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Efficacy of silymarin in treatment of COPD via P47phox signaling pathway

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Abstract

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide. To investigate effect of silymarin on chronic obstructive pulmonary disease (COPD). The serum samples of 20 healthy controls, 20 patients with acute exacerbation of COPD and 20 patients with stable COPD were collected. LPS and smoking were used to induce COPD mouse model. Our results showed that in patients with acute exacerbation of COPD, O2-level release from peripheral blood neutrophils were negatively correlated with forced expiratory volume in the first second (FEV1), FEV1 in predicted value, FEV1/forced vital capacity (FVC), and arterial partial pressure (PaO2). (r=-0.898, -0.878, -874, -0.890, all P<0.01). Compared with that in the control group, the phosphorylation of NADPH oxidase p47phox factor and peripheral blood neutrophil membrane protein in the stable COPD group and the acute exacerbation COPD group were significantly stronger. Silymarin can inhibit the inflammatory response and oxidative stress. In conclusion, silymarin reduces oxidative stress in phagocytic and non-phagocytic cells, thus decreasing the oxidative stress in COPD patients

Keywords: P47phox; silymarin; chronic obstructive pulmonary disease; oxidoreductase.

Practical Application: Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide. Our study showed that silymarin reduces oxidative stress in phagocytic and non-phagocytic cells, thus decreasing the oxidative stress in COPD patients. Our study will provide a potential drugs for the treatment of COPD.

1 Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide (World Health Organization 2014). COPD mainly affects the lung parenchyma and peripheral airways, eventually leading to progressive and irreversible damage of the lung tissues, and even more severe complications, such as gastric ulcer, respiratory failure, pulmonary heart disease and right heart failure, *etc*. COPD is mainly composed of three phenotypes: emphysema, small airway obstruction, and chronic bronchitis (Debbabi et al., 2013; Rennard & Drummond, 2015; Merling et al., 2016). Although the application of reducing smoking, oxygen therapy, bronchodilators, and mediation therapy has greatly improved the efficacy in the treatment of COPD, no radical treatment of COPD has been identified. Therefore, it is of significance to explore the molecular and biological basis of COPD, aiming to identify effective drugs and therapeutic treatment (Bel & Brinke, 2017; Schejtman et al., 2017).

The pathogenesis of COPD mainly attributes to oxidative stress response, airway inflammation, protease-antiprotease imbalance and apoptosis, and autophagy (Barnes, 2013, 2016). Among them, oxidative stress is considered to be the most important link, which can control and induce the remaining three mechanisms (Bernardo et al., 2015). Environmental pollution, smoking and radiation can cause oxidative stress in the body (Thomson, 2018) mainly due to excessive accumulation of free radicals / reactive oxygen species (ROS) and nitrogen oxides (RNS) or antioxidant levels and antioxidants. Enzyme activity is low, resulting in DNA damage in respiratory airway cells causing COPD and other related complications. Consequently, effective interventions for oxidative stress may be a novel target for the treatment of COPD.

Although small molecule antioxidants have been widely applied in the treatment of various types of chronic inflammation including COPD, it is still difficult to obtain high clinical efficacy due to dose, mode of administration and the form of antioxidants, *etc*. Recent studies have confirmed that nicotinamide adenine dinucleotide phosphate oxidases (NADPH oxidases, NOXs) are the main source of intracellular reactive oxygen species (ROS) found in vascular endothelial cells, especially the lung. When NADPH oxidase is activated, it can rapidly produce a large amount of ROS, aggravate vascular and other endothelial tissue damage or inflammation (Wang et al., 2011). NOXs family includes NOX1-5, NUOX1, and NUOX2, of which NOX1 and NOX2 are mainly involved in the production of ROS.

NOXs are enzyme complexes composed of various subunits including the membrane component NOX2, p22phox and the cytosolic fraction p40phox, p47phox, p67phox and the small molecule GTP-binding protein Rac1/2 (Jackman et al., 2009).

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P47phox is an important subunit that regulates the activity of NOXs and plays a crucial role in the production of ROS. When exposed to endogenous factors or external stimuli, p47phox is phosphorylated, its conformational change binds to SH3 at the p67phoxC end, and translocates to the membrane, binding to p22phox in the membrane to complete the complex plasma membrane. Integration, which activates NADH oxidative activity, produces large amounts of ROS (Fan et al., 2009). Gene therapy and immunotherapy has been adopted for p47phox in chronic granulomatous, cardiovascular and renal diseases (Montezano & Touyz, 2013; Li et al., 2017a; Liu et al., 2017; Manichaikul & Nguyen, 2017). Therefore, we hypothesized that NADPH oxidative activity plays an extremely important role in the pathogenesis of COPD (Brandes et al., 2016; Rezende et al., 2018), primarily by alleviating oxidative stress in COPD patients, blocking the p47phox signaling pathway as well as inhibiting phagocytic and non-phagocytic NOXs activity.

