



4-octyl Itaconate Attenuates Acute Pancreatitis and Associated Lung Injury by Suppressing Ferroptosis in Mice

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Abstract

Acute pancreatitis (AP) is a common gastrointestinal emergency requiring hospitalization. In recent years, several studies have demonstrated a role for 4-octyl itaconate (4-OI) in anti-inflammatory and oxidative stress injury. However, the potential effects of 4-OI in AP have not been investigated. Caerulein and LPS were used to induce experimental AP models in mice and AR42J cells and then studied by histopathology, biochemical, and molecular analysis. Ferroptosis inhibitor ferrostatin-1 effectively improves pancreatic injury and reduces lipid peroxidation products in experimental AP mice. 4-OI treatment significantly alleviated pancreatic and AP-associated lung injury and inflammation in experimental AP mice by inhibiting ferroptosis. The ferroptosis activator Erastin blocked the protective effect of 4-OI against pancreatic injury in AP, validating that 4-OI alleviates pancreatitis injury through ferroptosis. In vitro experiments further confirmed that 4-OI treatment ameliorated AP-induced pancreatic injury by inhibiting ferroptosis. Our study, for the first time, found that 4-OI ameliorates AP and AP-related lung injury by inhibiting ferroptosis in experimental AP mice, providing a new therapeutic target for alleviating AP.

Keywords Acute pancreatitis · 4-octyl itaconate · Ferroptosis · Inflammation

Introduction

Acute pancreatitis (AP) is one of the most common gastrointestinal diseases leading to acute hospitalization, with considerable morbidity, mortality, and socioeconomic burden [1, 2]. As the acute inflammatory process of the pancreas, the disease is characterized by a local and systemic

inflammatory response [3], which not only injures local peripancreatic tissue but also leads to multisystem organ dysfunction, particularly acute lung injury (ALI), that is the cause of death in the majority of patients in the early stages of AP [4]. It is urgent to provide strategies to inhibit the early response of systemic inflammatory response syndrome (SIRS) and prevent subsequent organ failure [1], with increasing incidence globally and with continued rates of high mortality in patients progressing to severe AP [5].

Ferroptosis, a recently recognized form of regulated cell death, has features of oxidative cell death [6, 7]. Iron metabolism and lipid peroxidation are critical mediators driving ferroptosis [8]. Mechanistically, ferroptosis is controlled by iron-catalyzed lipid radical formation and glutathione-dependent lipid hydroperoxidase glutathione peroxidase 4 (GPX4), which is induced through extrinsic or intrinsic pathways and can be inhibited by iron chelators and lipophilic antioxidants [9]. Although ferroptosis has a relatively straightforward relationship with tumors and ischemia–reperfusion injury, there are limited studies on its correlation with AP [7]. Studies have suggested that ferroptosis facilitates inflammation and reactive oxidative stress (ROS) during AP and AP-induced multiple organ failure [8, 10, 11].

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Targeting ferroptosis may serve as a novel potential therapeutic strategy for AP.

Recent years have shed light on intracellular metabolic changes, opening new avenues for inflammation treatment [12, 13]. Itaconate is an essential intermediate metabolite isolated from the Tricarboxylic acid (TCA) cycle, which is the core of cellular metabolism [12]. Additionally, itaconate is readily cleared from the organism, and its toxicity is likely very low [12]. Previous studies have shown that Itaconate and its derivatives have anti-inflammatory effects in sepsis, viral infections, ischemia/reperfusion injury, and pulmonary fibrosis, pointing to possible itaconate-based therapeutics for various inflammatory diseases [13]. Given that itaconate is weak cell-permeability to satisfy further research, 4-octyl itaconate (4-OI), a cell-permeable itaconic acid derivative, makes it a suitable cell-permeable itaconate surrogate [14]. It is reported that 4-OI can directly modify JAK1, alkylates kelch-like associated protein 1 (KEAP1) activating Nrf2, and inhibits NLRP3 inflammasome activation to exert anti-oxidant and anti-inflammatory effects [14–16]. It is worth noting that, He et al. found that 4-OI can inhibit ferroptosis to alleviate LPS-induced ALI [17]. To date, no studies have explored the function of 4-OI in pancreatitis, especially in AP.

Therefore, we hypothesized that 4-OI could attenuate AP and first dissected its effect on experimental AP mice. Meanwhile, the ferroptosis inhibitor ferrostatin-1 and the activator of ferroptosis Erastin were utilized in this study to investigate the role of 4-OI in modulating ferroptosis in the AP.

Materials and Methods

Antibodies

The different antibodies used herein were antibodies against GPX4 (Abcam, ab125066), antibodies against Tfr (Invitrogen, JF0956), antibodies against PTGS2 (CST, #12282S), antibodies against FTL (Abcam, ab69090), antibodies against TNF- α (Proteintech, 17,590–1-AP), antibodies against IL-1 β (Abcam, ab9722), and antibodies against β -actin (Proteintech, 66,009–1-Ig).

Animal Model and Treatment Groups

All animal experiments were carried out by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and the Ethics Committee of Wuhan Third Hospital (SY2022-040). C57BL/6J wild-type male mice (8 weeks old) (weight: 20 ± 2 g) were obtained from the Hubei Provincial Center for Disease Control and Prevention (Hubei, China). The mice

were conditioned in-house for 1 week after arrival and provided with tap water and a commercial diet.

AP model in mice was induced by administered intraperitoneal injections of caerulein (50 μ g/kg, MedChemExpress, HY-A0190) seven times at an interval of one hour, and a single LPS (10 mg/kg) was intraperitoneal injected at the same time as the last intraperitoneal injection of caerulein [11]. The control group was administered 0.9% NS. In addition, 50 mg/kg 4-OI (InvivoChem, V30953, CAS:3133–16-2) was administered to mice by intraperitoneal injection before the first caerulein injection. The 4-OI dose (50 mg/kg) used for the *in-vivo* experiments in this study was determined based on previous literature [18–20]. The ferrostatin-1 (1 mg/kg, MedChemExpress, HY-100579) and the Erastin (15 mg/kg, MedChemExpress, HY-15763) [21] were administered to mice by intraperitoneal injection 1 h before modeling. After the LPS administration for 24 h, mice were euthanized via administration of sodium pentobarbital followed by cervical dislocation. Mice blood samples and histological samples were collected. Serum was further collected after centrifugation at 10,000 g for 5 min at 4°C for subsequent analysis. Tissue samples were collected, partially fixed in formalin, paraffin-embedded sections were used for subsequent experiments, and the remaining tissue was snap-frozen in liquid nitrogen and stored at -80 °C.

