Biochemical and Biophysical Research Communications 572 (2021) 72–79

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of hypoxia-inducible factor-1α alleviates acinar cell necrosis in a mouse model of acute pancreatitis

Qinhao Shen [a, 1], Xiaolei Shi [a, 1], Lide Tao [b, 1], Qingtian Zhu [a], Weiming Xiao [a], Yanbing Ding [a], Weijuan Gong [a], Guotao Lu [a, ***], Mei Wang [a, **], Guanghuai Yao [a, *]

[a] Pancreatic Center, Department of Gastroenterology, The Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, Jiangsu, China
[b] Department of Surgery, The Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, Jiangsu, China

ARTICLE INFO

Article history:
Received 18 June 2021
Received in revised form
9 July 2021
Accepted 12 July 2021
Available online 3 August 2021

Keywords:
Acute pancreatitis
Acinar cell
Hif1α
Necroptosis

ABSTRACT

Hypoxia-inducible factor-1α (Hif1α) is activated in hypoxia and is closely related to oxidative stress, immunity and cell metabolism. Recently, it is reported that Hif1α is involved in atherosclerosis, ischemia-reperfusion (I/R) injury, alcoholic liver disease and pancreatic tumors. In this study, we found that Hif1 signal pathway is significantly changed in pancreas of acute pancreatitis (AP) mice. Meanwhile, we verified that the high expression of Hif1α injured pancreatic tissues of cerulean-induced AP mice, which prompting that Hif1α participated in the progress of histopathology on AP. We applied a Hif1α inhibitor PX478 and observed that it could alleviate histological injury of pancreas as well as the levels of serum amylase, lipase and proinflammatory cytokine in the murine model of AP induced by caerulein. In addition, PX478 could reduce the formation of necrosome (RIP3 and p-MLKL) and the generation of reactive oxygen species (ROS) in AP mice. Correspondingly, we further confirmed the effectiveness of PX478 in vitro and found that inhibiting Hif1α could mitigated the necrosis of pancreatic acinar cells via reducing the RIP3 and p-MLKL expression and the ROS production. In conclusion, inhibiting Hif1α could protect against acinar cells necrosis in AP, which may provide a new target for the prevention and treatment of AP clinically.

1. Introduction

Acute pancreatitis (AP) is one of the commonest digestive inflammatory diseases, of which the etiologies include the gallstones and excessive alcohol consumption [1]. Most cases of AP are mild disease, while several AP patients may develop into severe acute pancreatitis (SAP), characterized by persist organ failure and pancreatic necrosis [2–4]. As an acute non-infectious inflammatory disease, AP was closely related to the premature activation of the proenzyme on the pancreatic injury tissues, as well as the necrosis of pancreatic acinar cells and the release of inflammatory mediators, causing local and systemic inflammatory responses [5]. Currently, acinar cell injury and death were remarkable interrelated with the severity and prognosis of AP. The early stage of AP is only accompanied by apoptosis, whereas SAP is accompanied with necrosis of the local or surrounding tissues of the pancreas, and distant organ dysfunction. Animal experiments have shown that inhibiting acinar cell necrosis during the early stage of AP could ameliorate the prognosis of AP [6,7], proven that the necrosis of pancreatic acinar cells is one of the important mechanisms for the development of AP.

Necroptosis is one of the major ways of acinar cells death and regulated the pathological processes of AP [8], a part of cell programmed necrosis, which is modulated by a set of cellular signal pathways. There is one wildly accepted theory that necroptosis is dependent on a kinase cascade consisting of the receptor-interacting protein 3 (RIP3) and mixed lineage kinase domain-like protein (MLKL). Moreover, MLKL is a functional RIP3 substrate and is phosphorylated by RIP3 at the Thr357 and Ser358 sites [9–11]. Activation of MLKL could cause the decomposition of plasma membrane, resulting in cell death, which is essential for necroptosis. Inhibiting necroptosis may be a potential therapeutic target for AP in clinic [12].

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: gtlu@yzu.edu.cn (G. Lu), wangmei@yzu.edu.cn (M. Wang), ghyao@yzu.edu.cn (G. Yao).
[1] Qinhao Shen, Xiaolei Shi and Lide Tao contributed equally to this work.

https://doi.org/10.1016/j.bbrc.2021.07.043
0006-291X/

Hypoxia-inducible factor-1 (HIF-1), a master regulator of injury responses, has divergent roles in different cells. Hypoxia Inducible Factor 1(Hif1) is an oxygen-sensitive transcriptional activator consisting of Hif1α and Hif1β [13,14]. Under normoxic conditions, Hif1α is degraded by the ubiquitin-proteasome system. In hypoxia, as the degradation of Hif1α is inhibited, Hif1α accumulates and forms a dimer with Hif1β, binding to the downstream gene and recruiting coactivators [15–17]. It has been reported that the acinar cell-specific deficiency of Hif1α mice exhibited less fibrinogen-γ dimer (Fib-γD) accumulation and amylase release which was induced by caerulein, compared with wild-type mice [18]. In our study, we analyzed the RNA-seq data of public database and found significant high expression of Hif1α in the pancreatic tissues of AP mice. Notably, we applied Hif1α inhibitor PX478 and found that PX478 could improve pathological characteristics and reduce serum amylase and lipase levels in caerulein-induced AP mice. Meanwhile, we verified that PX478 could protect acinar cell from necrosis via necroptosis signal pathway.

