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Baicalin Targeting ALOX15 Inhibits Ferroptosis of Lung Epithelial Cells and Alleviates Chronic Obstructive Pulmonary Disease

Kezhen Chen

Heweiskent School, 619th Yonghe West Road, Zhenhai, Ningbo, China

kk1122558@foxmail.com

Abstract. Chronic obstructive pulmonary disease (COPD) is a progressive lung disorder characterized by chronic inflammation and irreversible airflow limitation. Ferroptosis has been implicated in the pathogenesis of COPD. In this study, we investigated the potential of baicalin, a natural compound with anti-inflammatory properties, to target ALOX15 and inhibit ferroptosis in lung epithelial cells, thereby alleviating COPD. Overexpression ALOX15 of BEAS-2B cells was induced by RSL-3, an activator of ferroptosis, to mimic the inflammatory environment in COPD. The effects of baicalin on ferroptosis were assessed by measuring cell viability, lipid peroxidation, lipid ROS, and ferrous ion (Fe2+). The expression of OH-1, ALOX15, and GPX4 was evaluated using Western blotting. Baicalin treatment significantly increased cell viability in RSL-3 induced epithelial cells. Our findings also demonstrate that baicalin significantly increases cell viability and reduces lipid ROS and Fe2+ levels, thereby inhibiting ferroptosis and improving cell viability, irrespective of ALOX15 expression Furthermore, baicalin upregulated the expression of GPX4, a key regulator of ferroptosis. Importantly, baicalin treatment inhibited ALOX15 expression, a critical enzyme involved in lipid peroxidation. Our study demonstrates that baicalin targeting ALOX15 inhibits ferroptosis of lung epithelial cells and alleviates COPD. These findings provide a basis for further research and development of baicalin-based therapies for COPD patients, with the potential to improve their clinical outcomes and quality of life.

Keywords: COPD, baicalin, ALOX15, ferroptosis, ROS.

1. Introduction

Chronic obstructive pulmonary disease (COPD), is a type of respiratory disorder, which can be characterized by persistent cough, chest distress, and breathing difficulties [1]. The risk for COPD is various and can be briefly divided into 4 dimensions -- Noxious exposures, including smoking exposures, and the amount of air pollutants from indoor and outdoor spaces. Social-economic factors, including access to local healthcare, and racial and ethnic disparities [1]. General surrounding environments, including climate conditions [2]. Habit influences, including early respiratory infections and growth trajectories [3]. Recently, COPD has become the third deadly disease in China, which causes over 1 million people deaths annually, with an average of 2.5 people dying per minute. According to the survey result of the "Chinese Adult Lung Health Study", there are approximately 100 million COPD patients in China, and the prevalence rate of people over 60 years old has exceeded 27% [4]. The common treatment for COPD involves inhalation therapy, which includes Long-Acting Muscarinic Antagonists

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(LAMA) and Long-Acting Beta-Agonists (LABA) drugs, such as bronchodilators and glucocorticoids. The primary function of these drugs is to dilate the trachea and relieve airway spasms [5]. Additionally, LAMA and LABA can be utilized individually in treatments. However, in cases of severe symptoms, these two drugs are often combined to treat patients effectively. Moreover, LAMA and LABA treatments can also solve the comorbidity with COPD, such as heart disease, osteoporosis, and pulmonary hypertension. Besides, patients can improve their body fitness by quitting smoking, changing their diet, and doing exercises. However, LAMA and LABA have potential disadvantages in clinical use. Common adverse effects of LAMAs include xerostomia, constipation, and urinary retention. Additionally, LAMAs may cause blurred vision and tachycardia in some patients. There is also an elevated risk of acute narrow-angle glaucoma, particularly in individuals with preexisting ocular conditions. In the treatment of asthma, the use of LABAs in isolation (without the concomitant use of inhaled corticosteroids) may heighten the risk of asthma-related mortality and severe exacerbations. Moreover, there is a potential for developing tolerance to LABAs over time, which could diminish their therapeutic efficacy.

The pathogenesis of COPD is highly intricate, involving a multitude of cellular and molecular mechanisms. Key contributing factors include damage to small airway epithelial cells, loss of secretory and ciliated cells, and the metaplasia of squamous and goblet cells. These alterations disrupt normal lung function and play a pivotal role in the development and progression of COPD [1, 6]. Recent research has found that ferroptosis also plays a significant role in the progression of COPD PMID: 36687445. Multiple studies are considering ferroptosis as a new way of cell death, which differs from apoptosis, necrocytosis, and autophagy to elicit COPD [7]. The essence of ferroptosis is the lipid peroxide accumulation caused by the depletion and activity declination of glutathione peroxidase (GPX4), which causes lipid oxides not to be metabolized through the GPX4 catalyzed glutathione reductase reaction, [8]. Ferrostatin-1 is an inhibitor of ferroptosis that prevents the accumulation of lipid peroxides, thereby protecting cells from undergoing ferroptosis. In contrast, RSL-3 (Ras Selective Lethal 3) is a small molecule that induces ferroptosis by inhibiting GPX4 thereby protecting cells from undergoing ferroptosis [9]. There are 2 main mechanisms that cause COPD of ferroptosis. One of the mechanisms involved in Fe^{2+} catalyze, which causes the damage of cell membrane, and the other mechanism is ferroptosis. Due to the disadvantages of LAMA and LABA described above, ferroptosis can be a new target in researching therapeutic agents of COPD. The information on ferroptosis and GPX4 in curing COPD is scarce, these 2 elements have an ultimate space to discover.