Cell Model and Treatment Groups

Pancreatic AR42J were obtained from the China Center for Type Culture Collection (Wuhan, China) and were cultured in Ham's F-12 K medium (Procell Life Science&Technology Co., Ltd., Wuhan, China) in a 37 °C humidified incubator containing 5% CO₂ and 95% air. The cells were divided into five groups as follows: (1) control group; (2) control group + 4-OI; (3) AP group; (4) AP group + 4-OI; and (5) AP group + 4-OI + Erastin. The cells in the AP group were treated with caerulein (10 nmol/L) and LPS (10 μ g/ml) for 24 h. In the 4-OI treatment group, the cells were pre-incubated with 250 μ M 4-OI for 24 h. Erastin (10 μ M) was administered to incubate the cells for 24 h.

Histopathological Evaluation

The 5- μ m-thick paraffin sections were subjected to hematoxylin and eosin (H&E) staining for histological analysis. Histopathological changes in the pancreas and lungs were scored according to scoring criteria [22, 23]. The 5- μ m-thick paraffin sections were subjected to 4-hydroxy-2-nonenal (4-HNE) (Bioss-6313R) and MPO (Servicebio-GB150006) for immunohistochemical staining and were purchased from Bioss (Beijing, China). Slides were imaged under a light microscope (Olympus, Japan). Experienced pathologists were blinded to the experimental groups before examining

the histological samples and carried out pathological assessments independently.

Lung Wet-to-Dry Ratio

The degree of pulmonary edema was determined by calculating the wet-to-dry ratio between the initial weight of the lung lobes (wet weight) and the weight after 24 h of drying in a 60–70 °C drying oven (dry weight).

Evaluation of Amylase, Lipase, TNF- α , and IL-1 β

The amylase and lipase levels in serum were assessed using an Amylase assay kit (TE0203) from Leagene Biotechnology (Beijing, China) and a Lipase assay kit (A054-2) from Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. The levels of TNF- α and IL-1 β in cellular supernatant from AR42J cells were detected with TNF- α ELISA Kit (EK182-96) and IL-1 β ELISA kit (EK101B-96) from Multi-Science Biotechnology (Hangzhou, China) according to the manufacturer's instructions.

Detection of MDA, GSH-Px Activity, Iron

The Malondialdehyde (MDA), and glutathione peroxidase (GSH-Px) activity levels in serum and in AR42J cell lysates were measured by malondialdehyde assay kit (A003-1), and the glutathione peroxidase (GSH-Px) (A005) purchased from Jiancheng Bioengineering Institute (Nanjing, China) according to manufacturer's instructions. The iron concentration levels in fresh pancreatic tissues and AR42J cells were measured by an iron assay kit (C016) purchased from Changchun Huili Biotech Co., Ltd. (Changchun, China).

ROS Assay

The pancreatic tissue's reactive oxidative stress (ROS) activity was assayed with dihydroethidium (DHE) staining. The ROS activity of the cells was measured by a fluorescent probe (DCFH-DA) (D6470, Solarbio, Beijing, China). Cells in 6-well plates were incubated with 10 μ M/L of DCFH-DA for 30 min at 37 °C. Subsequently, cells were washed three times with serum-free medium. Images were obtained using a fluorescence microscope (Olympus, Japan).

Cell Viability

For the Cell Counting Kit-8 (CCK8) assay, cells (1×10^3 /well) were seeded in 96-well plates. Cells were treated with 4-OI (0, 15.6, 31.25, 62.5, 125, 250, 500 μ M) for 48 h. CCK8 reagent was added to the corresponding 96-well plates for incubation. The absorbance at 450 nm was measured using

a microplate spectrophotometer (Victor3 1420 Multilabel Counter, Perkin Elmer, USA) according to the instructions (CCK8, Beyotime, China).

Western Blotting (WB)

Homogenized mice pancreas samples or collected AR42J cells were lysed with RIPA lysis buffer (Biosharp, China), phosphatase inhibitors (Servicebio, China), and PMSF (Servicebio, China). An SDS/PAGE gel (Bio-Rad) was used to separate the proteins, which were then electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The PVDF membrane was blocked with skimmed milk for one hour at room temperature and then washed with TBST. The PVDF membrane was incubated with specific primary antibodies overnight at 4 °C and then washed with TBST 3 times. The PVDF membrane was incubated with a secondary antibody for 1 h at room temperature. The scanning imaging of protein bands was performed with the ChemiDoc Imaging System (BIO-RAD USA). The protein bands were quantified based on densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was conducted using SPSS V.23 and GraphPad Prism 7. Continuous variables normally distributed are expressed as the mean $\pm$ SD, and categorical variables are expressed as counts (%). Independent sample t-tests were applied for normally distributed continuous data, and χ^2 test or Fisher's exact tests were used for categorical data. $P < 0.05$ was considered statistically significant.

Results

Ferrostatin-1 Significantly Ameliorates Pancreatic Injury and AP-associated Lung Injury in AP Mice

We established the caerulein + LPS-induced AP, the most widely used protocol to induce AP in mice [24], for subsequent investigation of AP and AP-induced lung injury. As shown in Fig. 1A and C–F, the representative signs of pancreatic injuries, such as edema, inflammatory cell infiltration, alveolar necrosis, and hemorrhage, were observed in the AP group compared with the control group. Significant representative signs of pancreatitis-related lung injury, such as alveolar edema (thickness of the alveolar wall) and inflammatory cell infiltration, were observed in the lung tissue of the AP group compared with the control group (Fig. 1B and G–J). In addition, amylase and lipase serum

