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Potential role of ferroptosis in sterile acute lung injury induced by a non-hypoxic ischemia-reperfusion insult

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ABSTRACT

Objective(s): Ferroptosis, a regulated form of necrotic cell death, is implicated in the pathogenesis of hypoxic lung ischemia-reperfusion injury (LIRI). However, its role in non-hypoxic LIRI, which occurs in common clinical conditions such as trauma and pulmonary embolism, remains unclear. Given that ferroptosis has been implicated in sterile inflammation and lipid peroxidation is a hallmark of this process, this study aimed to investigate the potential role of ferroptosis in inflammatory responses during non-hypoxic LIRI.

Materials and Methods: Non-hypoxic LIRI was induced in C57BL/6 mice by occluding the left pulmonary artery for 1 hr, followed by 30 min of reperfusion. The treatment group received liproxstatin-1 (Lip-1; 10 mg/kg, IP) 1 hr before surgery. IL-6 levels in plasma and lung tissue were measured by ELISA. For the *in vitro* model, normal human bronchial epithelial (NHBE) cells were exposed to nutritional ischemia for 1 hr and then reperfused for 2 hr. Cells were pretreated with Lip-1 (200 nM) 12 hr before ischemia. CXCL1 release was measured by ELISA, and lipid reactive oxygen species (ROS) accumulation was assessed by BODIPY 581/591 C11 fluorescence.

Results: Non-hypoxic LIRI markedly increased IL-6 levels in plasma and lung tissue, as well as CXCL1 levels and lipid ROS accumulation in NHBE cells. Pretreatment with Lip-1 significantly reduced these inflammatory responses and lipid ROS accumulation compared with untreated LIRI.

Conclusion: Our findings suggest that ferroptosis-associated lipid peroxidation is associated with sterile inflammatory responses during non-hypoxic LIRI in both systemic and epithelial compartments. Lip-1 attenuated these responses, highlighting a potential strategy for non-hypoxic LIRI.

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Introduction

Apoptosis, a non-inflammatory form of cell death, was once considered synonymous with regulated cell death (RCD). In contrast, necrosis, an inflammatory form of cell death, was thought to occur primarily in a random, unregulated manner (1). However, this view has been challenged by recent reports showing that certain forms of necrosis are, in fact, regulated. Among these, ferroptosis has emerged as a distinct form of regulated necrotic cell death (2).

Ferroptosis differs substantially from other forms of RCD both morphologically and biochemically. Morphologically, ferroptotic cells exhibit shrunken mitochondria with increased membrane density and reduced cristae (3). Biochemically, ferroptosis centers on a cytotoxic, iron-dependent accumulation of reactive oxygen species (ROS) generated by the oxidation of polyunsaturated fatty acids (PUFAs) in membrane phospholipids, ultimately leading to lipid peroxidation (4).

Since its discovery in 2012, ferroptosis has attracted worldwide attention for its critical role in various pathological conditions, including ischemia-reperfusion (IR)-related insults (5). In this regard, recent studies have highlighted a pivotal role for ferroptosis in the pathogenesis of lung ischemia-reperfusion injury (LIRI), a form of sterile acute lung injury (ALI) that remains a significant clinical challenge with high morbidity and mortality (6, 7).

LIRI is generally classified into two types based on the presence or absence of ventilation impairment: 1) Hypoxic LIRI, in which both pulmonary perfusion and ventilation are transiently interrupted, as occurs during lung transplantation or cardiopulmonary bypass; 2) Non-hypoxic LIRI, in which pulmonary perfusion is temporarily interrupted while ventilation is maintained, as seen in hemorrhagic shock with fluid resuscitation or in pulmonary embolism with thrombolysis (8, 9).

Although ferroptosis has been implicated in hypoxic LIRI, its role in non-hypoxic LIRI remains unclear. To address this important and clinically relevant gap, we designed our study to investigate the potential involvement

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of ferroptosis in both *in vivo* and *in vitro* models of non-hypoxic LIRI.