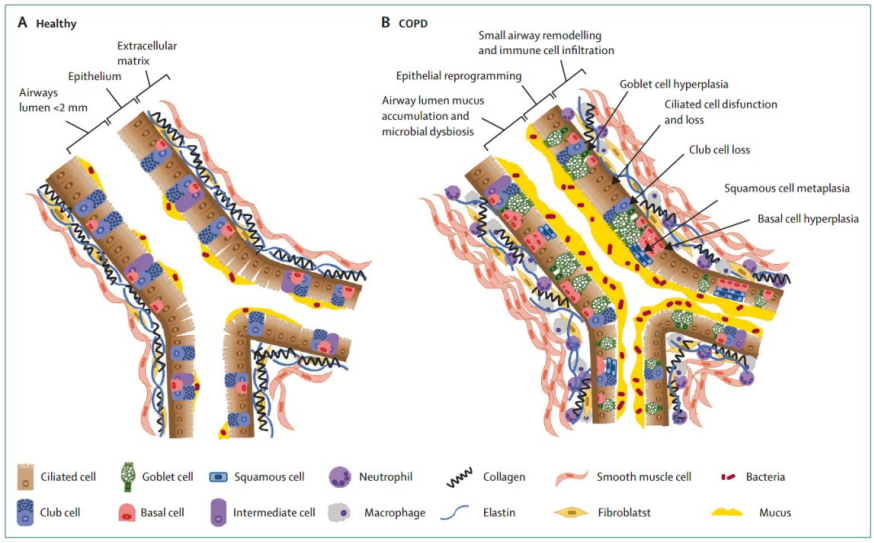

Figure 1. Small airway altering cell interactions in COPD [1]

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Lipoxygenases (LOX) are a group of enzymes that peroxidize lipids and play roles in various physiological processes as well as in the development of inflammatory, hyperproliferative, and neurodegenerative diseases [10]. The human genome contains six LOX genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOX5, and ALOXE3), each encoding a different isoform. One such isoform, arachidonic acid 15-lipoxygenase-1 (ALOX15), was first identified in rabbits in 1974, noted for its ability to oxidize membrane phospholipids during the breakdown of mitochondria in immature red blood cells. Over the subsequent decades, ALOX15 has been extensively studied, and its biological functions have been explored in numerous cellular in vitro systems and various diseases. The traditional understanding of the arachidonic acid cascade suggests that eicosanoid-synthesizing enzymes, such as ALOX5, perform their biological functions by generating bioactive signaling molecules like prostaglandins, leukotrienes, and lipoxins. This mechanism likely applies to ALOX15 as well, given the various biological activities reported for ALOX15 pathway products [11]. However, there are at least two additional mechanisms through which ALOX15 orthologs may exert their effects. One is ALOX15 orthologs can oxidize polyunsaturated fatty acids within ester lipids, even when they are part of biomembranes and lipoproteins, which suggests a role for ALOX15 in the restructuring of cellular organelles and the metabolism of lipoproteins [12]. The other is that, as an intracellular lipid peroxidizing enzyme, ALOX15 acts as a pro-oxidant, influencing the cellular redox balance. As a result, ALOX15's catalytic activity can regulate gene expression, thereby altering the functional phenotype of cells [13]. In the journal Nature, ALOX15 was identified as the primary mediator of phospholipid peroxidation caused by ischemia through multi-omics analysis. The study further investigated the involvement of ALOX15 in cardiac ferroptosis mediated by myocardial ischemia injury [14]. Zhang, Huang, Ding, Qin, Wang and Luo [15] found that ALOX15 is associated with ferroptosis in asthma, with its expression up-regulated in HDM/LPS-induced mice and cells. Silencing ALOX15 significantly reduced HDM/LPS-induced ferroptosis in 16HBE cells. Consequently, ALOX15 involved in phospholipid peroxidation is intricately linked with the occurrence of ferroptosis.

Scutellaria baicalensis Georgi, also known as camellia root or golden tea, is derived from the dry root of Scutellaria baicalensis Georgi. It is traditionally used for its therapeutic effects in clearing heat, drying dampness, purging fire, detoxifying, stopping bleeding, and placating fetuses in China [16]. Modern pharmacological research has also highlighted Baicalin, a compound derived from *Scutellaria baicalensis*, for its diverse pharmacological properties, including antibacterial, antiviral, liver protection, anti-tumor, anti-inflammatory, antioxidant, and lipid and glucose-lowering effects [17]. Specifically, Baicalin has shown therapeutic potential in chronic obstructive pulmonary disease (COPD) [18]. Studies indicate that Baicalin alleviates COPD by modulating the JNK pathway [19]. Additionally, research by Zhang, Liu, Jiang, Wu, Qi, Mohammadtursun, Li, Li, Zhang, Sun and Dong [20] suggests that Baicalin regulates the HDAC2/NF-κB/PAI-1 signaling pathway to mitigate airway inflammation induced by cigarette smoke in rats. These findings suggest that Baicalin may inhibit the onset of COPD. In addition, recent progress revealed that Baicalin was found to increase cell viability and inhibit ferroptosis in rat pheochromocytoma PC12 cells treated with hemin, elastin, and RSL3. Importantly, baicalin also exhibited anti-ferroptotic effects on primary cortical neurons (PCN) [21]. In addition, Scutellarein, a tetrahydroxy flavonoid, which found in many scutellaria plants, alleviates chronic obstructive pulmonary disease by chelating iron and interacting with 15-lipoxygenase, thereby inhibiting ferroptosis [22].