Silymarin is a natural flavonoid ligand compound, which is a natural active substance extracted from the dried fruits of milk plant and weed. The main component is silybin, which has a strong antioxidant function and protects liver cells from free radical damage. It has been widely applied in the management of hypolipidemic disorder, diabetes mellitus, liver failure and liver dysfunction (Saller et al., 2001; Rivière et al., 2006; Morishima et al., 2010; Ryu et al., 2015; Stolf et al., 2017). Wu et al. (2011) have found that silymarin has antioxidant and reactive carbonyl-inducing activities, which can down-regulate the expression level of p47phox protein, suggesting that its antioxidant activity may be mediated by p47phox (Wu et al., 2011). Besides, silymarin is capable of protecting PC12 cells from acrylamide-induced neurotoxicity through the Nrf2 signaling pathway[26]. Other studies have demonstrated that silymarin can suppress the effect of intracellular p47phoxS induced by NAFLD in mouse models with nonalcoholic fatty liver (NAFLD) (Li et al., 2017b). Therefore, we speculate that silymarin plays a significant role in the treatment of COPD.

In this investigation, silymarin's target and molecular mechanism were investigated from the clinical, cellular and animal levels, aiming to confirm the important role and application prospect of silymarin in the treatment of COPD in clinical practice.

2 Materials and methods

2.1 Baseline data

The serum samples of 20 healthy controls, 20 patients with acute exacerbation of COPD, and 20 patients with stable COPD were collected and retrospectively analyzed. The number of acute exacerbations and mMRC scores were counted within 1 year. The lung function, blood gas analysis and biochemical conditions were detected. The serum levels of NAPDH, ROS, AECA and VEGF in patients with COPD were detected by ELISA. The expression levels of NAPDH, ROS, p47phox, AECA, VEGF proteins were quantitatively detected by qRT-PCR and Western blot. Besides, the expression level of miRNA-23a was quantitatively measured.

2.2 Effect of silymarin on cytokines and oxidative stress in oxidative injury model of lung epithelial cells in vitro

A model of Beas-2 oxidative damage in lung epithelial cells was successfully established. the expression levels of IL-6, TNF-α, oxidative factor MDA, antioxidants SOD, GSH and VEGF in the serum of Beas-2 oxidative injury in lung epithelial cells were measured and statistically compared before and after silymarin treatment. The expression levels of downstream target genes were detected after p47phox knockout in the Beas-2 oxidative damage model. In addition, the expression levels of downstream NAPDH, ROS, NOX2, AECA, VEGF proteins were quantitatively detected. The expression of p47phox on the membrane of lung epithelial cells was detected by using immunofluorescent staining.

2.3 Effect of silymarin on cytokines and oxidative stress in COPD mouse models in vivo

A COPD mouse model was established by lipopolysaccharide (LPS) + smoking exposure. The expression of NAPDH, ROS, AECA and VEGF in the serum samples was detected. The expression levels of NAPDH, ROS, p47phox, AECA, VEGF proteins were detected by qRT-PCR and Western blot. The COPD mouse models were treated with silymarin and then the expression levels of NAPDH, ROS, AECA, p47phox and VEGF genes and proteins were detected and statistically compared before and after interventions.

2.4 Effect of silymarin on cytokines and oxidative stress in COPD mouse models after p47phox knockout

The mouse models with p47phox knockout were successfully established. Subsequently the expression levels of NAPDH, ROS, AECA and VEGF mRNA and proteins were quantitatively measured by qRT-PCR and Western blot. Then, the established mouse models were treated with silymarin and the expression levels of NAPDH, ROS, p47phox, AECA, VEGF mRNA and proteins were quantitatively detected.

2.5 Establishment of in vitro cell models

The p47phox plasmid, interference plasmid and empty vector plasmid were constructed. The cells in the model group were exposed to H_2O_2 of 200 μmol/L for 30 min and washed twice with cold PBS before incubation with H_2O_2 (Ni & Wang, 2016). The cell proliferation at 24, 48 and 72 h was assessed by MTT assay. The changes in the ROS levels were detected by flow cytometry. The levels of IL-6, TNF-α, MDA, SOD, GSH and VEGF were detected by ELISA before and after silymarin treatment. The expression level of p47phox was detected by immunofluorescent staining. The expression levels of NAPDH, ROS, NOX2, AECA, VEGF proteins were quantitatively measured.