Materials and Methods

Mouse maintenance

The *in vivo* ferroptosis studies were conducted using wild-type C57BL/6 male mice, either obtained from The Jackson Laboratory (Bar Harbor, ME, USA) or bred in the animal facility at the University of California, San Francisco, USA (UCSF). Male mice were exclusively used because ALI caused by traumatic events occurs disproportionately in human males. All purchased animals were acclimated for at least one week before the experiments. Mice were housed five per cage at an ambient temperature of 22 ˚C and 50% relative humidity under a 12 hr light/dark cycle. Food and water were available *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at UCSF and performed in accordance with all relevant regulatory standards.

Design of in vivo ferroptosis studies

Specific pathogen-free (SPF) C57BL/6 mice (12-15 weeks old, 25-30 g) were randomly divided into three groups: 1) Control, 2) IR, and 3) IR+Liproxstatin-1 (Lip-1). The sample size for each group was 10, as determined by previous studies in our laboratory (10). One hour prior to the induction of non-hypoxic LIRI, the ferroptosis inhibitor Lip-1 (S7699; Selleck, TX, USA), dissolved in 1% dimethyl sulphoxide (DMSO; Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) in phosphate-buffered saline (PBS; Gibco®, Thermo Fisher Scientific, Waltham, MA, USA), was administered to mice in the IR+Lip-1 group via intraperitoneal (IP) injection at a dose of 10 mg/kg (11). The timing of Lip-1 administration was chosen in accordance with previous IR and ferroptosis study (11) and was expected to provide sufficient systemic absorption of Lip-1 before ischemia induction. Mice in the Control and IR groups received an IP injection of vehicle (1% DMSO in PBS) only.

In vivo induction of non-hypoxic LIRI was achieved by unilateral occlusion of the left pulmonary artery (PA), as previously described (12). Briefly, mice were anesthetized with an IP injection of tribromoethanol (250 mg/kg, Avertin®; Sigma-Aldrich, St. Louis, MO, USA) and received buprenorphine (0.05 mg/kg IP; Henry Schein, Melville, NY, USA) for analgesia. Animals were then intubated and connected to a rodent ventilator (Harvard Apparatus, Holliston, MA, USA) with a tidal volume of 0.225 mL and a respiratory rate of 180 breaths/min. Body temperature was maintained throughout the surgical procedure using a heating pad. A left thoracotomy was performed via the intercostal space between the second and third ribs to expose the left PA, which was then ligated with a slipknot using a 7-0 Prolene monofilament suture. The free end of the suture was passed through a 27-gauge needle and externalized through the anterior chest wall to facilitate later release. After inflation of the left lung with positive end-expiratory pressure (PEEP), the chest was closed, and 3-4 drops of 0.25% bupivacaine (Sigma-Aldrich, St. Louis, MO, USA) were applied topically before skin closure. Mice were extubated and allowed to recover from anesthesia. This unilateral model induces non-hypoxic ischemia by occluding pulmonary blood flow while maintaining lung ventilation. During occlusion of the left PA, blood flow is redistributed to the right lung, thereby maintaining systemic gas exchange and animal viability. Because the current study aimed to investigate early IR injury in the previously occluded left lung, analyses focused on the left lung and did not include evaluation of the right lung. After 1 hr of ischemia, the slipknot ligature was released to allow reperfusion of the left lung for 30 min. Control animals underwent anesthesia and thoracotomy without vascular occlusion. At the end of the reperfusion period, all mice were euthanized.

Blood was collected by cardiac puncture with a heparinized syringe. Plasma was separated by centrifugation at 14,000 × g for 5 min at 4 ˚C and then stored at -80 ˚C. Left lung tissues were immediately harvested, washed in ice-cold PBS, and stored at -80 ˚C.