2. Materials and methods

2.1. Reagent

PX478 were purchased from MCE (MCE Co. Ltd., New Jersey, USA); KC7F2, IDF11774 were purchased from Selleck (Selleckchem, Texas, USA); caerulein, cholecystokinin (CCK), Dihydroethidium (DHE) and anti-GAPDH antibody were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA); DMEM High Glucose Medium, Fetal Bovine Serum, 0.25 % Trypsin and Penicillin-Streptomycin Solution were purchased from gibco. anti-RIP3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz,CA,USA); anti-Hif1α antibody, anti-MLKL antibody and anti-p-MLKL were purchased from Abcam (Abcam, Cambridge,UK); anti-rabbit and anti-mouse lgG secondary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA); the LDH Cytotoxicity Assay Kit was purchased from Beyotime Biotechnology (Beijing, China); Hoechst 33342 and propidium iodide (PI) were purchased from Solarbio (SolarbioScience&Technology, Beijing,China); amylase kits were purchased from BioSino (BioSino BioTechnology & Science Inc., Beijing, China) and lipase kits were purchased from Nanjing Jiancheng (Nanjing Jiancheng Corp, Nanjing, China).

2.2. Animals and ethics

Male ICR mice (6–8 weeks old, about 20–25g) were purchased from the Comparative Medicine Center of Yangzhou University (Yangzhou, Jiangsu, China). All mice were housed under specific-pathogen-free (SPF) conditions with suitable room temperature (20–25 °C), appropriate humidity animal facility and cycle lighting (12 h light/12 h dark), and fed with standard rodent chow and water. All methods were carried out in accordance with the principles of Laboratory Animal Care (NIH Publication No. 85Y23, revised 1996) and all studies were approved by the Science and Technology Commission of the Affiliated Hospital of Yangzhou University municipality.

2.3. RNA-seq

The gene expression profiling data was performed, based on the published literature and databases [19]. Statistical analyzes were performed with the aid of the R software [20]. The heatmap analysis and functional-enrichment analysis KEGG (Kyoto Encyclopedia of Genes and Genomes) were used to identify differentially expressed genes (DEGs) between two different groups by R software [21,22].

2.4. Construction of the AP model and Hif1α inhibitors administration

Intraperitoneal (i.p.) injection of caerulein to establish MAP model (100 μg/kg, 1 h interval, 7 times). PX478 was dissolved in PBS before use. At 0 h, mice were intraperitoneally injected with different concentrations of PX478 (25, 50, 100 mg/kg), KC7F2 (2.5 mg/kg) and IDF11774 (25 mg/kg). The animals were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg) prior to execution. Blood samples were collected for enzymology and enzyme-linked immunosorbent assay (ELISA) analysis. Partial pancreatic tissues were immediately collected in liquid nitrogen, then stored at −80 °C for further research. The remaining pancreatic tissues were fixed in 4 % paraformaldehyde for histological analysis.

2.5. Histological analysis

The fixed pancreatic tissues were embedded and sliced into 5 μm thick sections which were sticked on the glass slides. The slides were treated with dewaxing, dehydration and hematoxylin and eosin staining (H&E). Then slides were observed with a light microscope. The histopathological scoring analysis of the pancreatic tissues was performed blindly according to our previously described methods [23].

2.6. Immunohistochemical analysis

The fixed pancreatic tissues were embedded and sliced into 5 μm thick sections which were sticked on the glass slides. The slides were treated with dewaxing, dehydration, antigen retrieval, blocking endogenous peroxidase and blocking with normal goat serum. The slides were incubated overnight with anti-Hif1α antibody(1:100 dilution),anti-RIP3 antibody (1:100 dilution) and anti-p-MLKL antibody (1:100 dilution) in 4 °C. Then it was incubated with biotinylated secondary antibody(1:500 dilution) at room temperature for 15 min. Images were acquired through a light microscope.

2.7. Determination of serum enzymology and ELISA

Serum amylase, lipase and proinflammatory cytokines were analyzed according to kit instructions.

2.8. Determination of ROS in pancreatic tissues

The assays were performed according to our previously described methods [23]. The content of ROS in pancreatic tissue was detected by DHE fluorescent probe. The fresh tissues of the pancreas were embedded in optimal cutting temperature (OCT) compound, and samples were cut into 7 μm section. Tissues were incubated in the dark with DHE solution for 30 min at 37 °C. Wash the slides were washed with PBS (pH = 7.4, 3*5min). Then tissues were incubated by 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) solution at room temperature for 10 min and washed again. Finally, the slides were observed under the fluorescence microscope.

2.9. Cell culture and cell viability determination

The mouse pancreatic acinar cell line 266-6 was purchased from ATCC (Manassas, VA, USA). The 266-6 cells were cultured in DMEM medium containing 10 % fetal bovine serum(FBS), 100U/ml penicillin and 100U/ml streptomycin in 37 °C, 5 % CO_2 incubator. 266-6 cells were treated with different concentrations of PX-