In this study, ALOX15 was targeted by using baicalin to investigate the disease mechanism of COPD. To be specific, in the experiment, BEAS-2B, a type of bronchial epithelial cell of humans, is used to construct the model to study the function of an inhibitor called baicalin, which can disable the ferroptosis pathway, which ultimately causes COPD.

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2. Method

2.1. Cell culture

BEAS-2B cells were purchased from Beyotime (C6106). BEAS-2B cells were cultured in a 37℃ incubator with 5% CO_2. They were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) (10099141C, Gibco). To thaw BEAS-2B cells from liquid nitrogen, immediately transfer the vial to a 37℃ water bath until completely thawed. Transfer the cells into a 15 mL centrifuge tube and add 10 mL of DMEM/F12 medium, gently mix. Centrifuge the cells for 5 minutes at 1000 rpm. Carefully aspirate the supernatant and add 5 mL of DMEM/F12 medium, mix gently. Transfer the cells to a 10 cm culture dish and add 5 ml of DMEM/F12 medium, culture until reaching 70%-80% confluence. When BEAS-2B cells reach 70%-80% confluence, they can be subcultured. Use a 0.25% trypsin-EDTA (25200072, Gibco) mixture to detach the cells from the culture dish, then add DMEM/F12 medium for further culture. When BEAS-2B cells reach 80%-90% confluence, they can be passaged. Detach the cells and transfer them into a new culture dish for continued culture.

2.2. Vector construction

For the construction of ALOX15-overexpressing BEAS-2B, ALOX15 was cloned into the pCDH-CMV vector. Following the instructions provided by the ClonExpress MultiS One Step Cloning Kit (C113-01, Vazyme), primer pairs were designed. The pCDH-CMV vector was then linearized using the restriction endonucleases EcoRI (ER0271, ThermoFisher Scientific) and BamHI (ER0055, ThermoFisher Scientific). The PCR products were combined with the linearized pCDH-CMV to complete the cloning process. The recombinant pCDH-CMV-ALOX15 or plasmids were then transformed into DH5α bacteria, followed by plasmid extraction for subsequent use. For the construction of ALOX15-overexpressing BEAS-2B, ALOX15 was cloned into the pCDH-CMV vector. Following the instructions provided by the ClonExpress MultiS One Step Cloning Kit (C113-01, Vazyme), primer pairs were designed. The pCDH-CMV vector was then linearized using the restriction endonucleases EcoRI (ER0271, ThermoFisher Scientific) and BamHI (ER0055, ThermoFisher Scientific). The PCR products were combined with the linearized pCDH-CMV to complete the cloning process. The recombinant pCDH-CMV-ALOX15 or plasmids were then transformed into DH5α bacteria, followed by plasmid extraction for subsequent use.

2.3. Cell transfection

One day before transfection, seed approximately 200,000 to 700,000 cells per well (the exact number of cells depends on cell type, size, and growth rate) into the six-well plate. The goal is to achieve a cell density of approximately 70-90% the next day. Before proceeding with the following transfection steps, replace the medium in each well of the six-well plate with 2 ml of fresh culture medium (containing serum but without antibiotics). For each well of the six-well plate to be transfected, take two clean, sterile centrifuge tubes. Add 125 μl of antibiotic- and serum-free DMEM or Opti-MEM® Medium (Gibco) to each tube. In one tube, add 2.5 μg of DNA and gently mix by pipetting. In the other tube, add 5 μl of Lipo6000™ transfection reagent (Beyotine) and gently mix by pipetting. After allowing the mixtures to sit at room temperature for 5 minutes, gently add the DNA-containing medium to the medium containing Lipo6000™ transfection reagent. Gently invert the tube or mix by pipetting, and let it sit at room temperature for 5 minutes. Add 250 μL of the Lipo6000™ transfection reagent-DNA mixture to each well of the six-well plate and evenly distribute it across the entire well. Gently mix afterward. Replace the medium with fresh complete culture medium 4 hours after transfection. After continuing the culture for approximately 24 hours, assess the transfection efficiency using Western Blot.

2.4. Experimental treatment of cells

Seed BEAS-2B cells (human normal lung epithelial cells) into six-well plates and incubate overnight. Transfect the cells with human ALOX15 plasmid or vector using Lipo6000 reagent. After 6 hours, replace the medium. 24 hours post-transfection, treat the cells with baicalin (5 μM) or Ferrostatin-1 (5

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μM) for 1 hour, followed by RSL-3 (1 μM) treatment for 8 hours. Collect the cells and stain them with Liperfluo (1 μM) and FerroOrange (1 μM), incubating in serum-free RPMI 1640 medium at room temperature in the dark for 30 minutes. Wash and resuspend the cells in HBSS buffer, and analyze using a flow cytometer with excitation wavelengths of 488 nm and 560 nm.