Enzyme-linked immunosorbent assay (ELISA)

IL-6 and CXCL1 were assessed as indicators of early sterile inflammatory response during non-hypoxic LIRI. The concentrations of interleukin (IL)-6 and chemokine (C-X-C motif) ligand 1 (CXCL1) were measured in mouse plasma, lung tissue homogenates, and culture supernatants using the corresponding ELISA kits (R&D Systems, Minneapolis, MN, USA). All assays were performed according to the manufacturer's instructions. Standard curves were generated and used to quantify cytokine levels in each sample.

A schematic overview of the *in vivo* experimental design is shown (Figure 1).

Cell culture

The *in vitro* ferroptosis studies were conducted using normal human bronchial epithelial (NHBE) cells obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured according to the manufacturer's instructions in bronchial epithelial growth medium (BEGM), which consists of bronchial epithelial basal medium (BEBM; Lonza, Walkersville, MD, USA) supplemented with BEGM SingleQuot Kit growth factors

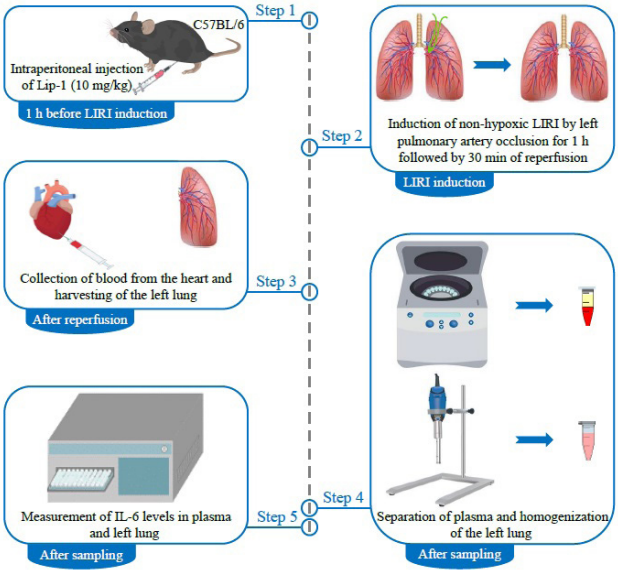

Figure 1. Schematic workflow of the *in vivo* experimental procedures in mice
Lip-1, liproxstatin-1; LIRI, lung ischemia-reperfusion injury; IL-6, interleukin-6

(Lonza, Walkersville, MD, USA), excluding antibiotics. Cultures were maintained in T75 Corning™ flasks (Fisher Scientific Co LLC, Pittsburgh, PA, USA), precoated with 4.5 ml of matrix per 75 cm^2 surface area. The matrix was composed of 0.01 mg/ml fibronectin (Sigma-Aldrich, St. Louis, MO, USA), 0.03 mg/ml bovine collagen (Sigma-Aldrich, St. Louis, MO, USA), and 0.01 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), all dissolved in BEBM. Cells were incubated at 37 ˚C in a humidified atmosphere containing 5% CO_2.

Design of in vitro ferroptosis studies

NHBE cells were seeded at 10,000 cells/well in matrix-coated 96-well Nunc™ Cell-Culture Treated Multidishes (Thermo Fisher Scientific, Waltham, MA, USA) and incubated in BEGM for 24 hr. Wells were then randomly assigned to three groups: 1) Control, 2) IR, and 3) IR+Lip-1. Each group comprised five parallel wells within a single experimental run, consistent with our laboratory's previous protocol (10). Twelve hours before inducing non-hypoxic (nutritional) ischemia, cells in the IR+Lip-1 group were pretreated with 200 nM Lip-1 in 0.1% DMSO in BEGM (11), whereas the IR group received an equivalent volume of vehicle control (0.1% DMSO in BEGM) only. This pretreatment interval was based on a previous IR and ferroptosis study (11) and was expected to allow sufficient intracellular accumulation of Lip-1 before inducing cellular stress.

