GSDMD in the endoplasmic reticulum exacerbates acute pancreatitis through TXNIP/HIF-1α pathway

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Abstract

Endoplasmic reticulum stress (ERS) and GSDMD mediate acute pancreatitis (AP), but little is known about their mutual influence in AP. The GSDMD accumulation in ER was firstly found in this study, which caused ERS of acinar cells. GSDMD inhibitor DSF (Disulfiram) notably decreased the expression of GSDMD in ER and TXNIP/HIF-1α signaling. TXNIP inhibitor RES (Resveratrol) significantly inhibited TXNIP/HIF-1α, but not down-regulated the accumulation of GSDMD in ER. The molecular docking study indicated that there was a potential interaction between Drug D (Diosgenin derivative D) and GSDMD. Our results showed that Drug D significantly inhibited necrosis of acinar cells dose dependently, and we also found that Drug D moderated pancreatic necrosis and systemic inflammation with the GSDMD down-accumulation in ER. Furthermore, the level of p-IRE1α (a marker of ERS) was also down-regulated by Drug D in a dose-dependent manner in AP. We also found that Drug D alleviated TXNIP up-regulation and oxidative stress in AP. Moreover, our results revealed that $GSDMD^{-/-}$ mitigated AP by inhibiting TXNIP/HIF-1α. Our study reveals a new pathological mechanism for AP.

1. Introduction

Acute pancreatitis (AP) is the most common cause of hospitalisation for gastrointestinal diseases with extremely high morbidity and mortality [1, 2]. Currently, there is no specific treatments for AP. Cleaved GSDMD punches holes in mice acinar cell membrane, resulting in cell death and inflammatory factor release in AP [3, 4]. Endoplasmic reticulum stress (ERS) is a key initiating factor in the development of AP [5], which also causes the release of cell contents and inflammatory cascade. Although both ERS and GSDMD regulate the release of inflammatory cytokines from acinar cells in AP mice, their effects on the pathogenesis of AP remain unclear.

Thioredoxin-interacting protein (TXNIP) [6–10] is an endogenous thioredoxin negative modulator that plays an important role in keeping the redox balance in cells. TXNIP has been widely studied in other disease models, such as pancreatic cancer [11, 12] and diabetes [13, 14]. In addition, TXNIP is related to reactive oxygen species (ROS) bursts in cells. Studies have shown that the accumulation of GSDMD in mitochondria, causing ROS bursts [15]. Whether the gathering of GSDMD in the ER of acinar cells also regulates AP through the TXNIP signaling pathway is controversial.

Acute lung injury is a common serious complication of AP, which leads to hypoxemia in clinical and experimental AP [16]. HIF-1α (Hypoxia-inducible factor-1α) is a transcription factor that is over-expressed in the presence of hypoxia [16]. Studies have shown that TXNIP regulates diabetic peripheral neuropathy by regulating HIF-1α [16]. Therefore, whether ERS regulates the occurrence of oxidative stress through TXNIP and HIF-1α in AP mice is unclear.

Therefore, it is necessary to investigate whether the accumulation of GSDMD in ER of acinar cells regulates ERS, and then regulates oxidative stress through the TXNIP/HIF-1α signaling pathway in AP.

Diosgenin is a kind of natural steroidal sapogenin extracted from Dioscorea zingiberensis, which has excellent anti-inflammatory properties [17, 18]. A relevant study [18] has shown that Diosgenin prevents AP through mitochondrial protection and PI3Kγ/Akt inhibition. In our research, we further study the mechanism of Diosgenin derivative D (Drug D) in AP.

In our study, we found that ERS induced by GSDMD up-regulation in ER, aggravated AP trough TXNIP/HIF-1α signaling pathway. The GSDMD accumulation of ER was firstly detected in AP. Through GSDMD inhibitor DSF (Disulfiram) and TXNIP inhibitor RES (Resveratrol), we further found that GSDMD regulated AP through TXNIP/HIF-1α pathway. Finally, using GSDMD knockout mice, we explored the regulatory effect of Drug D on GSDMD and TXNIP/HIF-1α pathways, and proved its excellent activity in AP. In summary, our study reveals a new pathological mechanism for AP, and develops a new type of anti-AP drug, laying the foundation for clinical research.

2. Materials And Methods

2.1. Reagents and antibodies

L-Arg monohydrochloride, Dihydroethidium (DHE) dye and DAPI were from Sigma-Aldrich (St. Louis, MO, USA). GSDMD antibody was from Affinity (Shanghai, China), REDD1 Antibody (AF18) and p-NF-Kb (p-P65) were from Santa Cruz Biotechnology (CA, USA). Resveratrol was from Aladdin (Shanghai, China). HIF-1α was from Abcam (Boston, USA). Malondialdehyde (MDA) assay kit, catalase (CAT) assay kit, total glutathione peroxidase (GPX) assay kit with NADPH and anti-β-actin antibody were purchased from Beyotime Biotech (Shanghai, China). Mouse IL-1β elisa kit was purchased from Elabscience Biotechnology Co. Ltd (Wuhan, China). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental animal

Healthy wild type C57BL/6 mice and healthy GSDMD$^{-/-}$ C57BL/6 mice (male, 8 weeks old, weighing 22–25 g) were used to establish the animal model in the experimental, all of which were separately purchased from animal feeding center of Sichuan University Health Science Center (Chengdu, China) and Shanghai Model Organisms (Shanghai, China). Under specific-pathogen-free (SPF) conditions, all mice were kept in individually ventilated cages with wooden blocks as a bed (5 mice per cage) at 25°C and 50% humidity at 12 hours of dark/light cycle. All mice were kept for 7 days before the experiment. Mice were provided standard diet and free tap water. The animal experiment procedure was carried out in accordance with the Guide of Laboratory Animal Care and Use from the United States National Institution of Health and was approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan University, China (20211363A).