2.6 Establishment of COPD mouse models in vivo

For the establishment of LPS + smoking-induced COPD mouse models, C57BL/6J mice (6 weeks, 20-25 g) were exposed to cigarette: 3R4F reference cigarette 35 mL/time/min, each lasting for 2 s, 8 cigarettes per day, and LPS, 10 μg / 30 μL, which was dissolved in PBS. Silymarin, 50 mg/kg/day, was dissolved

in 0.5% w/v CMC. A dose of 10μg LPS (dissolved in 30μL PBS) was administered in the nasal cavity on days 1 and 14, twice a day and smoking exposure on days 2-28. Compared with the normal control group, the mice in the model group developed weight loss, sparse hair, slow movement and irritated

2.7 BALF inflammation parameters

The alveolar cells of the unilateral lung tissues of the mice were lavaged with 0.6 mL physiological saline for 6 times. The supernatant of the first 3 BALFs was retained after centrifugation.

2.8 Pathological examination of lung tissues

On the 28th day, the mice were sacrificed 24 hours after gavage, and the lung tissues on both sides were taken for pathological section. The remaining lung tissues without lavage were fixed with 4% paraformaldehyde and 4% sucrose solution for 24 h, washed with PBS, embedded in paraffin, routine pathological section, HE staining and Masson staining. Pathological changes of the lung, the small arteries and lung glands were observed under light microscope.

2.9 Silymarin intervention

LPS + smoking COPD mouse models, control group, LPS + smoking COPD mouse models + silymarin group, LPS + smoking COPD mouse models + p47phox interference group, LPS + smoking COPD mouse models + p47phox interference + silymarin group, LPS + smoking COPD mouse models + empty group, p47phox interference group and empty group were established. Silymarin intervention was delivered at a dose of 50 mg/kg/day, once daily

3 Results

3.1 Pulmonary function and blood gas analysis

The level of O_2- in peripheral blood neutrophils in patients with acute exacerbation of COPD was significantly higher than that in the first second forced expiratory volume (FEV1), FEV1 in predicted value, FEV1/forced vital capacity (FVC), and arterial partial pressure (PaO_2). There was a significant negative correlation (r = -0.898, -0.878, -874, -0.890, all $P < 0.01$). The $PaCO_2$ values did not significantly differ among different groups (all $P > 0.05$) (Table 1).

3.2 Neutrophil membrane protein and p47phox factor phosphorylation level

Peripheral blood neutrophil membrane protein and the phosphorylated level of NADPH oxidase p47phox in the stable COPD group and the acute exacerbation COPD group were significantly stronger than those in the normal group (Figure 1). The phosphorylated level of the NADPH oxidase p47phox did not significantly differ among the stable COPD (Figure 2), acute exacerbation of COPD and normal control groups, as illustrated in Figure 3 and 4.

Table 1. O_2- and pulmonary function and blood gas analysis of COPD patients (x ± s), n = 60.

Group	Number of cases	O_2^-(%)	FEV1(L)	FEV1 accounted for the expected value (%)	FEV1\FVC	PaO_2 (mmHg)	$PaCO^2$ (mmHg)
Normal	20	100 ± 2.2	2.74 ± 0.42	87.3 ± 3.4	77.8 ± 3.4	89.3 ± 9.4	38.9 ± 4.1
Stable COPD	20	120 ± 11.9	1.85 ± 0.37	56.9 ± 2.7	65.1 ± 3.8	70.2 ± 5.2	40.2 ± 6.5
Acute exacerbation of COPD	20	170 ± 9.9	1.58 ± 0.34	49.1 ± 2.5	59.3 ± 4.5	65.7 ± 4.8	41.5 ± 8.3

