0.05) (see Table 1).

Group	Cases	TNF-α (pg/mL)
Normal group	8	276.33±22.78
Positive control group	8	275.26±25.89
Model group	8	1392.59±77.20*#
EGFR inhibitor group	8	881.58±66.03*#△

Table 1: Effect of EGFR inhibitors on serum TNF-α in ALI mice.
*Note: Compared with the normal group *P<0.05; compared with the positive control group #P<0.05; compared with model group △P<0.05.*

Effect of EGFR inhibitors on ERK1/2 and P38 phosphorylation of MAPK signaling pathway

The phosphorylation levels of ERK1/2 and P38 in the model group and the EGFR inhibitor group were significantly higher than those in the normal group (P<0.05).

In addition, the phosphorylation levels of ERK1/2 and P38 in the EGFR inhibitor group were significantly lower than those in the model group (P<0.05). The difference was statistically significant (see Figure 3 and Table 2).

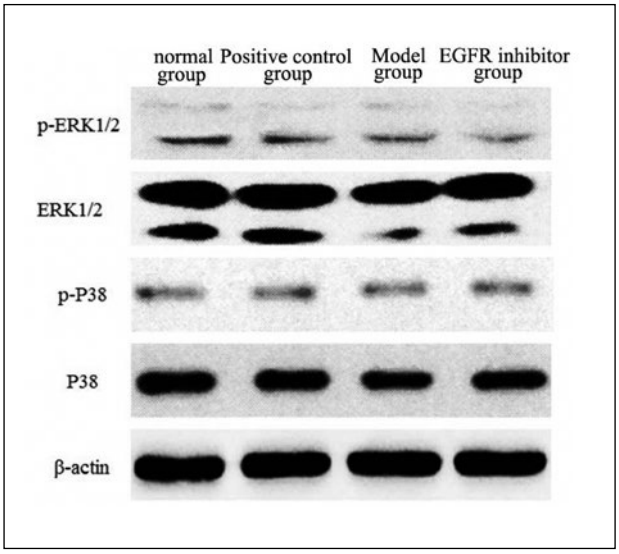

Figure 3: Effect of EGFR inhibitors on ERK1/2 and P38 phosphorylation of MAPK signaling pathway.

Group	Cases	p-ERK1/2/ ERK1/2	p-P38/P38
Normal group	8	0.56±0.05	0.58±0.06
Positive control group	8	0.54±0.04	0.56±0.05
Model group	8	1.76±0.13*#	0.88±0.07*#
EGFR inhibitor group	8	0.98±0.07*#△	0.65±0.08*#△

Table 2: Effect of EGFR inhibitors on ERK1/2 and P38 phosphorylation of MAPK signaling pathway.
*Note: Compared with normal group *P<0.05; compared with positive control group #P<0.05; compared with model group △P<0.05*

Discussion

ALI is a critical and severe disease with a high clinical morbidity, with a complex pathogenesis and high mortality. The onset of ALI is often accompanied by many pathological changes, such as excessive control of pulmonary inflammatory response and reduction of alveolar surfactant. At present, various internal and external factors of the lungs cause the release of a variety of inflammatory mediators, and the participation of a large number of inflammatory cells has become a research hotspot[7]. Exogenous zinc ions can stimulate multiple signaling molecules such as EGFR and mitogen-activated protease of respiratory epithelial cells, but their mechanism of action is still controversial[8, 9]. EGFR displays ligand-mediated tyrosine kinase activity. It is a multifunctional transmembrane glycoprotein that is widely present in epithelial tissues and is an important regulator of cell growth, differentiation, proliferation, and migration. In addition, EGFR can also directly participate in the repair of respiratory epithelial cell membranes, which plays an important role in the development of respiratory tract inflammatory diseases[10]. Studies have shown that high tidal volume mechanical ventilation can induce EGFR phosphorylation to be activated in rat lung tissues, indicating involvement in the occurrence of lung injury caused by mechanical ventilation[10]. There have also been reports that EGFR plays an important regulatory role in airway immunity[11].