In vitro non-hypoxic LIRI was induced as described in our previous study (10). Because pulmonary ischemia can develop despite preserved alveolar oxygenation, this model was designed to mimic nutrient deprivation under normoxic conditions rather than oxygen-nutrient deprivation. Accordingly, cells were maintained in a normoxic incubator throughout the experiment, and hypoxic conditions were not induced. Ischemia was induced by washing NHBE cells three times with Dulbecco's phosphate-buffered saline (DPBS; Gibco®, Thermo Fisher Scientific, Waltham, MA, USA), followed by replacement with BEGM. DPBS contains inorganic salts but lacks metabolic substrates such as glucose, amino acids, and lipids, thereby transiently eliminating extracellular nutrients while preserving oxygen availability. This approach was intended to mimic the metabolic conditions of a ventilated but non-perfused lung during PA occlusion. After 1 hr of ischemia, BEGM was reintroduced for a 2-hour reperfusion period. At the end of reperfusion, culture supernatants were collected and stored at -80 ˚C.

C11 BODIPY 581/591 analysis

For the confocal imaging experiment, 150,000 NHBE cells per well were seeded in matrix-coated 6-well plates (Corning Costar, Corning, NY, USA), each containing a 22 mm^2 glass slide. The following day, the aforementioned interventions were performed on the cells. Subsequently, BEGM was removed, and the cells were gently washed once with Hanks' Balanced Salt Solution (HBSS; Gibco®, Thermo Fisher Scientific, Waltham, MA, USA). The cells were then incubated at 37 ˚C for 10 min with a labeling solution containing 5 µM BODIPY 581/591 C11 (Thermo Fisher Scientific, Waltham, MA, USA) dissolved in HBSS, to detect membrane-associated reactive oxygen species (ROS). After incubation, the labeling solution was removed, and 1 ml of fresh HBSS was added to each well. The glass slide was carefully transferred onto a microscope slide preloaded with 25 µl of fresh HBSS, and the cells were imaged using a Zeiss Axio Observer microscope equipped with a Yokogawa spinning-disk confocal head (Yokogawa, Tokyo, Japan).

A schematic overview of the *in vitro* experimental design is shown (Figure 2).

Statistical analysis

Data are presented as mean±standard deviation (SD). Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. A P-P<0.0 of < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA).

Results

Ferroptosis-associated lipid peroxidation is associated with systemic and pulmonary inflammation

Given the reported association between ferroptosis and sterile inflammation across various disease contexts (13), we investigated whether inhibiting ferroptosis could mitigate sterile inflammation in non-hypoxic LIRI. Since ferroptosis is thought to occur predominantly at the beginning of the reperfusion phase rather than during ischemia (11), we designed a non-hypoxic LIRI model that incorporates 1 hr of ischemia followed by 30 min of reperfusion to detect downstream ferroptotic events shortly after reperfusion. To evaluate whether ferroptosis may contribute to sterile