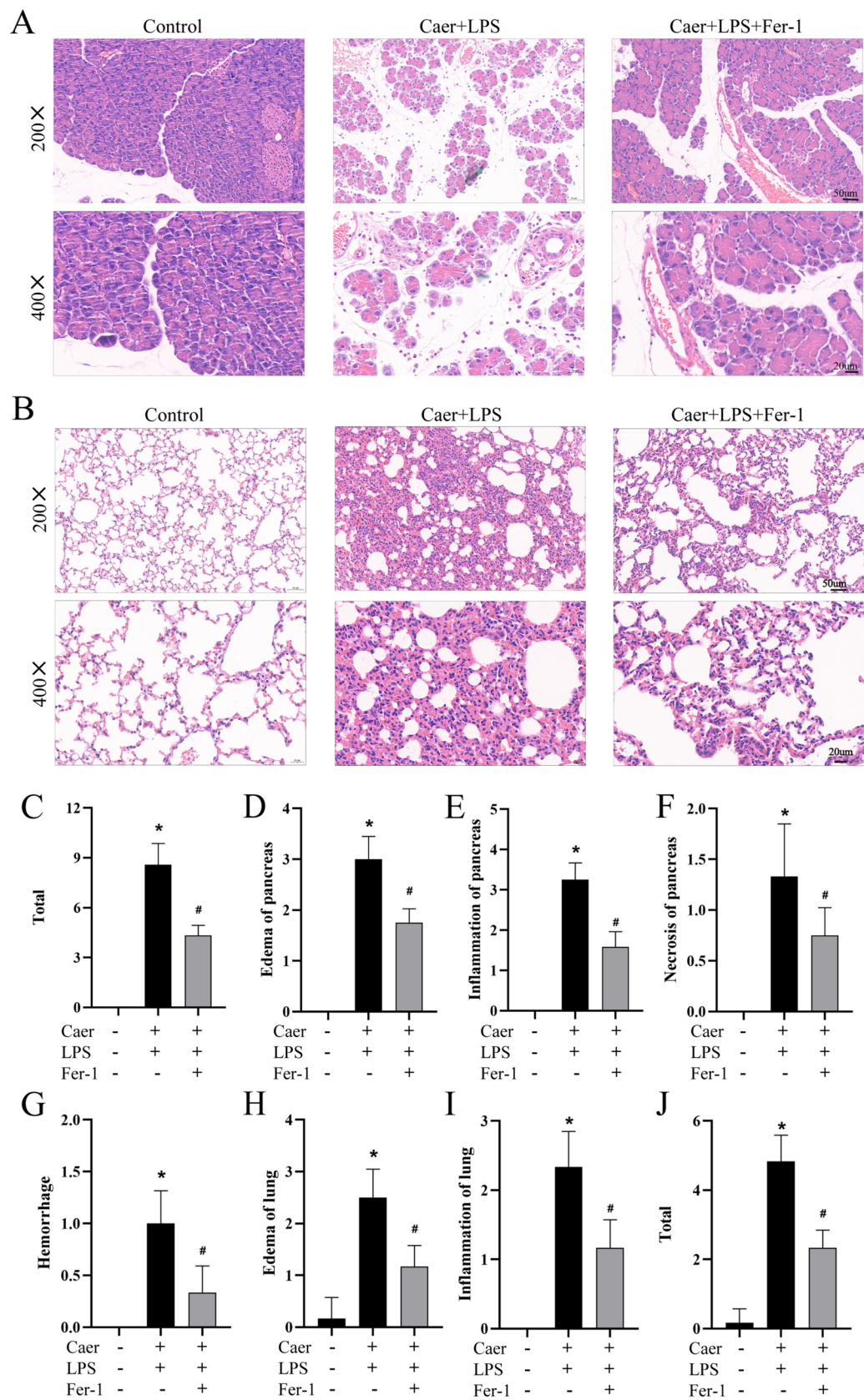


Fig. 1 Effects of Fer-1 on pancreas injury and lung injury in experimental AP mice. **A** Representative images of H&E-stained pancreas pathology ($\times 200$ and $\times 400$). **B** Representative images of H&E-stained lung pathology ($\times 200$ and $\times 400$). **C–G** Pathological scores of the pancreas injury ($n=8$). **H–J** Pathological scores of the lung injury ($n=8$). * $P < 0.05$ vs. control group, # $P < 0.05$ vs. AP group

levels were significantly increased compared with the control group (Fig. 2A). The above features indicate that we successfully established an AP model induced by caerulein and LPS.

Then, we verified the role of ferroptosis in the context of AP. Ferrostatin-1, a ferroptosis antagonist, was used to further validate the part of ferroptosis in AP and AP-induced lung injury. As shown in the figure, ferrostatin-1 significantly ameliorated pancreatic injury and AP-induced lung injury, as evidenced by a reduction in histologic scores (Fig. 1). The assays of amylase and lipase results showed that the increased serum amylase and lipase in the AP model could be significantly alleviated by administering ferrostatin-1 (Fig. 2A). The serum MDA levels declined by the treatment with ferrostatin-1 (1 h before AP) (Fig. 2B). It is well known that GPX4 and Prostaglandin-endoperoxide synthase 2 (PTGS2) are the key regulators in ferroptosis[6]. The WB result shows that the levels of PTGS2 protein were significantly increased, and the expression of GPX4 protein was significantly decreased in the AP groups compared with the control group (Fig. 2C). Administration of ferrostatin-1 significantly alleviated the aberrant protein expression of GPX4 and PTGS2 (Fig. 2C). These results validated that ferroptosis plays a critical role in AP and AP-induced lung injury.

4-octyl Itaconate Ameliorates Pancreatic Injury and AP-associated Lung Injury in AP Mice

To investigate whether 4-OI can protect against AP damage, 4-OI (50 mg/kg) was administered in intraperitoneal injections before the first caerulein injection [17, 25, 26]. The control + 4-OI group was used to evaluate whether the 4-OI had potential toxic effects (Fig. 3). The impact of 4-OI on pancreatic injury in AP mice was also assessed. As shown in Fig. 3A–F, the AP + 4-OI group mice have less edema, acinar cell necrosis, inflammatory cell infiltration, and hemorrhage in the pancreatic tissues compared with the AP group. The degree of alveolar edema (thickness of the alveolar wall) and inflammatory cell infiltration in the lung tissues of the AP + 4-OI group exhibited a significant reduction compared to that observed in the AP group. (Fig. 3G, I–K). Similarly, the results of lung MPO levels and lung wet-to-dry ratios in mice further suggested that 4-OI could alleviate AP-induced lung injury (Fig. 3H, L–M). In addition, 4-OI decreased serum amylase and serum lipase compared with the AP model (Fig. 4A). As shown in Fig. 5B, the increased levels

of TNF- α and IL-1 β in the AP model could be significantly alleviated by administering 4-OI. The results suggest that 4-OI treatment improved tissue injury and inflammation of the pancreas and lungs in experimental AP mice.

4-octyl Itaconate Alleviated Ferroptosis in the Experimental AP Mice

As shown in Fig. 4B, the serum MDA and non-heme iron content in pancreatitis tissue were significantly suppressed in the 4-OI treatment group compared with the AP group. The GSH-Px activity was increased dramatically in the 4-OI treatment group compared with the AP group (Fig. 4B). There is strong evidence to suggest that 4-OI may regulate ferroptosis, potentially providing valuable protection against experimental AP. The DHE labeling and 4-HNE staining, as indicators to assess superoxide content and lipid peroxidation, were also measured. DHE labeling (Fig. 4C) and 4-HNE (Fig. 4D) staining revealed that superoxide content and lipid peroxidation were significantly elevated in the pancreatic tissues of AP mice, while 4-OI significantly suppressed them (Fig. 4C–D).