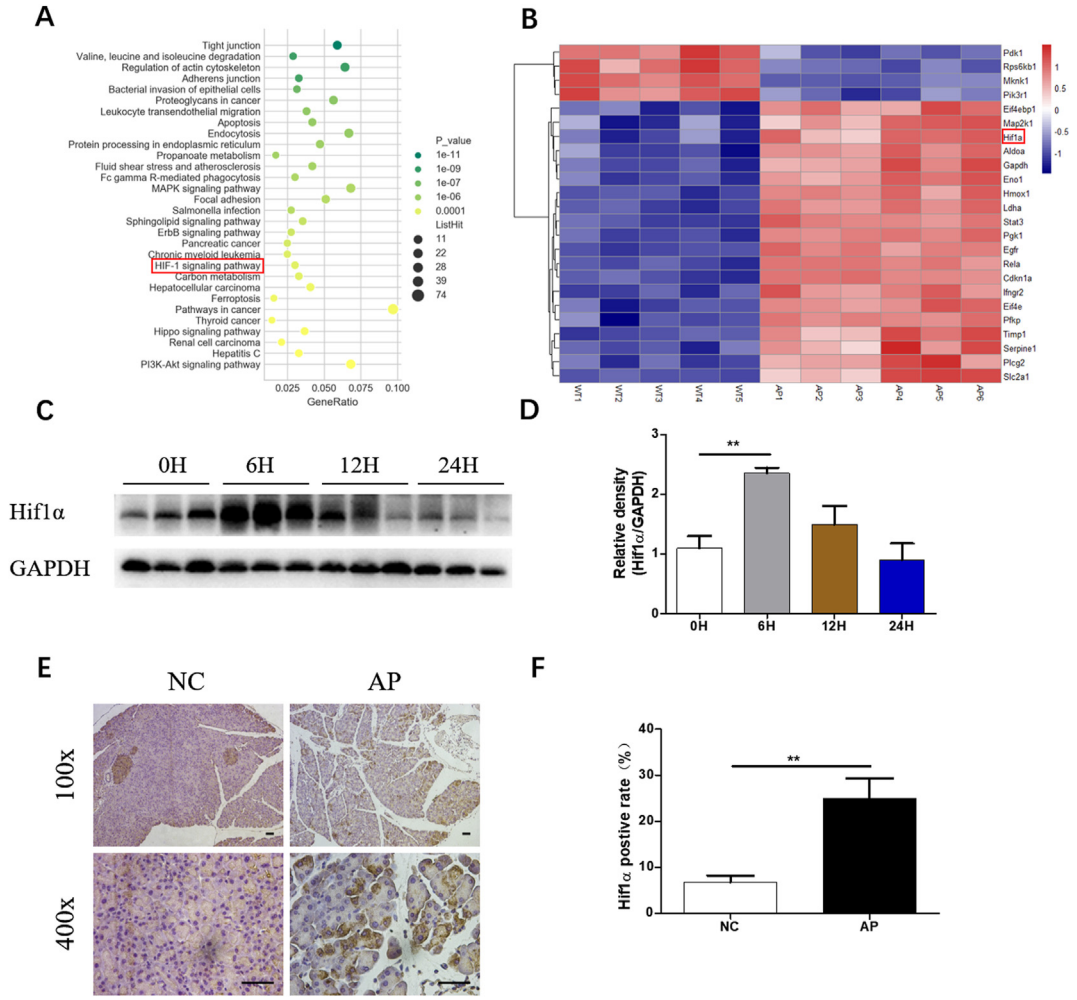

Fig. 1. Hif1α is significantly overexpressed in the pancreatic tissue of AP mice.
(A) KEGG pathway enrichment analysis of all differentially expressed genes of RNA-seq data, (AP versus WT). (B) Heatmap analysis of differentially expressed genes of HIF1 signaling pathway from the pancreas of WT and AP mice. (C) Protein levels of Hif1α in pancreatic tissues were analyzed by western blotting (D) Relative density of Hif1α. GAPDH was used as control for protein loading, N = 3 each group. (E) Representative immunohistochemistry images for Hif1α expression in the pancreas at 6 h in caerulein-induced AP mice. Scar Bar = 50μM. N = 4 each group.(F) Hif1α positive rate of pancreatic acinar cells. *P < 0.05 and **P < 0.01.

478(1,5,10 μM) for 30min, and then stimulated with 5 μM CCK for 12 h to establish AP cell injury model in vitro. The 266-6 cells of the control group were treated with 0.1%DMSO. Finally, the cells were collected for subsequent western blot and flow cytometry analysis. These cells were incubated with Propidium Iodide (PI) or DHE fluorescent probe for flow cytometry analysis. The cell supernatant was collected to detect the LDH according to the LDH release kit.

2.10. Western blot

The protein was extracted by RIPA lysate (Beyotime Biotechnology, Beijing,China) containing protease inhibitor (Roche, Shanghai, China) and phosphatase inhibitor (1:50 dilution, Beyotime Biotechnology, Beijing, China). Protein concentrations were measured via BCA kit (Thermo Fisher Scientific,MA,USA). Protein samples were subjected to 10 % SDS- PAGE, transferred to PVDF membranes and membranes were blocked with 5 % skim milk at room temperature for 2 h. The membranes were incubated overnight with primary antibody,anti-Hif1α (1:1000 dilution), anti-GAPDH(1:2000 dilution),anti-RIP3(1:1000 dilution),anti-MLKL(1:1000 dilution),anti-p-MLKL(1:1000 dilution) in 4 °C. In the second day, wash the membranes with TBST(3*15 min), and the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies(1:5000 dilution), anti-rabbit or anti-mouse lgG, at room temperature for 2 h. Wash the membranes with TBST(3*10 min) and use the ECL Plus chemiluminescent system to detecte the protein bands. The gray value of images was analyzed by ImageJ.

2.11. Immunofluorescent staining

The 266-6 cell were collected for immunofluorescent staining by incubating with Hoechst33342(10μg/ml,37 °C, 20min) and PI(10 μm PI working liquid,37 °C, 15min) according to kit instructions.

2.12. Statistical analysis

Statistical analysis was performed by GraphPad Prism 6 software (GraphPad, San Diego, CA, USA) and the results were presented as mean ± standard deviation (SD). The difference between two groups was analyzed by *t*-test, and the difference between more than two groups was analyzed by one-way ANOVA test. P < 0.05 was considered statistically significant (two-tailed).