In order to investigate its expression changes in ALI, this study used the western blot to detect the expression of EGFR and phosphorylated EGFR. This examination found that EGFR was expressed in normal mice and ALI model mice. In the latter, the p-EGFR protein expression in the model group was significantly higher than that in the control group, and the p-EGFR/EGFR ratio was significantly higher than that in the control group (P<0.05). In addition,

EGFR inhibitors were shown to significantly reduce lung tissue damage in mice, producing a large number of normal alveolar structures. This suggested that the increase of the EGFR level is involved in the occurrence and development of ALI, and inhibition of EGFR expression can significantly reduce the degree of lung tissue damage.

TNF-α is the main cytokine that mediates ALI, and it is mainly secreted by activated monocytes/macrophages, lymphocytes, and so forth. It can induce lung endothelial cell activation, leukocyte migration, capillary leakage, and the like. The accumulation of edema fluid hinders the perfusion of alveolar cells and oxygen exchange, causing ARDS[12, 13]. The synthesis and release of inflammatory mediators such as TNF-α and the production of oxygen free radicals lead to a series of pathophysiological changes that play an important role in the occurrence and development of ALI. In addition, studies have shown that EGFR is a key step in the production of TNF-α by lipopolysaccharide in cardiomyocytes[14]. In this study, by analyzing the serum TNF-α levels of mice in each group, it was found that the expressions of serum TNF-α in the model group and EGFR inhibitor group were significantly higher than that in the normal group and positive control group ($P<0.05$), and serum TNF-α expression was significantly lower than that of the model group ($P<0.05$). This suggested that TNF-α was overexpressed in ALI mice, while EGFR inhibitors could significantly inhibit its overexpression. MAPK is one of the most important signaling molecules regulating the production of TNF-α in sepsis. As important members of the MAPK signaling pathway, ERK1/2 and P38 are closely related to the regulation of inflammation and stress response, and EGFR affects the production and release of TNF-α by activating the downstream MAPK signaling pathway[15]. As a specific inhibitor of EGFR, erlotinib can reversibly inhibit the phosphorylation of EGFR, which has an inhibitory effect on the activation of MAPK and other proteins in its downstream signaling pathway[16].

In this study, This study revealed that the phosphorylation levels of ERK1/2 and P38 in the model group and EGFR inhibitor group were significantly higher than those in the normal group ($P<0.05$). Beyond this, the phosphorylation levels of ERK1/2 and P38 in the EGFR inhibitor group were significantly lower than those of the model group ($P<0.05$), which suggested that EGFR can mediate the synthesis and release of TNF-α by activating the MAPK signaling pathway ERK1/2 and P38 levels.

In summary, EGFR participates in the production and release of TNF-α induced by ALI, and its mechanism may occur through the activation of ERK1/2 and P38 levels of the MAPK signaling pathway. Furthermore, the phosphorylation of ERK1/2 and P38 promote the occurrence of ALI.