The expression of critical proteins in ferroptosis, including ferroptosis-specific marker GPX4 and ferritin light chain (FTL) was significantly decreased in the AP group compared with the control group (Fig. 5A). PTGS2 and transferrin receptor (TfR) proteins were significantly increased in the AP group compared with the control group (Fig. 5A). Meanwhile, treatment with 4-OI rescued the decreased levels of GPX4 and FTL proteins in AP mice (Fig. 5A). 4-OI treatment significantly ameliorates the abnormal protein expression of PTGS2 and TfR proteins in acute pancreatitis (Fig. 5A). These results demonstrate that 4-OI effectively reduces ferroptosis to improve AP in mice.

4-octyl Itaconate Blocked Ferroptosis Induced by AP *In-vitro*

We then confirmed the effect of 4-OI on AR42J cells treated with caerulein and LPS for 24 h. The AR42J cell line was chosen for the *in-vitro* study of 4-OI efficacy because it has been widely used for cell validation in acute pancreatitis as a rat-derived pancreatic exocrine cell line. The AR42J cells were incubated with the 4-OI (0, 15.6, 31.25, 62.5, 125, 250, 500 μ M) for 48 h to detect the optimal and safest therapeutic concentration of 4-OI in cells (Fig. 6A). The cell viability assays showed that treatment at 4-OI (250 μ M) did not affect cell viability, but at high concentrations (500 μ M), it depressed cell viability at 48 h (Fig. 6A). Concerning previous studies and the effect of 4-OI on AR42J cells' activity, we selected 4-OI (250 μ M) pretreatment *in-vitro* experiments to evaluate 4-OI. Our results showed that compared to the AP group, pretreatment with 4-OI significantly suppressed the increased

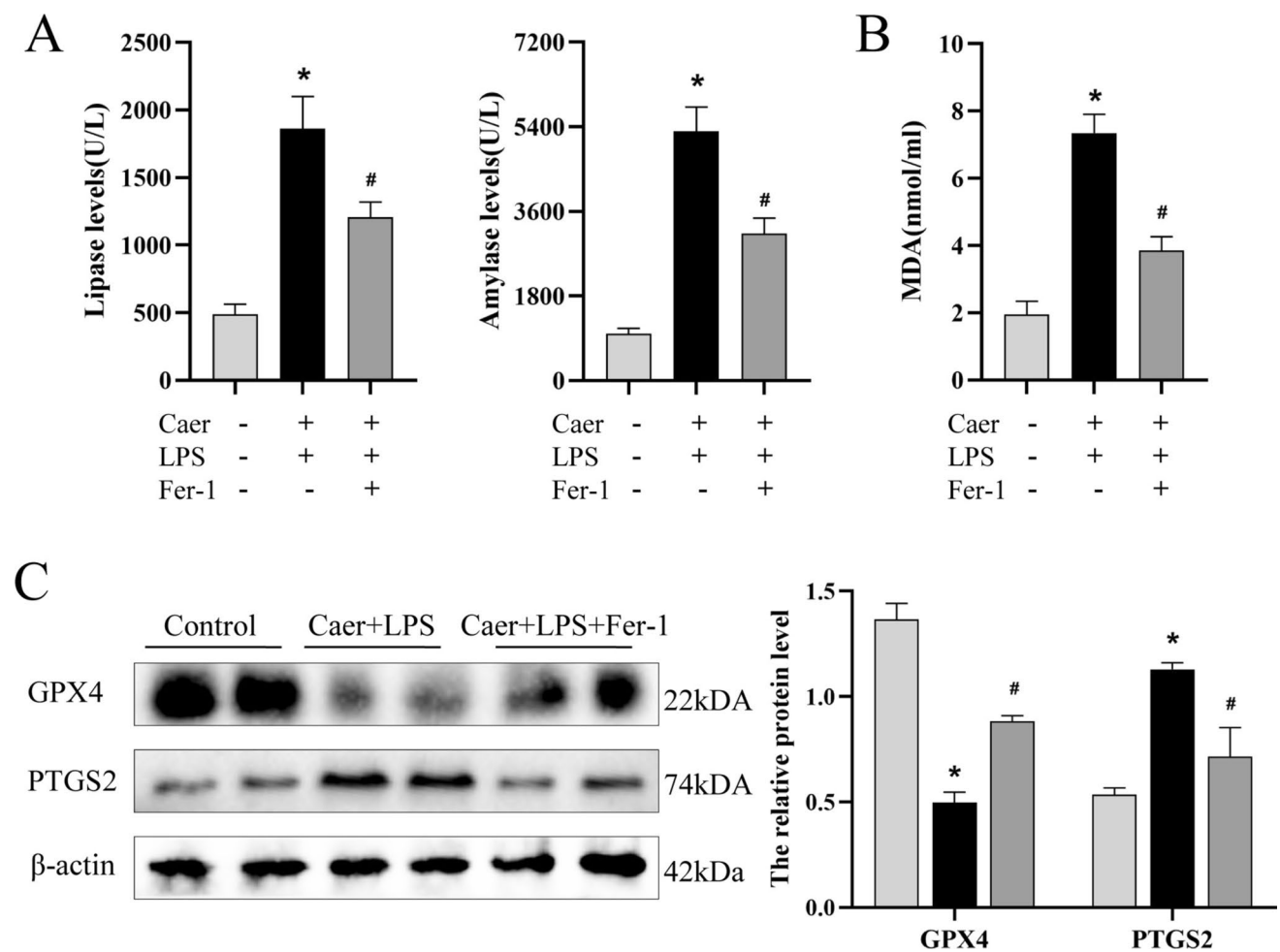


Fig. 2 Effects of Fer-1 on ferroptosis proteins, serum MDA, amylase, and lipase in AP mice. **A** The effects of Fer-1 on serum amylase and lipase activities in AP mice ($n=8$). **B** Fer-1 treatment down-regulated

the MDA content of the pancreas ($n=8$). **C** Western blot analysis of GPX4 and PTGS2 in pancreas tissue ($n=4$). * $P<0.05$ vs. control group, # $P<0.05$ vs. AP group

expression of TNF- α and IL-1 β in AR42J cells induced by caerulein and LPS (Fig. 6B). The MDA content and iron were reduced considerably in the 4-OI pretreatment group compared with the AP group (Fig. 6C). The GSH-Px activity was elevated in the 4-OI pretreatment group compared to the AP group (Fig. 6C). The ROS activity of the cells decreased in the 4-OI pretreatment group compared with that in the AP group (Fig. 6D). Meanwhile, the expression of GPX4 and FTL was increased by the pretreatment with 4-OI compared with the AP group (Fig. 6E). The levels of PTGS2 and TfR proteins were suppressed by the pretreatment with 4-OI compared with the AP group (Fig. 6E). The above results suggested that 4-OI alleviated pancreas injury and inflammation *in-vitro*.