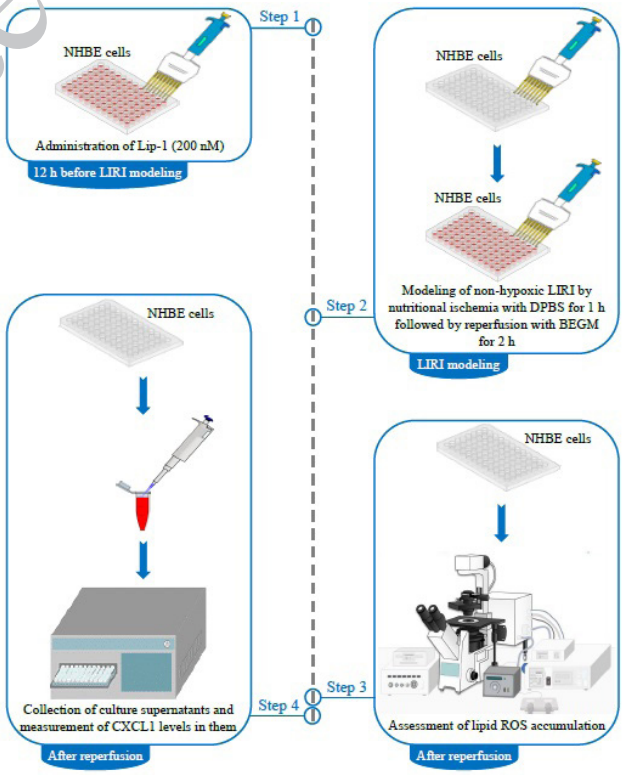

Figure 2. Schematic workflow of the *in vitro* experimental procedures in mice
NHBE, normal human bronchial epithelial; Lip-1, liproxstatin-1; LIRI, lung ischemia-reperfusion injury; DPBS, Dulbecco's phosphate-buffered saline; BEGM, bronchial epithelial growth medium; ROS, reactive oxygen species; CXCL1, chemokine (C-X-C motif) ligand 1

inflammation in this model, mice were administered Lip-1, a widely used ferroptosis inhibitor known to attenuate IR-related injury (14), via IP injection 1 hr before non-hypoxic LIRI induction. Our findings demonstrated that non-hypoxic LIRI significantly increased the levels of the pro-inflammatory cytokine IL-6 in both plasma (P<0.0001) (Figure 3A) and lung tissue (P<0.0001)(Figure 3B). Notably, pre-treatment with Lip-1 markedly reduced these elevations (P<0.0001)(Figure 3, A and B), suggesting an association between ferroptosis-associated lipid peroxidation and sterile inflammatory responses during non-hypoxic LIRI.

Ferroptosis-associated lipid peroxidation is associated with inflammatory responses and lipid ROS accumulation

To corroborate our *in vivo* results with complementary *in vitro* studies, LIRI was induced in NHBE cells under non-hypoxic conditions. NHBE cells were selected because epithelial cell death plays a central role in the pathogenesis of ALI, and patient prognosis largely depends on the extent of epithelial repair and regeneration (15). In line with our *in vivo* findings, *in vitro* nutritional IR injury significantly elevated the levels of the pro-inflammatory chemokine CXCL1 in the culture supernatants (P<0.0001), and treatment with Lip-1, administered 12 hr before the induction of nutritional ischemia, markedly suppressed this increase (P<0.0001)(Figure 4A). To further examine the potential involvement of ferroptosis in non-hypoxic LIRI, we assessed the accumulation of lipid ROS, a hallmark of ferroptosis (16), using the fluorescent lipid ROS probe C11 BODIPY 581/591. Confocal microscopy revealed robust lipid ROS accumulation in the cells subjected to IR injury, which was effectively attenuated by Lip-1 pretreatment (Figure 4B). These findings support a potential association between ferroptosis, lipid ROS accumulation, and inflammatory responses in non-hypoxic LIRI at the epithelial cell level.

Discussion

To the best of our knowledge, the current study is one of the first to investigate the potential role of ferroptosis in non-hypoxic LIRI. Collectively, these results support the potential involvement of ferroptosis-associated lipid peroxidation in non-hypoxic LIRI and suggest that this