2.5. Cell counting kit-8 assay

For cytotoxicity assays, add 100 μL of 5000 cells per well (the specific number of cells per well should be determined based on factors such as cell size and cell proliferation rate). According to experimental needs, cultivate and provide 0-10 μL of the specific drug stimulus. Add 10 μL of CCK-8 solution to each well. If the initial culture volume is 200 μL, add 20 μL of CCK-8 solution; adjust accordingly for other volumes. Use wells with the appropriate amount of cell culture medium and CCK-8 solution but without cells as blank controls. If there is concern that the drug used may interfere with the assay, set up wells with the appropriate amounts of cell culture medium, drug, and CCK-8 solution but without cells as blank controls. Continue incubation in the cell incubator for 0.5-4 hours; typically, 1 hour is sufficient for most cases. The duration depends on the cell type and cell density, among other experimental conditions. In preliminary experiments, measure absorbance at 0.5, 1, 2, and 4 hours using a microplate reader, and select the time point with the most suitable absorbance range for subsequent experiments. Measure the absorbance at 450 nm. If a 450 nm filter is not available, use a filter in the range of 420-480 nm. A wavelength greater than 600 nm, such as 650 nm, can be used as a reference wavelength for dual-wavelength measurement. Seed varying numbers of BEAS-2B cells (100 μL culture medium per well) into a 96-well plate. After allowing cells to adhere properly, add 10 μL of CCK-8 solution to each well, incubate for 2 hours, and then measure the absorbance at 450 nm. Refer to Figure 2 for a sample detection effect. The detection results are for reference only; actual data may vary depending on the detection instrument used.

2.6. The LDH level detection

Seed an appropriate number of cells into a 96-well cell culture plate based on cell size and growth rate, ensuring that the cell density does not exceed 80-90% confluency at the time of detection. Aspirate the culture medium and wash the cells once with PBS. Replace with fresh culture medium (recommended: low-serum medium containing 1% serum or appropriate serum-free medium). Divide the wells into the following groups: wells with no cells (background blank control), control cells without drug treatment (sample control), cells without drug treatment for subsequent lysis (sample maximum enzyme activity control), and drug-treated cells (drug-treated sample wells), and label accordingly. Apply appropriate drug treatments as needed, such as adding 0-10 μL of specific drug stimulus, and set different concentrations and treatment times. Add an appropriate drug solvent to the control wells if needed. Continue with routine culture. One hour before the scheduled detection time, remove the cell culture plate from the incubator. Add 10% of the original culture volume of the LDH release reagent provided by the kit to the "sample maximum enzyme activity control wells." Mix thoroughly by pipetting up and down several times, then continue incubation in the cell incubator. At the designated time, centrifuge the cell culture plate at 400 g for 5 minutes using a microplate centrifuge. Transfer 120 μL of the supernatant from each well to the corresponding wells of a new 96-well plate, and proceed with sample measurement.

2.7. Western blot

After the BEAS-2B cells were treated as above mentioned, total cell protein was extracted using cell lysis buffer containing phenylmethylsulfonyl fluoride (PMSF, Sigma), protease inhibitor cocktail (Amresco, USA), and phosphostop (Roche Diagnostics, USA) as recommended by the protocol. A total of 30 μg of protein lysates was separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (80 V, 120 min; Beyotime) and transferred to polyvinylidene difluoride membranes (250 mA × 60 min, Millipore, MA, USA). After blocking with 5% milk, membranes were incubated overnight at 4°C with the following primary antibodies: anti-GPX4, anti-ALOX15, anti-HO-1, and anti-GAPDH (1:1000

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dilution), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) at room temperature for 1 h. Signals were detected using enhanced chemiluminescence (KPL, MD, USA). GAPDH was used as the loading control. Western blot was repeated three times for each cell batch.

2.8. The detection of Fe^{2+}

In a tube containing 24 μg of FerroOrange, add 35 μL of DMSO, and mix thoroughly using a pipette to prepare a 1 mmol/L FerroOrange solution. Dilute the 1 mmol/L FerroOrange solution with HBSS to prepare a 1 μmol/L FerroOrange working solution. Seed BEAS-2B cells into a fluorescence culture dish and incubate with a treatment vector or drug overnight at 37°C in a 5% CO2 incubator. Discard the supernatant and wash the cells 3 times with HBSS or serum-free medium. Replace with medium containing the drug and incubate at 37°C in a 5% CO2 incubator. Optimize the incubation time based on the characteristics of the drug. Remove the medium and wash the cells 3 times with HBSS or serum-free medium. Add the 1 μmol/L FerroOrange working solution and incubate at 37°C in a 5% CO2 incubator for 30 minutes. No washing is needed; proceed directly to observation after incubation. Observe the cells under a fluorescence microscope.

2.9. Statistical Analysis

The obtained results were subjected to statistical analysis using GraphPad Prism 6.02 software. Descriptive statistics were used to summarize the data, and the results were presented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was performed to assess the differences between groups. The statistical significance level was set at $p < 0.05$.

3. Results

3.1. Optimization of baicalin concentration for effective inhibition of ferroptosis in vitro

To determine the optimal concentration of baicalin for inhibiting ferroptosis, an LDH Release assay was performed. RSL-3 is an inhibitor of GPX4 and an activator of ferroptosis. It decreases the expression of GPX4, thereby inducing ferroptosis to establish a research model. The chemical structure of baicalin is illustrated in Figure 2A. Cells were treated with varying concentrations of baicalin, and the results showed a dose-dependent decrease in LDH release, indicating reduced cell damage and ferroptosis (Figure 2B). The black bar represents the untreated control group, which exhibited no significant differences in LDH release across different groups, confirming the baseline levels. The grey bars represent cells treated with RSL-3 and the blank bars represent the control group. Cells treated with Ferrostatin-1 (5 μM), a known ferroptosis inhibitor, were used as a positive control. The data clearly demonstrate that 5 μM baicalin significantly inhibited ferroptosis induced by RSL-3, as evidenced by the reduced LDH release. This concentration of baicalin was thus identified as optimal and was selected for use in subsequent investigations. These findings highlight the potential of baicalin as an effective agent in mitigating ferroptosis, providing a strong foundation for further studies on its therapeutic applications.