The Ferroptosis Activator Erastin Prevents the Protective Effect of 4-octyl Itaconate *In-vivo* and *In-vitro*

Furthermore, the ferroptosis activator Erastin was used to activate ferroptosis in AP further to validate that 4-OI alleviates pancreatitis injury through ferroptosis. It was found that Erastin blocked the protective effect of 4-OI against pancreatic injury in AP mice (Figs. 3–5). Using Erastin in the 4-OI-treated group with AP caused a rekindling of amylase and lipase inhibited by 4-OI treatment (Fig. 4A). The reduction in superoxide content and lipid peroxidation caused by 4-OI treatment were also canceled out by the effect of Erastin (Fig. 4B–D). The increased levels of GPX4 and FTL proteins due to 4-OI treatment could be abolished by adding Erastin to counteract the impact of 4-OI on these proteins (Fig. 5A). The inhibited levels of PTGS2 and TfR protein by 4-OI treatment were also restored using Erastin (Fig. 5A). In addition, the serum inflammatory mediators TNF- α and

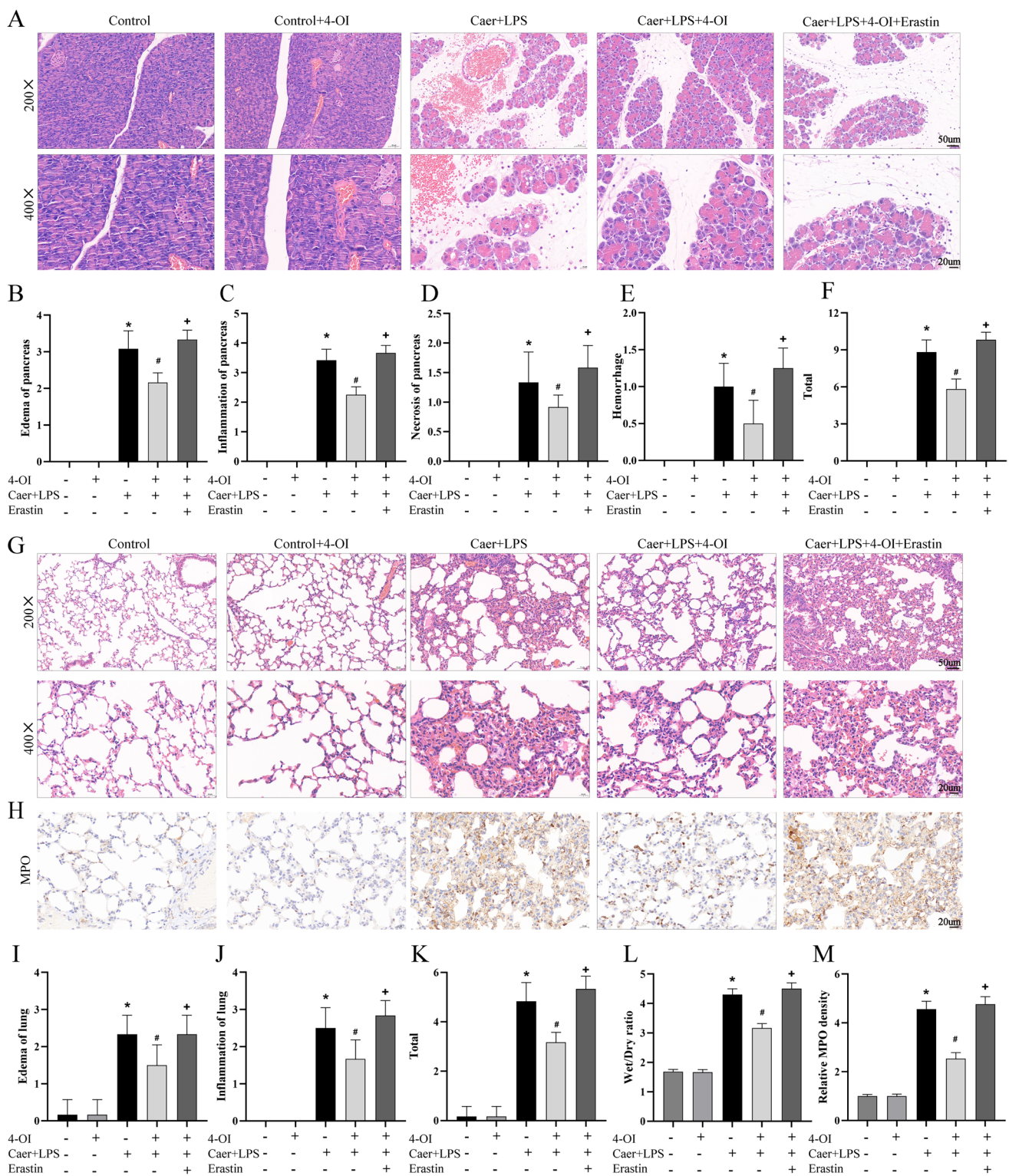


Fig. 3 4-OI protected against the experimental AP in mice. **A** Representative images of H&E-stained pancreas pathology (×200 and ×400). **B–F** Pathological scores of the pancreas injury (n=8). **G** Representative images of H&E-stained lung pathology (×200 and ×400). **H** Representative images of the lung MPO levels (×400).

(I–K) Pathological scores of the lung injury (n=8). **(L–M)** The relative MPO density and lung wet/dry ratios in experimental AP-induced lung injury (n=8). * $P < 0.05$ vs. control group, # $P < 0.05$ vs. AP group, + $P < 0.05$ vs. AP+4-OI group