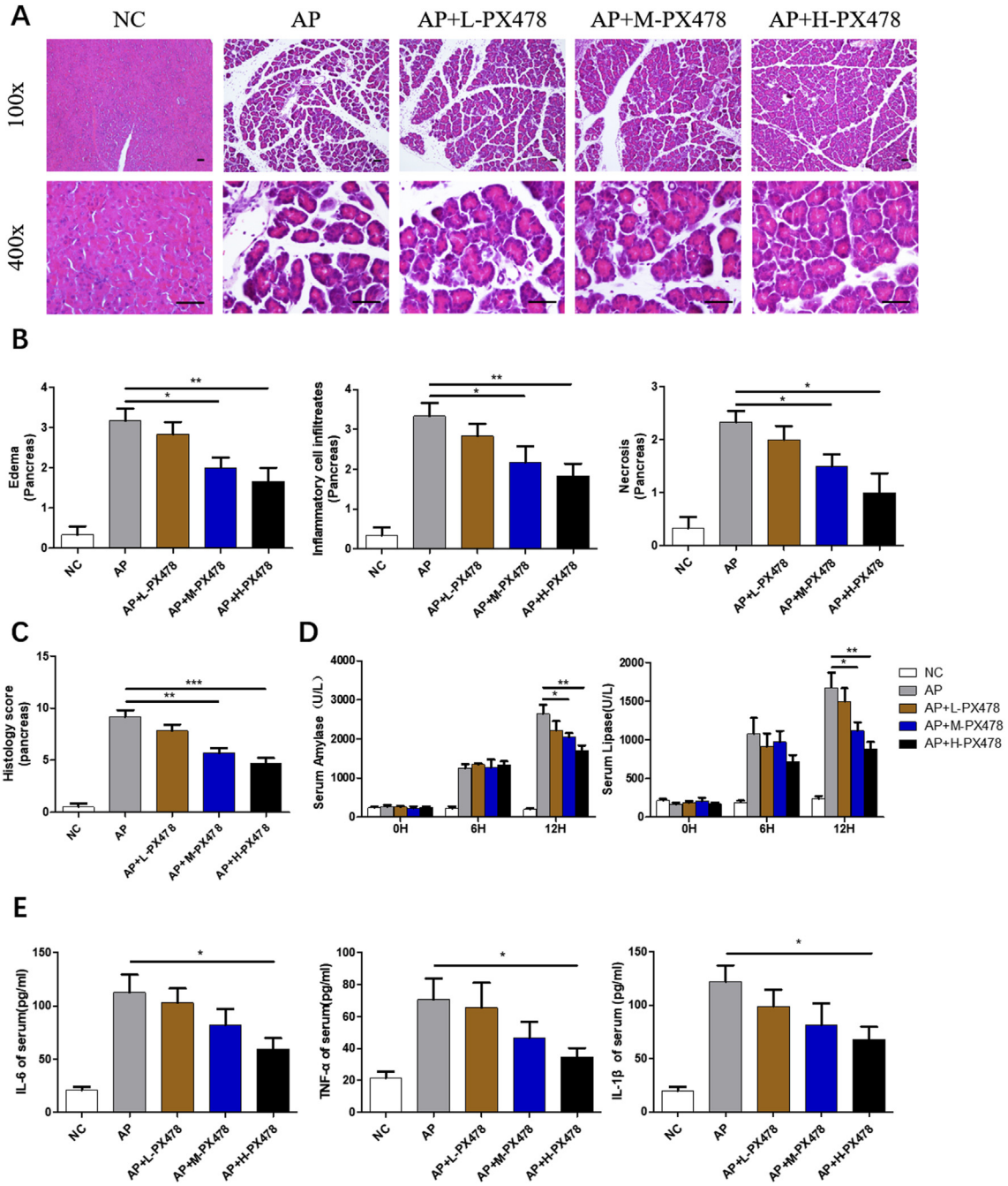

Fig. 2. Hif1α inhibitor PX478 could alleviate caerulein-induced AP in mice.
(A) Representative HE staining of pancreatic tissues in magnifications 100x and 400x.Scar Bar = 50μM. (B–C) The pathological scores of pancreatic tissues. (D) Serum levels of amylase and lipase. (E) The serum levels of IL-6, TNF-α and IL-1β were detected by ELISA. N = 7 each group. *P < 0.05,**P < 0.01 and ***P < 0.001. L, M and H represent low-dose (25 mg/kg), medium-dose (50 mg/kg) and high-dose PX478 (100 mg/kg).

3. Results

3.1. Hif1α is significantly overexpressed in the pancreas of AP mice

As shown in Fig. 1A and B, we analyzed RNA-seq data through public database and found that pancreatic tissues of WT mice had significantly changed in Hif1 signaling pathway compared with those of AP mice and Hif1α was significantly upregulated in pancreatic tissues of AP mice. Subsequently, we verified the high expression of Hif1α in the pancreatic tissues of AP mice by western blot and IHC staining shown in Fig. 1C–F.

3.2. Hif1α inhibitor PX478 could alleviate caerulein-induced AP in mice

In order to clarify the role of Hif1α on AP mice, we applied the Hif1α inhibitor PX478 to inject intraperitoneally into AP mice at 0 h. As shown in Fig. 2A–C, pancreatic tissues in AP group showed significant edema, inflammatory cells infiltration and acinar cell necrosis compared with NC group. Among them, the low-dose PX478 (L-PX478,25 mg/kg) group had insignificant protective effect compared with the AP group. However, pathological injury of pancreas was improved in the medium-dose PX478 (M-