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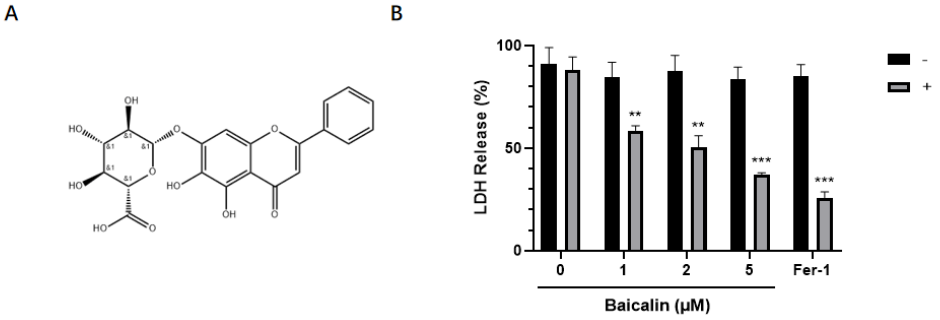

Figure 2. Baicalin inhibits the LDH release of *ferroptosis* in BEAS-2B cells. A) Chemical structure of Baicalin. B) RSL-3 induced *ferroptosis* and then baicalin treatment with different concentrations or ferrostatin-1 (*ferroptosis* inhibitor)) treated for 16 h. Medium was collected and LDH content was detected. **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.2. Baicalin increases cell viability of RSL-3-induced BEAS-2B cells.

In order to verify whether the potential target gene of baicalin is ALOX15, we assessed cell viability following treatment with 5 μM baicalin, and a CCK-8 assay was performed. Two types of BEAS-2B cells were used: vector control (Vector) and wild-type over-expressed ALOX15 (OE-ALOX15). The expression of ALOX15 in these cells was confirmed by Western blot analysis. A distinct 75 kDa band was observed in the BEAS-2B (OE-ALOX15) cells, confirming the expression of the ALOX15 protein (Figure 3A). In contrast, no band was detected at the 75 kDa position in the Vector cells, indicating that the ALOX15 gene had been completely knocked out. The histogram of the CCK-8 assay results showed that cell viability was slightly increased in OE-ALOX15 cells compared to Vector cells, but the difference was not statistically significant. Interestingly, after treatment with baicalin (5 μM), an improvement in cell viability was observed in both groups (Figure 3B). This suggests that baicalin may have a protective effect on cell viability, regardless of ALOX15 expression. The results indicate that while ALOX15 may not significantly impact cell viability under basal conditions, baicalin enhances cell survival in both OE-ALOX15 and Vector cells, potentially offering a therapeutic benefit by supporting cell health.

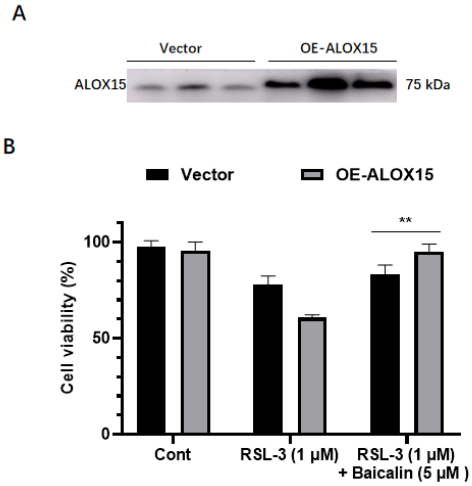

Figure 3. The effect of baicalin treatment on cell viability. A) Western blot to verify the ALOX15 expression in Vector and OE-ALOX15 group. B) RSL-3 induced *ferroptosis* and then baicalin treatment with different concentrations or ferrostatin-1 (*ferroptosis* inhibitor)) treated for 16 h then cell viability was detected. **P < 0.01, ***P < 0.001, ****P < 0.0001.

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3.3. Baicalin attenuates lactate dehydrogenase (LDH) in RsL-3-induced BEAS-2B cells.

To assess LDH levels, WST-8 was employed as a probe to detect LDH. The study involved two types of BEAS-2B cells: Vector cells and OE-ALOX15 cells. The resulting histogram indicated that there was a slight improvement in LDH release between the Vector and OE-ALOX15 cells treated with RSL-3 (1 μM), suggesting that OE-ALOX15 alone does not significantly influence LDH. However, upon treatment with baicalin (5 μM), a significant decrease of LDH was observed in the BEAS-2B (OE-ALOX15) cells, as indicated by the statistical analysis (Figure 4). The data suggest that while OE-ALOX15 may not drastically alter LDH release under normal conditions, its presence is crucial in mediating the protective effects of baicalin against lipid peroxidation, thereby contributing to the inhibition of ferroptosis.

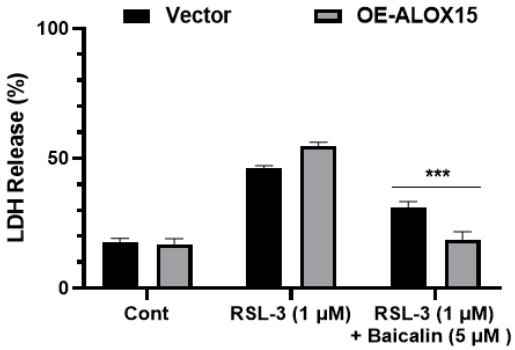

Figure 4. Baicalin inhibits the LDH release of *ferroptosis* in BEAS-2B cells. RSL-3 induced *ferroptosis* and then baicalin treatment with different concentrations for 16 h then LDH release was detected. Medium was collected and LDH content was detected.