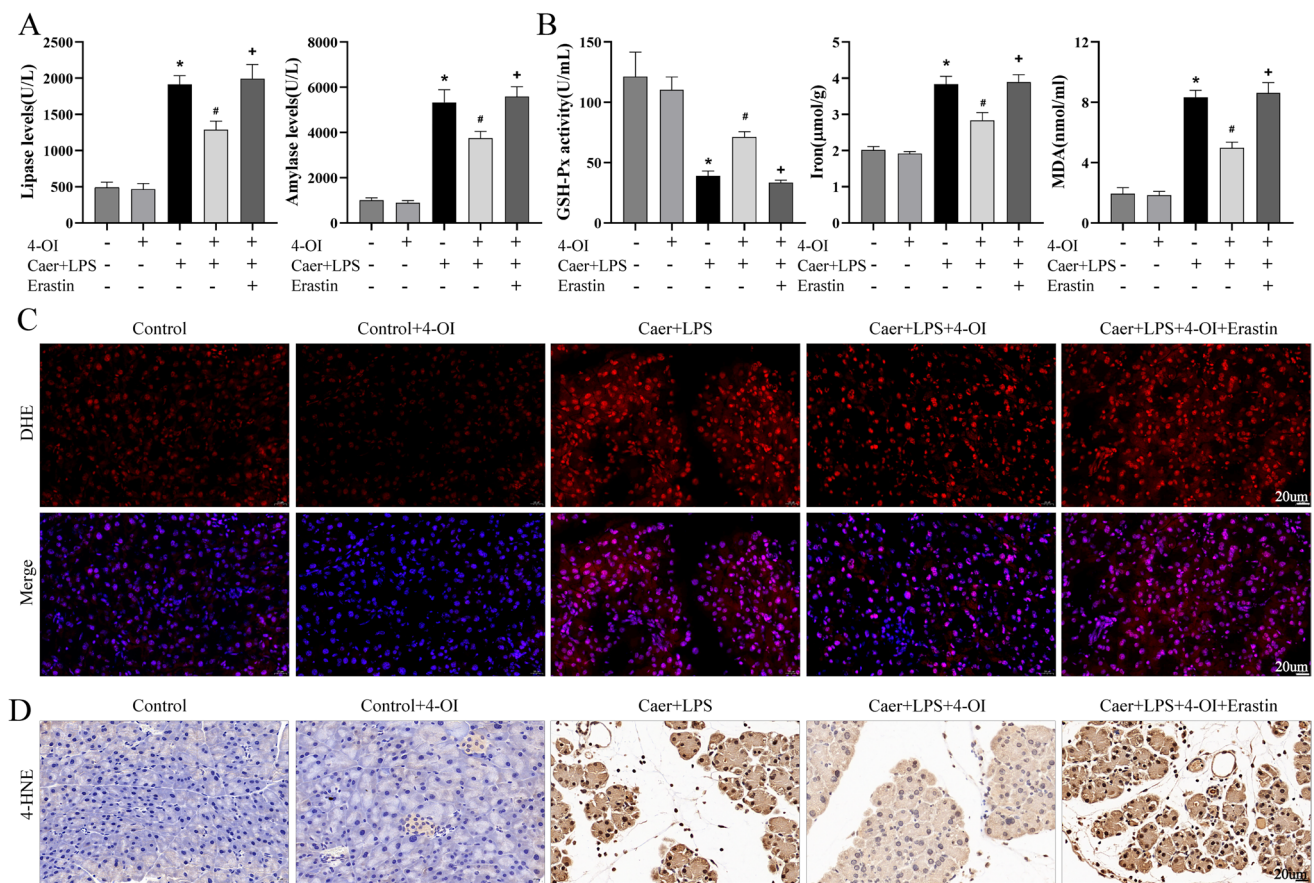


Fig. 4 Effects of 4-OI on ferroptosis and serum biochemical indicators in experimental AP mice. **A** The effect of 4-OI on serum amylase and lipase in AP mice ($n=8$). **B** The effect of 4-OI on iron, serum MDA, and serum GSH-Px in AP mice ($n=8$). **C** The pancreas super-

oxide content was detected by DHE labeling ($n=6$). **D** The lipid peroxidation was assessed by 4-HNE staining in AP mice ($n=6$). * $P<0.05$ vs. control group, # $P<0.05$ vs. AP group, + $P<0.05$ vs. AP+4-OI group

IL-1 β levels, which declined after 4-OI treatment, were significantly elevated with the addition of Erastin (Fig. 5B).

Meanwhile, we also used the activator Erastin in AR42J cells. The results showed that Erastin could cancel the inhibitory effect of 4-OI on the level of inflammatory factors TNF- α and IL-1 β in AR42J cells (Fig. 6B). The reduced MDA content, iron, and ROS activity induced by 4-OI pretreatment could be abrogated by adding Erastin (Fig. 6C–D). The elevated GSH-Px activity in the 4-OI pretreatment group could be reduced by Erastin administration (Fig. 6C). Furthermore, the increased expression of GPX4 and FTL induced by the 4-OI treatment can be suppressed by the Erastin administration (Fig. 6E). The lower levels of PTGS2 and TfR induced by the 4-OI treatment could be promoted by the Erastin administration (Fig. 6E). Therefore, we could conclude that 4-OI provides protection against experimental AP mice by regulating ferroptosis *in-vivo* and *in-vitro*.

Discussion

The present study identified that ferroptosis is hyperactivated in AP and exacerbates AP and AP-associated lung injury. For the first time, we investigated whether 4-OI treatment suppresses pancreatic injury and inflammatory response in the experimental model of AP induced by caerulein and LPS. It was also demonstrated that 4-OI treatment reduced the accumulation of lipid peroxidation products and iron in pancreatic tissues. 4-OI mechanistically protects against pancreatic and lung injury by inhibiting the ferroptosis pathway.

AP is an inflammatory disease of the pancreas in which pancreatic necrosis and activation of the inflammatory cascade response can easily lead to SIRS [27]. However, the etiology of pancreatitis is complex, and the exact pathogenesis must be fully clarified. Cell death and inflammation are critical pathologic responses of AP, and cell death-mediated damage-associated molecular pattern molecules (DAMP) release initiate and extend sterile inflammation [28].