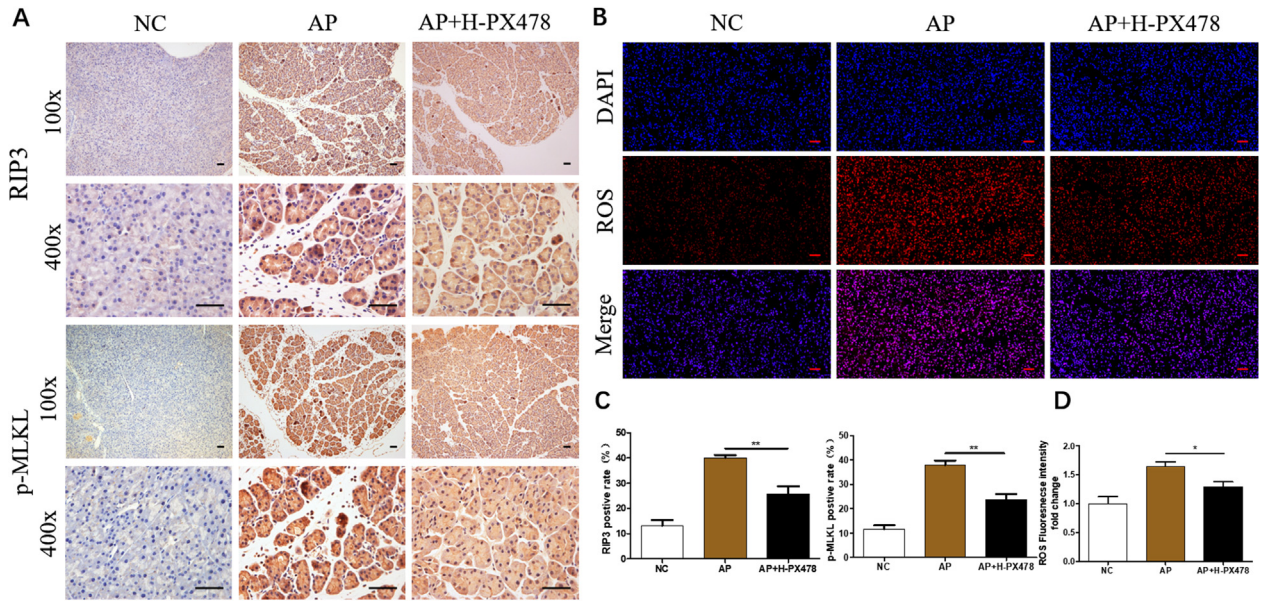

Fig. 3. Hif1α inhibitor PX478 reduced acinar cell necrosis and ROS production in mice.
(A) Representative immunohistochemistry images for RIP3 and p-MLKL expression in the pancreas in magnifications 100x and 400x. Scar Bar = 50μM. (B) Representative immunofluorescence image of DHE in magnification 200x. Scar Bar = 50μM. (C) RIP3 and p-MLKL positive rate of pancreatic acinar cells. N = 4 each group. (D) Densitometric analysis of DHE fluorescence. N = 4 each group. *P < 0.05 and **P < 0.01. H represent high-dose PX478 (100 mg/kg).

PX478,50 mg/kg) group and the levels of amylase and lipase in serum were also significantly decreased. And the high-dose PX478 (H-PX478,100 mg/kg) had best protective effect, as shown in Fig. 2A–D. In addition, We further measured serum levels of pro-inflammatory cytokines IL-6, TNF-α and IL-1β to assess systemic inflammatory response of AP mice. As shown in Fig. 2E, PX478 reduced the serum levels of pro-inflammatory cytokines IL-6, TNF-α and IL-1β compared with AP group, which was in accordance with the degree of pancreatic injury. All results indicated the Hif1α inhibitor PX478 was dose-dependent to protect pancreatic tissue from damage of caerulein-induced AP in mice. Therefore, We used high-dose PX478 for detecting the protection and mechanism. In addition, We applied two other Hif1α inhibitors, KC7F2 (2.5 mg/kg) and IDF11774 (25 mg/kg), which also significantly protected against caerulein-induced AP in mice(Supplementary Fig. 1A-1D). All of these results indicated that target inhibiting Hif1α could protect against caerulein-induced AP in mice.

3.3. Hif1α inhibitor PX478 reduced acinar cell necrosis and ROS production in mice

Increasing amounts of evidence support that necroptosis regulated the occurrence and development of AP [24], so we examined the proteins of necroptosis (RIP3 and p-MLKL) and the production of ROS. As shown in Fig. 3A and C, IHC staining showed that RIP3 and p-MLKL were significantly elevated in the pancreatic tissues of AP mice, which were significantly decreased after high-dose of PX478 treatment. In addition, DHE staining showed a significant increase in ROS production in AP mice, and high dose of PX478 could reversed this increase, which is was consistent with IHC staining results, as shown in Fig. 3B and D. All results indicated that Hif1α inhibitor PX478 could attenuate pancreatic acinar cell necroptosis and ROS production in vivo.

3.4. The protective effect of Hif1α inhibitor PX478 on 266-6 cells in vitro

The mouse pancreatic acinar tumor cell line 266-6 were widely applied to conduct cell experiments for mechanistic studies in pancreatic disease [25–27]. To further clarify the protective effect of PX478 in AP, we used cholecystokinin (CCK) and 266-6 cell lines to establish an AP model in vitro. As shown in Fig. 4A–E, we found that both 5 and 10 μM PX478 could reverse 266-6 cell death, ROS production and LDH release induced by CCK, and 10 μM PX478 had the best protective effect. We applied 10 μM PX478 for the study of mechanism. Hoechst/PI IF staining showed 10 μM PX478 protected 266-6 cells from CCK-induced damage, as shown in Fig. 4F and H. Besides, PX478 could reduce the expression of necroptosis pathway related proteins RIP3 and p-MLKL, which is increased by CCK, as shown in Fig. 4G and I. All of these results indicated that PX478 could protect pancreatic acinus cells and reduce the generation of ROS through necroptosis signal pathway in vitro.