References

1) Zimmermann KK, Spassov SG, Strosing KM, Ihle PM, Engelstaedter H, et al. Hydrogen sulfide exerts anti-oxidative and anti-inflammatory effects in acute lung injury. Inflammation. 2018; 41: 249-259.
2) Barranco R, Castiglioni C, Ventura F, Fracasso T. Immunohistochemical expression of P-selectin, SP-A, HSP70, aquaporin 5, and fibronectin in saltwater drowning and freshwater drowning. Int J Legal Med. 2019; 133: 1461-1467.
3) Zhou HM, Ling XY, Ni YJ, Wu C, Zhu ZP. Pre-cardiopulmonary bypass administration of dexmedetomidine decreases cardiac troponin I level following cardiac surgery with sevoflurane postconditioning. J Int Med Res. 2019; 47: 3623-3635.
4) Marchiori LLM, Dias ACM, Gonçalvez AS, Poly-Frederico RC, Doi MY. Association between polymorphism of tumor necrosis factor alpha (TNFα) in the Region-308 G/A with tinnitus in the elderly with a history of occupational noise exposure. Noise Health. 2018; 20: 37-41.
5) Hosseini SA, Zahrooni N, Ahmadzadeh A, Ahmadiangali K, Assarehzadegan MA. The effect of CoQ₁₀ supplementation on quality of life in women with breast cancer undergoing tamoxifen therapy: A double-blind, placebo-controlled, randomized clinical trial. Psychol Res Behav Manag. 2020; 13: 151-159.
6) Fujii K, Watanabe R, Ando T, Kousaka J, Mouri Y, et al. Alterations in three biomarkers (estrogen receptor, progesterone receptor and human epidermal growth factor 2) and the Ki67 index between primary and metastatic breast cancer lesions. Biomed Rep. 2017; 7: 535-542.
7) Flori H, Sapru A, Quasney MW, Gildengorin G, Curley MAQ, et al. A prospective investigation of interleukin-8 levels in pediatric acute respiratory failure and acute respiratory distress syndrome. Crit Care. 2019; 23: 128.
8) Garon EB, Siegfried JM, Stabile LP, Young PA, Marquez-Garban DC, et al. Randomized phase II study of fulvestrant and erlotinib compared with erlotinib alone in patients with advanced or metastatic non-small cell lung cancer. Lung Cancer. 2018; 123: 91-98.
9) El-Azab AS, Abdel-Aziz AA, Bua S, Nocentini A, AlSaif NA, et al. S-substituted 2-mercaptoquinazolin-4(3H)-one and 4-ethylbenzensulfonamides act as potent and selective human carbonic anhydrase IX and XII inhibitors. J Enzyme Inhib Med Chem. 2020; 35: 733-743.

10) Isla D, Massuti B, Lázaro M, de Alda LR, Gordo R, et al. Cost analysis of the management of brain metastases in patients with advanced ALK+ NSCLC: alectinib versus crizotinib. Lung Cancer Manag. 2020; 9: 28.
11) Minutti CM, Drube S, Blair N, Schwartz C, McCrae JC, et al. Epidermal growth factor receptor expression licenses T helper-2 cells to function in a T cell receptor-independent fashion. Immunity. 2017; 47: 710-722.
12) Kia A, McAvoy K, Krishnamurthy K, Trotti D, Pasinelli P. Astrocytes expressing ALS-linked mutant FUS induce motor neuron death through release of tumor necrosis factor-alpha. Glia. 2018; 66: 1016-1033.
13) Jing NS, Li XN. Dihydromyricetin attenuates inflammation through TLR4/NF-kappaB pathway. Open Med. 2019; 14: 2391-5463.
14) Kessler H, Jiang K, Jarvis JN. Using chromatin architecture to understand the genetics and transcriptomics of juvenile idiopathic arthritis. Front Immunol. 2018; 9: 2964.
15) Xu K, Sha Y, Wang S, Chi Q, Liu Y, et al. Effects of Bakuchiol on chondrocyte proliferation via the PI3K-Akt and ERK1/2 pathways mediated by the estrogen receptor for promotion of the regeneration of knee articular cartilage defects. Cell Prolif. 2019; 52: 12666.
16) Landeros RV, Pastore MB, Magness RR. Effects of the catechol and methoxy metabolites of 17β-estradiol on nitric oxide production by ovine uterine artery endothelial cells. Reprod Sci. 2019; 26: 459-468.

Acknowledgement:
This paper was supported by the Fujian Provincial Natural Science Foundation of China (No. 2019J0118) and the Fujian Provincial Medical Innovation Project of China (No. 2016-CX-2).

Corresponding Author:
Jian Cheng
Email: gurx7p@163.com
(China)