P < 0.01,*P < 0.001,****P < 0.0001

3.4. Baicalin inhibits lipid reactive oxygen species (ROS) in RsL-3 induced BEAS-2B cells.

To assess reactive oxygen species (ROS) levels, Liperfluo fluorescence was employed as a probe to detect lipid ROS. The study involved two types of BEAS-2B cells: Vector cells and OE-ALOX15 cells. The resulting histogram indicated that there was little difference in ROS levels between the Vector and OE-ALOX15 cells under basal conditions, suggesting that ALOX15 alone does not significantly influence ROS production in these cells. However, upon treatment with baicalin (5 μM), a noticeable decrease of approximately 10% in ROS levels was observed in the BEAS-2B (OE-ALOX15) cells, as indicated by the statistical analysis (Figure 5A). This reduction in ROS levels following baicalin treatment highlights the potential role of ALOX15 in modulating oxidative stress and inhibiting ferroptosis. The data suggest that while ALOX15 may not drastically alter ROS levels under normal conditions, its presence is crucial in mediating the protective effects of baicalin against lipid peroxidation, thereby contributing to the inhibition of ferroptosis. This finding provides valuable insight into the molecular mechanisms by which baicalin exerts its antioxidative effects, particularly in the context of ferroptosis regulation.

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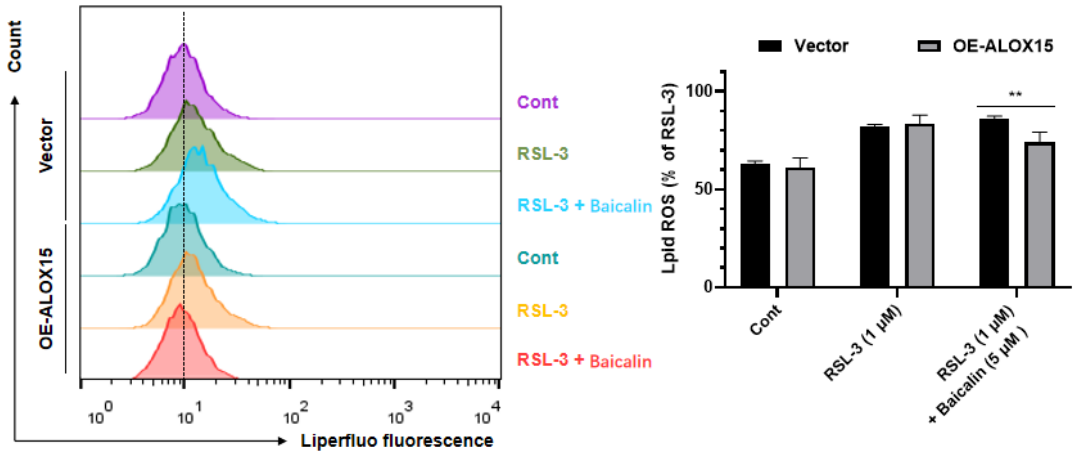

Figure 5. Baicalin inhibits the lipid reactive oxygen species (ROS) of *ferroptosis* in BEAS-2B cells. A) Detect the ROS level using a flow cytometer. B) RSL-3 induced *ferroptosis* and then baicalin treatment with different concentrations for 16 h then ROS was detected. **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.5. Measurement of ferrous ion (Fe^{2+})

To assess the Fe levels in cells, FerroOrange fluorescence was utilized as a probe, offering a reliable method for detecting intracellular Fe. In this experiment, we used two types of BEAS-2B cells: Vector cells and OE-ALOX15 cells. Flow cytometry analysis (FACS) provided a clear depiction of the Fe levels under different treatment conditions. The histogram from the FACS results illustrated a significant decrease ($p < 0.05$) in Fe levels in BEAS-2B (OE-ALOX15) cells following treatment with baicalin (Figure 6). This indicates that baicalin effectively reduces Fe accumulation in the presence of ALOX15. In contrast, cells treated solely with RSL-3, a known ferroptosis inducer, exhibited no significant change in Fe production, maintaining similar levels as untreated controls (Figure 6). These findings suggest that ALOX15 plays a crucial role in the regulation of ferroptosis, as its presence appears to facilitate the protective effects of baicalin, thereby inhibiting ferroptosis. This study underscores the potential therapeutic value of baicalin in modulating ferroptosis by targeting ALOX15-related pathways, offering new insights into the molecular mechanisms underlying iron homeostasis and cell death regulation.