4. Discussion

In summary, we applied a Hif1α inhibitor PX478 and found it could alleviate caerulein-induced AP in vivo. Then, we confirmed that PX478 reduced acinar cells necrosis during AP both in vivo and in vitro. Therefore, Hif1α may be a potential target for the prevention and treatment of AP clinically in the future.

Pancreatic acinar cells, the major cell type of the pancreas, often suffer injury in AP. And its degree of injury correlates with the severity and prognosis of AP. Clinically, mild AP takes the majority. However, several AP patients would progress to severe AP, which begins with the regulated cell death, then initiating local inflammation, forming inflammatory cascade and eventually leading to systemic inflammatory response syndrome (SIRS) and organ failure. Nowadays, there are no specific drugs preventing mild AP

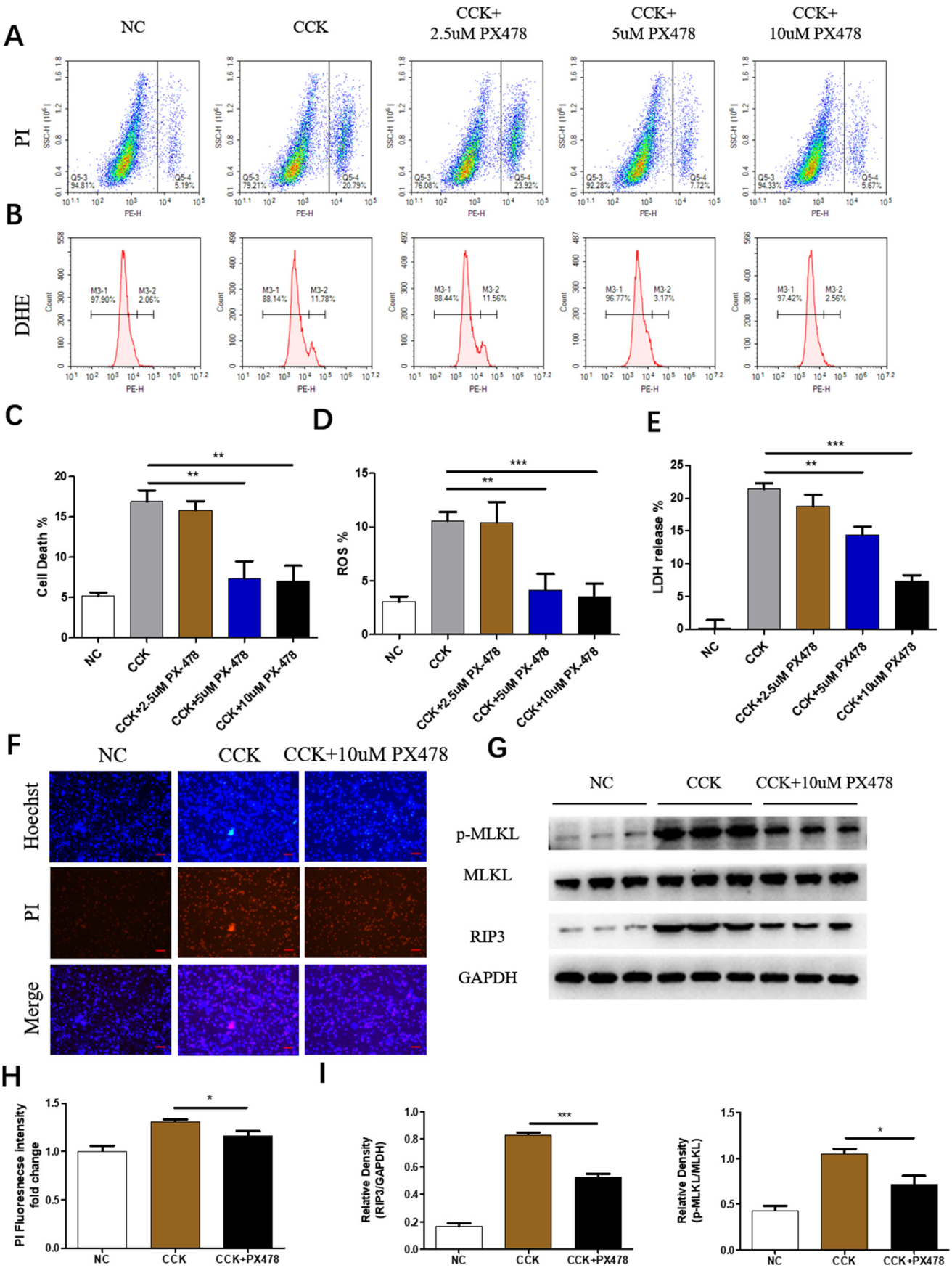

Fig. 4. The protective effect of Hif1α inhibitor PX478 on 266-6 cells in vitro.
(A) PI was detected by flow cytometry. (B) The reactive oxygen species (ROS) was detected by DHE fluorescent probe.(C)The cell death of 266-6 cells.(D)The ROS production of 266-6 cells.(E)The release of LDH of 266-6 cells.(F and H) Representative immunofluorescence image of PI in magnification 200x and densitometric analysis of PI fluorescence. Scar Bar = 50μM. (G) Protein levels of RIP3 and p-MLKL in 266-6 cells were analyzed by Western blotting.(I) Relative protein expression of RIP3 and p-MLKL. GAPDH and MLKL were used as control for protein loading respectively. *P < 0.05,**P < 0.01 and ***P < 0.001. CCK: cholecystokinin octapeptide.

progressing to severe AP, and aimed at the window between acinar cell injury and cell death, inflammation and organ failure [28]. However, inhibiting acinar cell necrosis may be a potential therapeutic target to prevent further cell injury.