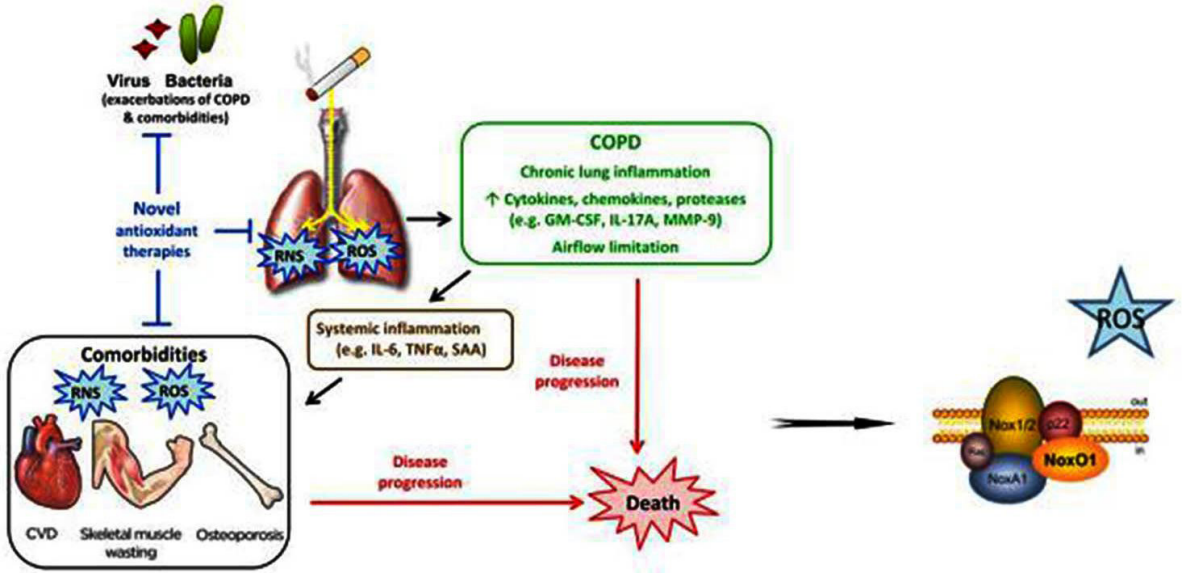

Figure 1. Treatment points of COPD and its complications and molecular mechanism of ROS production[9].

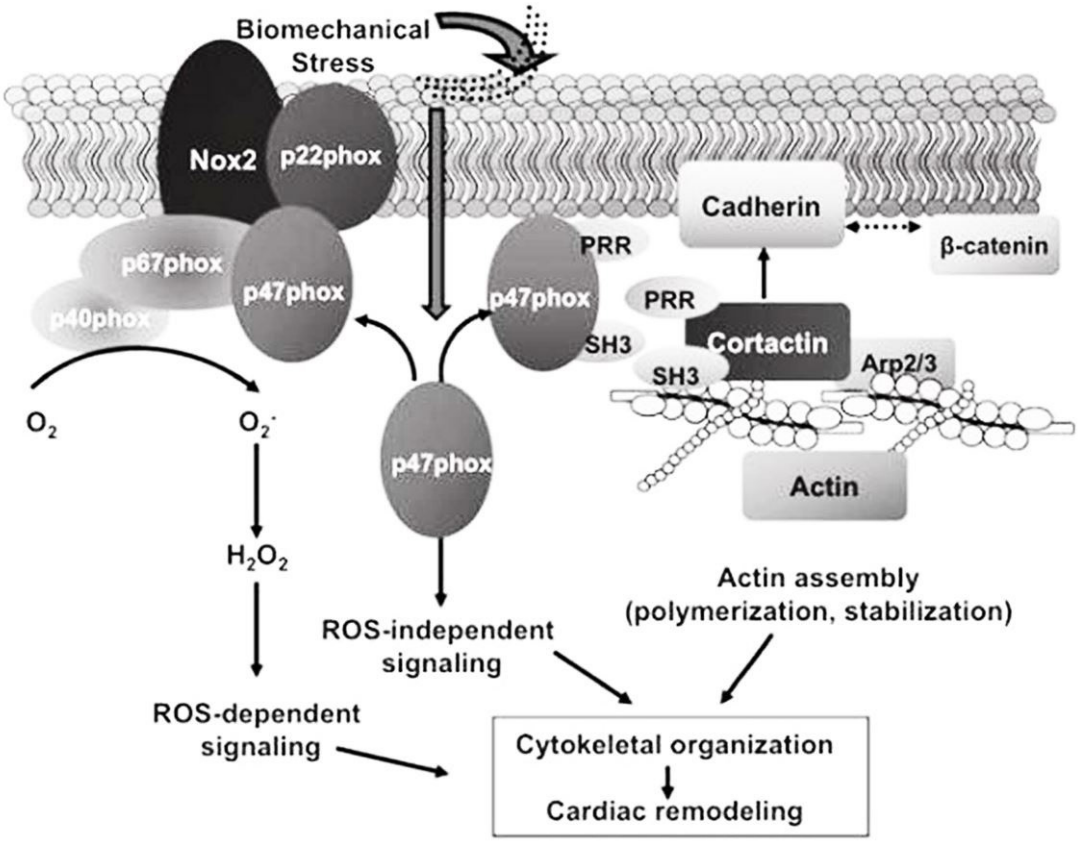

Figure 2. Potential molecular mechanism of ROS oxidation and p47phox[14].