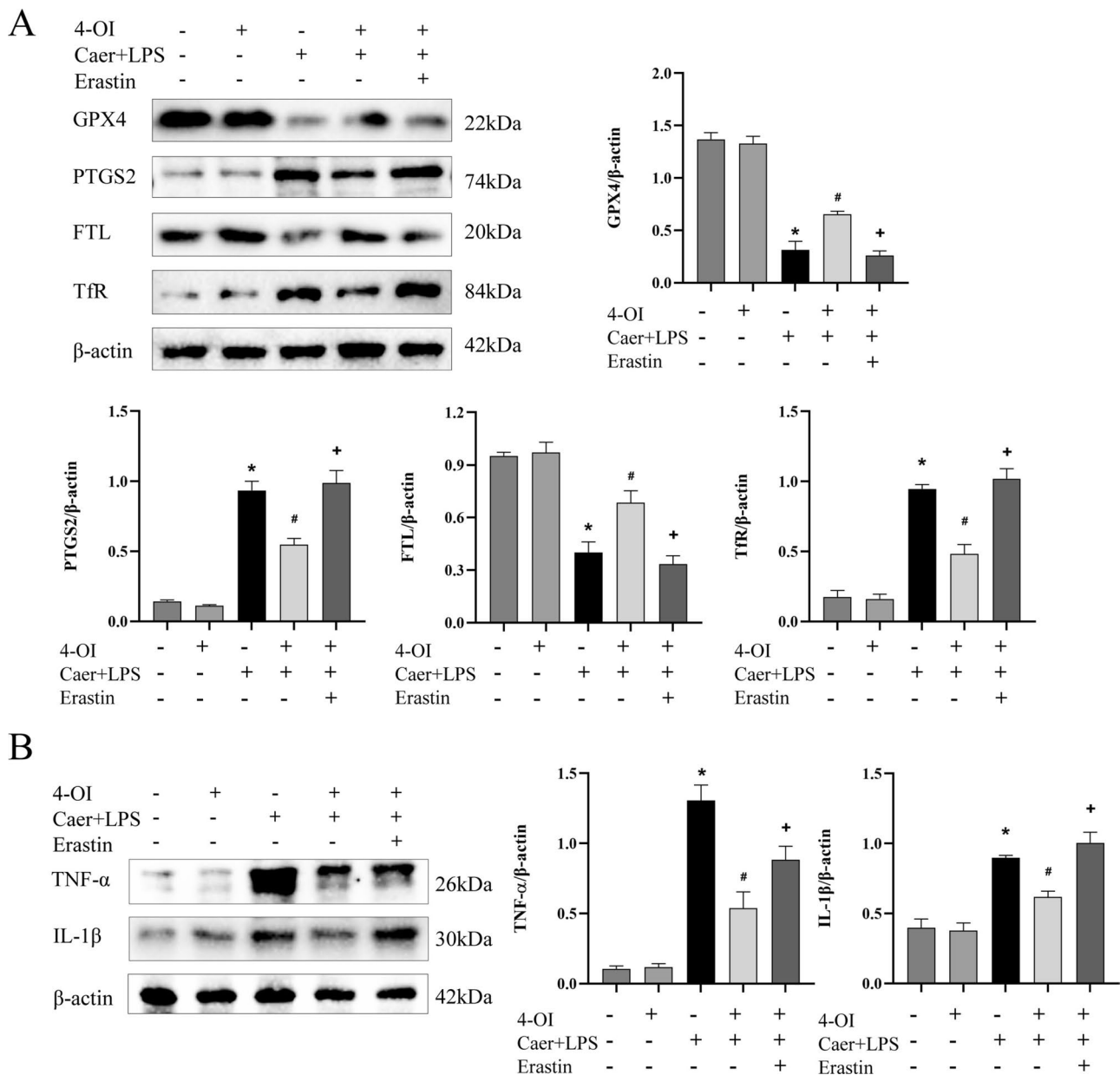


Fig. 5 Effects of 4-OI on ferroptosis and inflammation proteins in experimental AP mice. **A** Western blot analysis of critical proteins of ferroptosis in AP mice ($n=4$). **B** Western blot analysis of TNF- α and

IL-1 β in AP mice ($n=4$). * $P<0.05$ vs. control group, # $P<0.05$ vs. AP group, + $P<0.05$ vs. AP+4-OI group

Recently, itaconate has emerged as an important modulator of immunity and inflammation, one of the most highly upregulated metabolites during macrophage pro-inflammatory (M1) activation [16]. Many studies of itaconate involve the derivative compound 4-OI because OI can enhance cell permeability and intracellular conversion to itaconate [29]. Previous studies highlighted 4-OI immunomodulatory effects in multiple inflammations and infectious disease models, including airway inflammation, sepsis, peritonitis, SARS-CoV-2, and cardiovascular disease [17, 29–32]. 4-OI was demonstrated to alleviate the inflammatory process of

the disease by targeting JAK1 to inhibit macrophage activation and NLRP3 inflammatory vesicle activation [15, 16, 33]. The present anti-inflammatory effects of itaconic ester are primarily seen in macrophages and peripheral inflammatory immune responses [15, 16, 33], while its role in the pancreas and AP is not clarified. Thus, we aimed to explore the effect of 4-OI on AP. The pancreas and lungs in the OI-treated group had significantly lower edema, inflammatory cell infiltration, and necrosis than in the AP group. In addition, the protective effect of 4-OI against oxidative stress in pancreatic exocrine cells AR42J cells has been demonstrated