Hif1α was discovered in the 3′ enhancer of the erythropoietin (EPO) and forms a helix-loop-helix structure with Hif1β [13]. It has been reported that Hif1α knockdown plus glutamine supplementation attenuated the predominance of necrosis over apoptosis via relieving the intracellular energy stress in AP [29]. In addition, Researchers found that Hif1α participated in necroptosis signal pathway in ischemic brain injury and Hif1α protein level could be downregulated by a RIP3 inhibitor GSK872 and RIP3 siRNA [30]. Hence, Hif1α may be involved in acinar cell necrosis in AP. In public database, we found that Hif1α was significantly upregulated in pancreatic tissues of AP mice, and verified that Hif1α participated in AP.

The formation of necrosome (RIP3 and p-MLKL) are biomarkers for the detection of necroptosis, a new form of necrosis [31]. Necrosulfonamide (NSA), a MLKL inhibitor, could attenuate lung ischemia-reperfusion injury (IRI) [32]. It has been reported that RIP3 and MLKL knockout mice significantly reduced acinar cell necroptosis in caerulein-induced AP compared with wild-type mice [33,34]. In addition, the generation of ROS may be increased in the injury pancreatic tissues of AP mice, which closely related to the occurrence and development of AP.

Researchers found that ethanol and the fatty acid palmitoleic acid (EtOH/POA) induced ROS production, zymogen activation and IL-6 expression, thus resulting in pancreatic acinar cell injury [35]. Recently, researchers found targeted inhibition of ROS production and necroptosis signaling pathways could improve the progression of AP [36,37]. In this study, we found that Hif1α inhibitor PX478 reduced the formation of necrosome (RIP3 and p-MLKL) and ROS production during AP both in vivo and in vitro.

These findings justify the Hif1α signal pathway is significantly upregulated in pancreas of AP, prompting that Hif1α participated in the progress of pathophysiology on AP. Moreover, we applied three Hif1α inhibitors to conduct animal experiments and found that all of them alleviated necrosis of pancreatic acinar cells in AP mice, which suggested that inhibition of Hif1α might be potential therapeutic target for AP. However, it remains unclear whether other typical types of cell death would be influenced by the Hif1α signal pathway, such as apoptosis, pyroptosis, autophagy, etc.

5. Conclusion

Our results demonstrated that inhibition of Hif1α may alleviate AP by reducing the generation of ROS and regulating necroptosis signal pathway. In the future, inhibition of Hif1α maybe a promising therapeutic treatment for AP clinically.

Declaration of competing interest

All authors of this paper have no conflicts of interest to disclose.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 81801970, 82070668); Social development projects of Yangzhou (No. YZ2018091); Major public health projects in Yangzhou: Screening projects of early gastrointestinal diseases (2018) and the National Natural Science Foundation of Yangzhou (No. 2018YXZX20184, Gastroenterology).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.07.043.