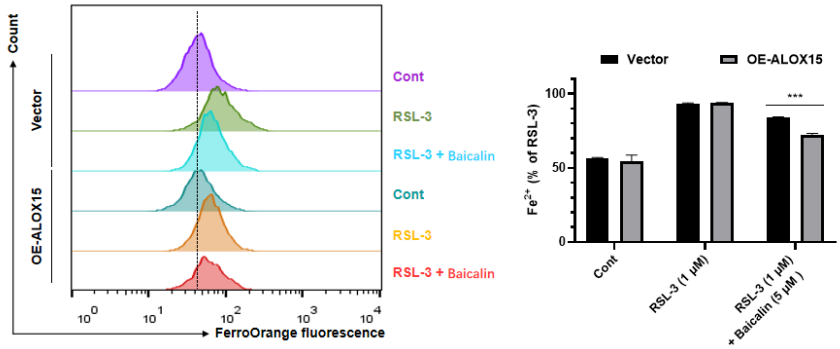

Figure 6. The Fe^{2+} level detection of *ferroptosis* in BEAS-2B cells. A) FACS to detect the Fe^{2+} level. B) RSL-3 induced *ferroptosis* and then baicalin treatment with different concentrations for 16 h then Fe^{2+} was detected using FerroOrange. **P < 0.01,***P < 0.001,****P < 0.0001.

3.6. Baicalin treatment modulates HO-1 and GPX4 expression.

HO-1 regulated ferroptosis, which is controlled by the Nrf2-SLC7A11-HO-1 hierarchy, through which ferrous ion accumulation and lethal oxidative stress cause cell death. GPX4 is closely associated with ferroptosis and functions as the primary inhibitor of this process. Together, GPX4 and ferroptosis contribute to the pathophysiology of several diseases, including sepsis, nervous system diseases,

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ischemia reperfusion injury, cardiovascular diseases, and cancer. Further, we used Western blot to investigate the HO-1 and GPX4 expression to understand the mechanism of ALOX15 inhibiting ferroptosis. Two cell lines, including untreated controls, were subjected to various treatments: RSL-3 (1 μM) induction alone, and a combination of RSL-3 (1 μM) and baicalin (5 μM). These treatments were applied to both empty Vector and OE-ALOX15 cells. Western blot analysis was used to detect the protein expression levels of HO-1 and GPX4 across these six experimental groups. The β-tubulin was used as the internal control to adjust the loading mass of protein, which also could be regarded as the house-keep protein expressing level control.

For the expression of HO-1, the protein levels in OE-ALOX15 cells induced by RSL-3 were significantly reduced following baicalin treatment compared to those in the Vector control group. However, the expression of GPX4 was significantly increased in OE-ALOX15 cells induced by RSL-3 after baicalin treatment compared with Vector. The GPX4 expression was reduced and HO-1 expression was slightly improved in different groups.

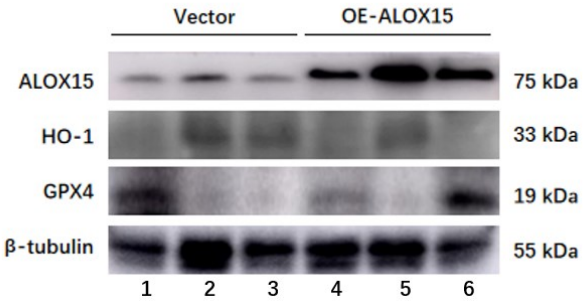

Figure 7. The effect of baicalin treatment on HO-1 and GPX4 protein expression. Bands 1-6 represent respectively Vector control (1); cells treated Vector and RSL-3 (1 μM) (2), cells treated Vector, RSL-3 (1 μM), Baicalin (5 μM) (3); OE-ALOX15 (4); cells treated OE-ALOX15 and RSL-3 (1 μM) (5); cells treated OE-ALOX15 and RSL-3 (5 μM) (6).

4. Discussion

Baicalin, a natural flavonoid compound, has been reported to possess various pharmacological properties, including anti-inflammatory and antioxidant effects. This study evaluates the efficacy of baicalin in inhibiting ferroptosis and elucidates its underlying mechanisms, focusing on its optimal concentration, effects on cell viability, oxidative stress, and iron homeostasis. Our findings provide significant insights into the potential therapeutic applications of baicalin in managing ferroptosis. The LDH release assay demonstrated a dose-dependent reduction in LDH levels, with 5 μM baicalin exhibiting significant inhibition of ferroptosis [23]. This concentration effectively reduced cellular damage induced by RSL-3, a known ferroptosis inducer, thereby establishing 5 μM as the optimal concentration for further studies. These results align with previous research indicating that baicalin can mitigate ferroptosis, reinforcing its potential as a ferroptosis inhibitor. The CCK-8 assay results showed that baicalin treatment improved cell viability in both Vector and OE-ALOX15 BEAS-2B cells. While ALOX15 expression did not significantly impact cell viability under basal conditions, baicalin's protective effect was evident across both cell types. The observed increase in cell viability supports the notion that baicalin has a cytoprotective effect, likely achieved through the modulation of ALOX15. Liperfluo fluorescence analysis revealed a 10% reduction in lipid ROS levels in OE-ALOX15 cells treated with baicalin. This reduction underscores baicalin's role in mitigating oxidative stress and highlights the potential involvement of ALOX15 in this process. Although ALOX15 does not significantly influence ROS levels under normal conditions, its presence appears crucial for baicalin's antioxidative effects. This finding suggests that ALOX15 may mediate the protective effects of baicalin against lipid peroxidation, contributing to the inhibition of ferroptosis. FerroOrange fluorescence and flow cytometry analysis indicated that baicalin treatment led to a significant decrease in intracellular

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Fe^{2+} levels in OE-ALOX15 cells. This reduction in Fe accumulation highlights the role of ALOX15 in ferroptosis regulation and supports the hypothesis that baicalin's ferroptosis-inhibiting effects are associated with reduced iron levels. Conversely, RSL-3 treatment alone did not alter Fe levels, suggesting that baicalin's action in modulating Fe^{2+} levels is specific and significant. Western blot analysis of HO-1 and GPX4 expression is ongoing to further elucidate the mechanisms by which baicalin inhibits ferroptosis. Both proteins are critical in the oxidative stress response and ferroptosis regulation. Changes in their expression could provide additional insights into how baicalin influences ferroptosis pathways, particularly in relation to ALOX15.