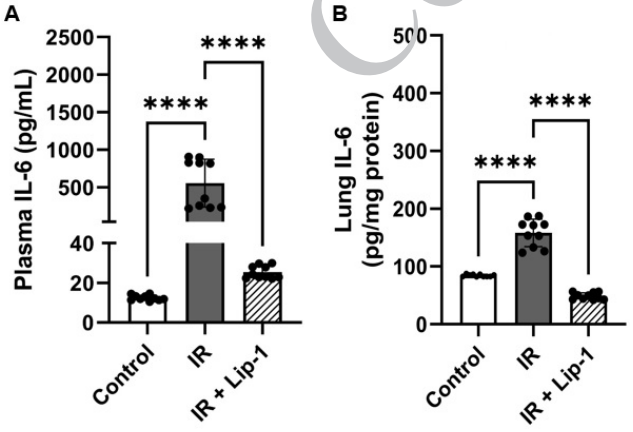

Figure 3. Liproxstatin-1 (Lip-1) attenuates interleukin-6 (IL-6) release during non-hypoxic lung ischemia-reperfusion injury (LIRI) *in vivo*
IL-6 levels in mouse plasma (A) and lung tissue (B) were measured by ELISA after 1 hr of ischemia followed by 30 min of reperfusion. P<0.0s are presented as mean±SD. ***P<0.001, ****P<0.0001 by one-way ANOVA. Each point represents an individual mouse

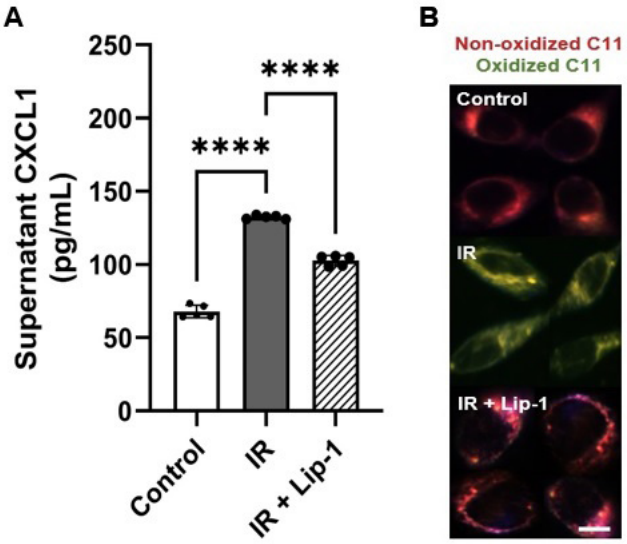

Figure 4. Liproxstatin-1 (Lip-1) reduces chemokine (C-X-C motif) ligand 1 (CXCL1) release and lipid reactive oxygen species (ROS) accumulation in NHBE cells subjected to non-hypoxic lung ischemia-reperfusion injury (LIRI)
CXCL1 levels in culture supernatants (A) were measured by ELISA, and lipid ROS accumulation (B) was assessed by BODIPY 581/591 C11 fluorescence (scale bar=10 µm). P<0.0s are presented as mean+SD. ****P<0.0001 by one-way ANOVA. Each point represents an individual culture well

process may be one of the contributing mechanisms, rather than the only underlying pathway, in the development of this injury. The key findings are as follows: 1) ferroptosis-associated lipid peroxidation may contribute to non-hypoxic LIRI in both *in vivo* and *in vitro* settings; and 2) ferroptosis-associated lipid peroxidation is associated with early sterile inflammatory response during this process.

Inflammation usually occurs in response to infection, serving to eliminate pathogens and promote tissue repair; however, in non-infectious conditions such as IR injury, sterile inflammation occurs, which plays a major role in driving both local and systemic tissue damage (17). This form of inflammation can be initiated or amplified by RCD pathways that may possess inflammatory characteristics. Unlike apoptosis, which is usually considered a non-inflammatory process, RCD pathways, such as ferroptosis, have been proposed to contribute to sterile inflammatory signaling under certain conditions (18).

More precisely, during apoptosis, the cell undergoes a regulated death process that preserves plasma membrane integrity, thereby largely limiting the release of inflammatory mediators. In contrast, ferroptosis is characterized by membrane damage associated with lipid peroxidation, a process that has been proposed to release endogenous damage-associated molecular patterns (DAMPs), thereby potentially contributing to inflammatory signaling (19-22). In the present study, IR-induced increases in IL-6 levels in mouse plasma and lung tissue, as well as increases in CXCL1 levels in cell culture supernatant, were attenuated by pretreatment with Lip-1. Although the findings support a potential link between ferroptosis-associated lipid peroxidation and early inflammatory activation in non-hypoxic LIRI, the study did not directly examine DAMP release, inflammasome activation, NF-κB signaling, or immune cell recruitment. Furthermore, although Lip-1 is widely used as a ferroptosis inhibitor, the observed protective