References

[1] S.D. Crockett, S. Wani, T.B. Gardner, Y. Falck-Ytter, A.N. Barkun, American Gastroenterological Association Institute Clinical Guidelines Committee, American gastroenterological association institute guideline on initial management of acute pancreatitis, Gastroenterology 154 (4) (2018) 1096–1101.
[2] P.G. Lankisch, M. Apte, P.A. Banks, Acute pancreatitis, Lancet 386 (9988) (2015) 85–96.
[3] S.M. van Dijk, N. Hallensleben, H.C. van Santvoort, et al., Acute pancreatitis: recent advances through randomised trials, Gut 66 (11) (2017) 2024–2032.
[4] N.J. Schepers, O.J. Bakker, M.G. Besselink, et al., Impact of characteristics of organ failure and infected necrosis on mortality in necrotising pancreatitis, Gut 68 (6) (2019) 1044–1051.
[5] A. Saluja, V. Dudeja, R. Dawra, R.P. Sah, Early intra-acinar events in pathogenesis of pancreatitis, Gastroenterology 156 (7) (2019) 1979–1993.
[6] C. Han, D. Du, Y. Wen, et al., Chaiqin chengqi decoction ameliorates acute pancreatitis in mice via inhibition of neuron activation-mediated acinar cell SP/NK1R signaling pathways, J. Ethnopharmacol. (2021) 114029.
[7] J. Luan, J. Kou, N. Huang, et al., Inhibition of CHRM3 alleviates necrosis via the MAPK-p38/miR-31-5p/RIP3 Axis in L-arginine-induced severe acute pancreatitis, Pancreas 49 (10) (2020) 1335–1341.
[8] J. Louhimo, M.L. Steer, G. Perides, Necroptosis is an important severity determinant and potential therapeutic target in experimental severe pancreatitis, Cell Mol Gastroenterol Hepatol 2 (2016) 519–535.
[9] J. Zhao, S. Jitkaew, Z. Cai, et al., Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis, Proc. Natl. Acad. Sci. U. S. A. 109 (14) (2012) 5322–5327.
[10] L. Sun, H. Wang, Z. Wang, et al., Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase, Cell 148 (1–2) (2012) 213–227.
[11] H. Wang, L. Sun, L. Su, et al., Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3, Mol Cell 54 (1) (2014) 133–146.
[12] G. Wang, F.Z. Qu, L. Li, J.C. Lv, B. Sun, Necroptosis: a potential, promising target and switch in acute pancreatitis, Apoptosis 21 (2016) 121–129.
[13] Q. Ke, M. Costa, Hypoxia-inducible factor-1 (HIF-1), Mol. Pharmacol. 70 (5) (2006) 1469–1480.
[14] G.L. Semenza, Hypoxia-inducible factor 1 (HIF-1) pathway, Sci. STKE 2007 (407) (2007) cm8.
[15] X. Li, Q. Zhang, M.I. Nasser, et al., Oxygen homeostasis and cardiovascular disease: a role for HIF, Biomed. Pharmacother. 128 (2020) 110338.
[16] T. Zhang, C. Suo, C. Zheng, H. Zhang, Hypoxia and metabolism in metastasis, Adv. Exp. Med. Biol. 1136 (2019) 87–95.
[17] A.A. Urrutia, J. Aragonés, HIF oxygen sensing pathways in lung biology, Biomedicines 6 (2) (2018).
[18] M.J. Park, S. Iyer, X. Xue, J. Bragazzi Cunha, S. Gu, D. Moons, S.W. Pipe, J.A. Williams, D.M. Simeone, Y.M. Shah, M.B. Omary, HIF1-alpha regulates acinar cell function and response to injury in mouse pancreas, Gastroenterology 154 (2018) 1630–1634.e3.
[19] K.J. Norberg, S. Nania, X. Li, H. Gao, P. Szatmary, R. Segersvärd, S. Haas, A. Wagman, U. Arnelo, R. Sutton, R.L. Heuchel, J.M. Löhr, RCAN1 is a marker of oxidative stress, induced in acute pancreatitis, Pancreatology 18 (2018) 734–741.
[20] R core Team, R:A Language and Environment for Statistical Computing, R Foundat ion for statistical computing, Vienna, Austria, 2020. https://www.R-project.Org/.
[21] H. wickham, ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag, New York, 2016.
[22] Raivo Kolde, pheatmap: pretty Heatmaps. R package version 1.0.12. https://CRAN.R-project.org/package=pheatmap, 2019.
[23] X. Liu, Q. Zhu, M. Zhang, et al., Isoliquiritigenin ameliorates acute pancreatitis in mice via inhibition of oxidative stress and modulation of the Nrf2/HO-1 pathway, Oxid Med Cell Longev 2018 (2018) 7161592.
[24] M. Boonchan, H. Arimochi, K. Otsuka, T. Kobayashi, H. Uehara, T. Jaroonwitchawan, Y. Sasaki, S.I. Tsukumo, K. Yasutomo, Necroptosis protects against exacerbation of acute pancreatitis, Cell Death Dis. 12 (2021) 601.
[25] P. Srinivasan, S. Nabokina, H.M. Said, Chronic alcohol exposure affects pancreatic acinar mitochondrial thiamin pyrophosphate uptake: studies with mouse 266-6 cell line and primary cells, Am. J. Physiol. Gastrointest. Liver Physiol. 309 (2015) G750–G758.
[26] P. Srinivasan, V. Ramesh, J. Wu, C. Heskett, B.D. Chu, H.M. Said, Pyridoxine and pancreatic acinar cells: transport physiology and effect on gene expression profile, Am. J. Physiol. Cell Physiol. 317 (2019) C1107–1107C1114.
[27] K.Y. Anandam, P. Srinivasan, T. Yasujima, S. Al-Juburi, H.M. Said, Proinflammatory cytokines inhibit thiamin uptake by human and mouse pancreatic

acinar cells: involvement of transcriptional mechanism(s), Am. J. Physiol. Gastrointest. Liver Physiol. 320 (2021) G108–108G116.
[28] E. Zerem, Treatment of severe acute pancreatitis and its complications, World J. Gastroenterol. 20 (2014) 13879–13892.
[29] L. Ji, X. Guo, J. Lv, F. Xiao, W. Zhang, J. Li, Z. Lin, B. Sun, G. Wang, Hypoxia-inducible factor-1α knockdown plus glutamine supplementation attenuates the predominance of necrosis over apoptosis by relieving cellular energy stress in acute pancreatitis, Oxid Med Cell Longev (2019) 4363672.
[30] X.S. Yang, T.L. Yi, S. Zhang, Z.W. Xu, Z.Q. Yu, H.T. Sun, C. Yang, Y. Tu, S.X. Cheng, Hypoxia-inducible factor-1 alpha is involved in RIP-induced necroptosis caused by in vitro and in vivo ischemic brain injury, Sci. Rep. 7 (2017) 5818.
[31] S. He, S. Huang, Z. Shen, Biomarkers for the detection of necroptosis, Cell. Mol. Life Sci. 73 (11–12) (2016) 2177–2181.
[32] S. Ueda, T.F. Chen-Yoshikawa, S. Tanaka, et al., Protective effect of necrosulfonamide on rat pulmonary ischemia-reperfusion injury via inhibition of necroptosis, J. Thorac. Cardiovasc. Surg. S0022-5223 (21) (2021), 00134-3.
[33] S. He, L. Wang, L. Miao, et al., Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha, Cell 137 (6) (2009) 1100–1111.
[34] J. Wu, Z. Huang, J. Ren, et al., Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis, Cell Res. 23 (8) (2013) 994–1006.
[35] J. Lee, J.W. Lim, H. Kim, Lycopene inhibits oxidative stress-mediated inflammatory responses in ethanol/palmitoleic acid-stimulated pancreatic acinar AR42J cells, Int. J. Mol. Sci. 22 (4) (2021).
[36] X. Xie, C. Yuan, L. Yin, et al., NQDI-1 protects against acinar cell necrosis in three experimental mouse models of acute pancreatitis, Biochem. Biophys. Res. Commun. 520 (1) (2019) 211–217.
[37] Y.P. Hong, J. Yu, Y.R. Su, et al., High-fat diet aggravates acute pancreatitis via TLR4-mediated necroptosis and inflammation in rats, Oxid Med Cell Longev 2020 (2020) 8172714.