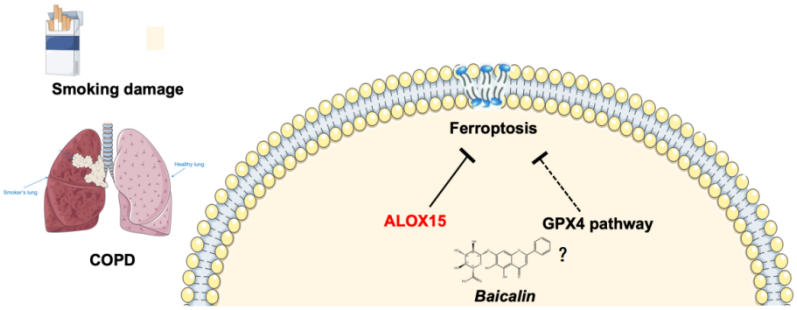

Figure 8. Illustration showing the potential mechanism of Baicailin regulating ferroptosis to inhibit COPD.

In recent years, studies have discovered that ferroptosis is closely related to the pathogenesis of COPD, suggesting that ferroptosis could be a potential therapeutic target for COPD [24]. A cigarette smoke exposure model using GPX4-deficient and GPX4-overexpressing mice has elucidated the critical role of GPX4-regulated cell death in COPD [25]. Nuclear transcription factor-2 is a key factor in maintaining oxidative/antioxidant balance and can prevent the development of COPD by combating oxidative stress and lung inflammation. Recent studies have reported that hypermethylation of promoter CpG sites leads to the downregulation of Nrf2 expression in the lung tissue of patients with COPD. Increased Nrf2 expression can enhance the expression of GPX4 and SOD while inhibiting ferroptosis and related inflammatory factors Further research has revealed that CS-/CSE-induced hypermethylation may lead to abnormal expression of Nrf2 [26]. Overactivation of Nrf2 results in the excessive induction of the rate-limiting enzyme HO-1, which catalyzes heme degradation to produce Fe^{2+} [27]. This process leads to iron-dependent lipid peroxidation. Previous studies have confirmed that RSL-3 can also induce overactivation of HO-1, thereby enhancing its iron-promoting activity [28]. After baicalin treatment, our results showed a significant down-regulation of HO-1 expression and a significant up-regulation of GPX4 expression. Therefore, we speculate that in addition to targeting ALOX15, baicalin may also exert a REDOX effect by modulating the expression of HO-1 through the NRF2 pathway, thereby inhibiting cellular ferroptosis. Other pathways also provided progress in the research field. The cystine/glutamate antiporter is located on the cell membrane and consists of SLC3A2 and SLC7A11, connected by a disulfide bond [29]. This system facilitates the exchange of cystine into the cell and glutamate out of the cell in a 1:1 ratio. Once inside, cystine is reduced to cysteine, which is a key precursor for synthesizing glutathione (GSH). GSH is an essential substrate for GPX4, which is responsible for degrading lipid peroxides (LPO) [30]. All these findings support our hypothesis. However, the detailed mechanism of this pathway needs further research. Although the ALOX15 appeared to be a potential target for developing therapeutic agents, the data collected all were concentrated at the cell level. In the future, the result described above should be verified in an animal disease model. In addition, other mechanisms might also be considered in this hypothesis, such as nuclear transcription factor-2 [31] and lipid metabolism pathway [32] to optimize the thesis.

In this experiment, we were limited by the available conditions and were only able to use cell-based experiments to verify our hypothesis. Animal experiments and related clinical trials were not conducted. Additionally, this study primarily focused on in vitro experiments and did not involve in vivo

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experiments, which may result in discrepancies when translating the data to a human context. Due to the limited laboratory conditions and the absence of relevant devices for smoke treatment, we used RSL-3 induced cells as the COPD cell model. In the future, we can enhance the project by conducting animal experiments. For instance, we could construct an in vivo animal model of smoke-induced COPD and verify the inhibitory effects of baicalin on inflammation through oral administration, to obtain experimental data in a biological context.

5. Conclusion

This study demonstrates that baicalin effectively inhibits ferroptosis, with 5 μM emerging as the optimal concentration for this purpose. The LDH release assay confirmed that this concentration significantly reduces ferroptosis induced by RSL-3, highlighting baicalin's potential as a therapeutic agent. Our findings indicate that baicalin enhances cell viability across different cell types, including both ALOX15 overexpression and wild-type BEAS-2B cells. Additionally, baicalin treatment led to a notable reduction in lipid ROS levels and intracellular Fe2+ accumulation, further supporting its role in mitigating oxidative stress and modulating iron homeostasis. The involvement of ALOX15 in this process appears crucial, as its presence facilitates the antioxidative effects of baicalin, thereby contributing to the inhibition of ferroptosis. Ongoing investigations into HO-1 and GPX4 expression will provide deeper insights into the molecular mechanisms by which baicalin exerts its ferroptosis-inhibiting effects. Overall, this study underscores baicalin's potential as a promising agent for managing ferroptosis-related conditions and highlights the need for further research to fully elucidate its therapeutic mechanisms.

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