effects may be due in part to reduced accumulation of lipid ROS and inflammatory signaling associated with oxidative stress, as well as to ferroptosis-specific mechanisms. Accordingly, the present findings should be interpreted as supportive rather than definitive evidence of a role for ferroptosis in non-hypoxic LIRI. Notably, these findings are consistent with previous reports showing that inhibition of ferroptosis attenuates inflammatory responses in hypoxic models of LIRI (14).

Fatty acids, including saturated fatty acids, monounsaturated fatty acids (MUFAs), and PUFAs, serve multiple cellular functions, acting as energy sources, signaling molecules, and modulators of enzymes and membrane channels (23). Among these, PUFAs have attracted particular attention in the context of ferroptosis due to their high susceptibility to oxidation. Specifically, arachidonic acid is prone to lipid peroxidation, a process that contributes directly to the initiation and progression of ferroptosis (24, 25). Therefore, it is not surprising that lipid peroxidation is a hallmark of ferroptosis and has been implicated in IR-induced tissue damage due to its harmful effects (16, 26). Fortunately, lipid peroxide scavengers such as Lip-1 can neutralize lipid peroxides by targeting lipophilic free radicals, thereby inhibiting ferroptosis (27). In our study, pretreatment of cells with Lip-1 significantly reduced IR-induced lipid peroxidation, as evidenced by a decrease in the green fluorescence intensity of the ROS lipid probe. This finding further supports the association between ferroptosis-associated lipid peroxidation and oxidative injury during non-hypoxic LIRI.

Conclusion

Taken together, the present findings support a potential link between ferroptosis-associated lipid peroxidation and early sterile inflammatory activation in non-hypoxic LIRI, and suggest that this process may contribute to the development of lung injury. The ability of Lip-1 to reduce lipid peroxidation and inflammatory markers also supports the potential importance of approaches targeting ferroptosis in IR-induced lung injury. It suggests the need for further investigation of these pathways.

Limitations and Future Perspectives

Several limitations should be considered when interpreting the present findings. Ferroptosis-associated lipid peroxidation was inferred primarily from the detection of lipid ROS and from pharmacological inhibition with Lip-1. Although Lip-1 is widely used as a ferroptosis inhibitor, reliance on a single pharmacological approach limits mechanistic specificity, and the observed anti-inflammatory effects may be due in part to broader modulation of oxidative or redox-sensitive pathways. Accordingly, the present findings support a link between lipid peroxidation and inflammatory activation rather than providing definitive evidence that ferroptosis is the primary causal factor in non-hypoxic LIRI.

Furthermore, this study focused on the early reperfusion period; therefore, it does not examine the time course of the evolution of inflammatory, immune, and tissue remodeling responses at later stages of injury. Also, the mechanistic identification of the processes was limited, as canonical ferroptosis-related markers, ultrastructural confirmations, extensive immune profiling, and comprehensive histological or physiological investigations were not systematically performed.

Furthermore, the *in vitro* experiments relied on within-experiment replicates and used only male mice, which limits the assessment of biological variability and potential sex-related differences. Future studies using complementary pharmacological and genetic approaches, longer reperfusion periods, and more extensive molecular and immunological analyses will be important for further elucidating the mechanistic and therapeutic significance of ferroptosis-associated lipid peroxidation in non-hypoxic LIRI.

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Authors' Contributions

A P and F K conceptualized and designed the study. F K, X T, and D M conducted the experiments and collected the data. F K performed the data analysis and drafted the manuscript. A P critically revised the manuscript and secured funding. All authors reviewed and approved the final version of the manuscript for submission.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

The authors used ChatGPT-5 to improve language clarity, then carefully reviewed the text and take full responsibility for the manuscript.

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