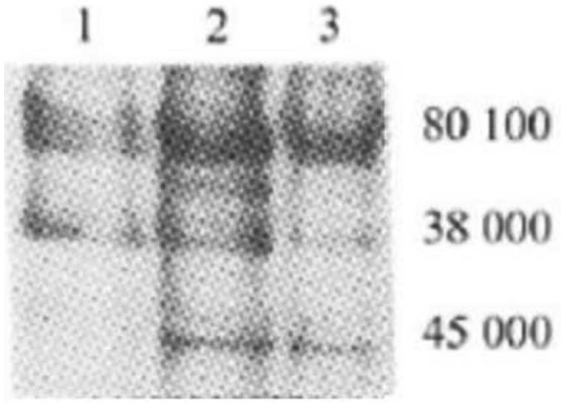

Figure 3. Phosphorylated level of blood neutrophil membrane proteins. Note: 1 normal control; 2 stable COPD patients; 3 acute exacerbation of COPD patients.

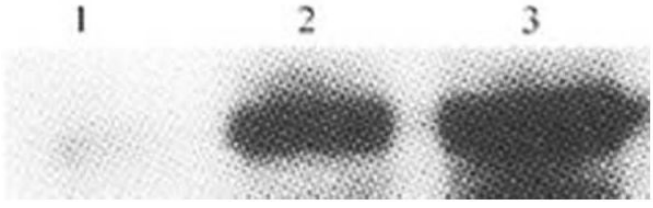

Figure 4. Western blot of eripheral blood NADPH oxidase p47phox. Note: 1 normal control; 2 stable COPD patients; 3 acute exacerbation of COPD patients.

4 Discussion

NADPH oxidase in the phagocytic cells is inactive at rest or produces merely a small amount of physiologically active ROS. When the body is stimulated by inflammatory factors, growth factors, calcium ions (Ca^{2+}), heavy metals, some drugs, it will be activated and generate a large amount of ROS (Wang & Tang, 2009; Casbon et al., 2012). Previous studies have confirmed that in the ischemic state of cardiomyocytes, intracellular ROS are significantly increased, and the expression of p47phox protein is consistent with the increase of reactive oxygen species (Hahn et al., 2011). When human umbilical vein endothelial cells were stimulated with high glucose of 20 mmol/L, the level of reactive oxygen species increased significantly compared with the control group (normal medium culture), and the cytoplasmic component p47phox was highly expressed in the membrane region (Bernard et al., 2014). In animal models where phagocytic NADPH oxidase is pharmacologically inhibited or the p47phox gene is absent, vascular oxidative stress is reduced (Mizrahi et al., 2006; De-Torres et al., 2015).

Oxidation-antioxidative imbalance exists because the oxidation-antioxidative imbalance can lead to protease inactivation, airway epithelial damage, excessive mucus secretion, increased accumulation of neutrophils in the pulmonary microvasculature, and gene expression in proinflammatory mediators (Santos et al., 2021; Simbine et al., 2021; Jouki et al., 2021). One of the important features is the pathogenesis of COPD (Barnes, 1999; Rusznak et al., 2000; MacNee, 2011).

Phosphorylation of p47-PHOX is the key to activation of ADPH oxidase. Our results showed that the amount of O^{2-} released from peripheral blood neutrophils in the stable COPD group and the acute exacerbation COPD group was significantly higher than that in the normal group, the stable phase COPD group and the acute exacerbation COPD group. NADPH oxidase p47-PHOX factor phosphorylation was significantly stronger than the normal group. The above studies suggest that COPD can activate NADPH oxidase to produce O^{2-}, and can also activate the tyrosine information channel and phosphorylate the important cytosolic component p47-PHOX of NADPH oxidase. The results of this study showed that the release of O^{2-} in peripheral blood neutrophils from patients with acute exacerbation of COPD was significantly negatively correlated with FEV1, FEV, FEV1/FVC, PaO_2, suggesting that peripheral blood neutrophil release in patients with COPD. The level of O^{2-} has a certain relationship with the progression of COPD.

P47phox is a subunit of phagocytic NADPH oxidase, which can regulate the activity of phagocytic NADPH oxidase. It plays a crucial role in the production of NADPH oxidase-derived reactive oxygen species. However, the role of p47phox in activating the NADPH oxidase inhibitors remains largely unknown.

Conflict of Interest

The author declare that they have no conflict of interest.

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