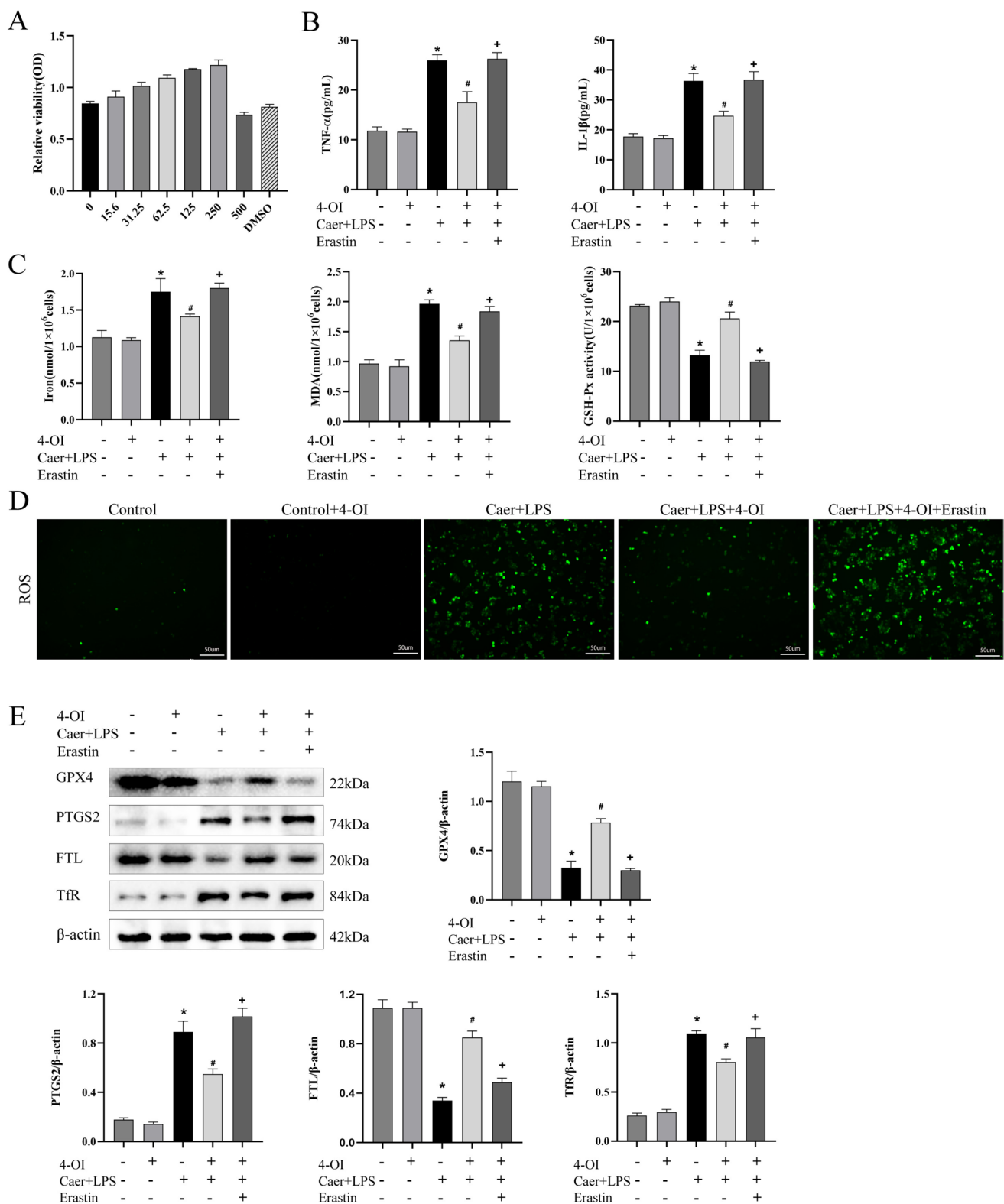


Fig. 6 4-OI blocked ferroptosis *in-vitro*. **A** The cell viability treated with 4-OI was determined by CCK8 assay ($n=6$). **B** The effect of 4-OI on the levels of TNF- α and IL-1 β in AR42J cells ($n=6$). **C** The effect of 4-OI on iron, MDA, and GSH-Px in AR42J cells

($n=6$). **D** The ROS activity of the cells was measured by a fluorescent probe (DCFH-DA) ($n=6$). **E** Western blot analysis of critical proteins of ferroptosis in AR42J cells ($n=4$). * $P<0.05$ vs. control group, # $P<0.05$ vs. AP group, + $P<0.05$ vs. AP+4-OI group

in-vitro. Several recent studies have found that, in addition to macrophages, 4-OI has also been shown to attenuate ROS and inflammation, including in Vero cells, hepatocytes cells, chondrocytes cells, aortic valve mesenchymal cells and the corresponding animal models [20, 31, 33, 34]. TNF- α and IL-1 β promote each other's secretion and induce an inflammatory cascade response. Blocking IL-1 β significantly reduces pancreatitis and organ failure [27]. Our study has also shown that 4-OI suppresses inflammation factors and superoxide content during AP *in-vivo* and *in-vitro*. Thus, our findings demonstrate the protective effect of 4-OI in experimental AP mice.

Ferroptosis, a pro-inflammatory type of cell death, is characterized by an iron-dependent increase in ROS, which disrupts lipid membranes and leads to a loss of cellular integrity [35]. Based on the accumulating evidence that ferroptosis plays an important role in multiple inflammatory diseases, therapeutic approaches targeting ferroptosis may have great potential in the treatment of inflammatory diseases [6]. In the study, we demonstrated that biomarkers of ferroptosis, including elevated MDA, iron, and decreased GSH-Px, were found in the AP group compared to blank controls, which is supported by Liu, Ke et al. [11]. The GPX4 prevents ferroptosis by converting lipid hydroperoxides into non-toxic lipid alcohol [36]. The decreased expression of GPX4 is essential for developing ferroptosis and is responsible for the induction of ferroptosis [9]. PTGS2, a gene encoding cyclooxygenase-2 (COX-2), is a biomarker for lipid peroxidation and is upregulated in ferroptotic cells [37]. The decreased expression of GPX4 and increased levels of PTGS2 were observed in experimental AP mice. Also, a ferroptosis inhibitor was employed to demonstrate that inhibition of ferroptosis protects against AP, supported by R. Fan et al. [10]. The treatment of AP with 4-OI significantly reduced the biomarkers of ferroptosis, including the elevated iron and MDA, and rescued the reduced GSH-Px in AP. Compared with the AP group, GPX4 and FTL protein levels were also significantly increased in the 4-OI-treated group. The expression of PTGS2 and TfR proteins was reduced considerably *in-vivo* and *in-vitro*. Ferritin, composed of two similar polypeptide chains: FTL and FTH, is a cellular iron storage protein upregulated when cellular iron levels increase during ferroptosis [38]. Intracellular iron homeostasis depends on the uptake of extracellular iron by the transferrin (Tf)/ TfR system, the discovered iron transport protein in mammals mediated iron export [39, 40]. The lipid peroxidation was also significantly reduced in the OI-treated group, as reflected by 4-HNE, which is consistent with the findings by He et al. [17]. Recently, it has been shown that 4-OI enhances the activity of GPX4 by alkylating it, thereby modulating ferroptosis [18]. In this study, we also found that 4-OI can affect ferroptosis by regulating the protein expression of GPX4, consistent with previous studies. In

addition, it has also been reported that 4-OI inhibits XPO1 (also known as chromosomal maintenance 1, CRM1) [41] which is a recently known positive activator of ferroptosis [42], but further research is needed on whether 4-OI regulates ferroptosis through XPO1, especially in the AP model. In this study, we preliminarily confirmed the effect of 4-OI on AP in the context of C57BL/6 mice. However, the impact of 4-OI in other species of animals is still unclear, especially the effect on humans, which needs further clinical experiments to confirm.

However, we recognize that this study has some limitations. First, previous studies have demonstrated that 4-OI can regulate in ferroptosis of macrophage in sepsis through KEAP1 ubiquitination and degradation of the Nrf2 pathway or other non-Nrf2-dependent pathways [14, 17]. Recently studies also showed that the 4-octyl tail of 4-OI interacted extensively with the hydrophobic groove of XPO1 as a recently identified target [41], which might be part of the mechanism by which 4-OI inhibits ferroptosis and pancreatitis. The molecular mechanism of 4-OI and ferroptosis in pancreatic cells needs further interpretation in future studies. Secondly, although 4-OI is a suitable cell-permeable itaconate surrogate for itaconate, whether there are differences between exogenous and endogenous itaconic acid derivatives needs to be further investigated. Finally, this study lacks clinical tissue samples to explore the link between ferroptosis and pancreatitis further.

Conclusion

In conclusion, our study provides important evidence to support the occurrence of ferroptosis in AP mice. It demonstrates for the first time that 4-OI ameliorates pancreatic and AP-associated lung injury in mice by inhibiting ferroptosis, providing a promising therapeutic target for alleviating AP.

Authors' Contributions Concept and design: Drs S. Lu and W. Dong. Performed, acquired, or interpretation of the experiments: Drs S. Lu, Y. Gong, M. Qi, P. He, and W. Dong. Drafting of the manuscript: Drs S. Lu, and W. Dong. Statistical analysis: Drs S. Lu and W. Dong. Supervision: Drs. W. Dong.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval All animal experiments were carried out by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and Ethics Committee of Wuhan Third Hospital (SY2022-040).

Competing Interests The authors declare no competing interests.

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