2.3. Experimental model

AP models were induced by L-Arg as described previously with minor modification [19, 20]. Mice were injected with 3 g/kg of 14% L-Arg solution intraperitoneally every hour for 3 hours. The L-Arg solution was

prepared in saline and filtered to sterilize. The mice in the control group were injected with sterile normal saline (0.85% sodium chloride). The mice were kept on the heating pad between injections and 4 hours after the last injection. The mice were euthanized 72 hours after the first injection of L-Arg.

2.4. Pancreatic primary acinar cells isolation

Mice pancreatic primary acinar cells were prepared by enzymatic digestion with collagenase IV as described previously [21]. In short, the pancreas from mice were taken out and digested with collagenase IV (200 U·mL-1) at 37°C for 19 minutes. After incubating with Collagenase IV, the separated cells were mechanically broken and filtered through a 100 μm cell strainer, and then centrifuged at 700 rpm for 2 minutes to obtain cell pellets. The cells were resuspended in an extracellular solution, which were stored in extracellular solution. The components [22] of the extracellular solution are: 140 mM NaCl, 4.7 mM KCl, 1.13 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], and 10 mM D-glucose at pH 7.35–7.45. The cells were then treated at room temperature and used within 4 hours after separation.

2.5. Endoplasmic reticulum protein extraction

ER proteins were prepared using the ER extraction kit (SIGMA, ER0100) according to the manufacturer's instructions.

2.6. Histopathological examination

Paraffin sections (5 μm) of pancreas tissues were stained with hematoxylin and eosin (H&E). The pancreatic histopathology score was blindly assessed by two pathologists for edema, inflammatory cell infiltration, and necrosis, from 0 to 3, assessed as previously described [18, 23]. Each slide was observed under an optical microscope (ZEISS, Jena, GMBH).

2.7. Immunofluorescence staining

Briefly, we prepared a paraffin section (3 μm) of the pancreas and dissolved blank goat serum in PBST containing 0.05% Tween 20 to make the concentration reach 5%, and then block for 0.5 hour. Slices were then incubated with anti-GSDMD (1:100 dilution) and anti-CALNEXIN (1:100 dilution) antibodies overnight at 4°C at the same time. Then slices were incubated with corresponding secondary antibodies (1 hour, 37°C) in dark. After washing, DAPI (4',6-diamidino-2-phenylindole) (1:2000 dilution) was used for nuclear staining (10 minutes, 37°C) in dark. Stained specimens were visualized with a fluorescence microscope (LEICA LAS X, Germany). The staining of TXNIP (1:100), HIF-1α (1:100), REDD1 (1:100) and p-IRE-1α (1:300) was as described above.

Isolated primary acinar cells were incubated with L-Arg for 50 minutes, then fixed with 4% paraformaldehyde for 1 hour, next incubated with anti-GSDMD (1:100 dilution) and anti-CALNEXIN (1:100 dilution) antibodies overnight at 4°C in 24-well-plate. Then cells were incubated with corresponding secondary antibodies (1 hour, 37°C) in dark. Then counterstained with DAPI for 10 minutes. The results were showed with a fluorescence microscope (LEICA LAS X, Germany).

2.8. Necrotic cell death measurement

Freshly primary acinar cells were treated with L-Arg (60 mM) and incubated at room temperature for 50 minutes with or without various concentrations of Drug D (50 μM, 100 μM and 200 μM). Then Hoechst 33342 (50 μg/mL) and propidium iodide (PI, 1 μmol/mL) were used to stain total nuclei and necrotic primary acinar cells characterized by plasma membrane rupture [24], respectively. Automatic ZEISS AX10 imager A2/AX10 cam HRC (Jena GmbH, Germany) was used to record the images. The total number of acinar cells showing PI uptake was calculated from each condition, with a minimum of 1000 cells counted, to provide the percentage (necrosis %) with five isolates per condition.

2.9. Oxidative stress measurement

According to the instructions, the activities of glutathione (GSH), Catalase (CAT), Glutathione peroxidase (GPX) and malondialdehyde (MDA) in pancreas tissues were detected by a commercial biochemical kit (built in Nanjing, China). The results were showed with a fluorescence microscope (LEICA LAS X, Germany), and select a representative field of view for application.

2.10. IL-1β level measurement

According to the instructions, the level of IL-1β in pancreatic tissues was detected by a commercial biochemical kit.

2.11. Western blot analysis

Protein lysates were prepared from pancreatic tissues or isolated primary acinar cells by homogenizing in RIPA buffer containing protease and phosphatase inhibitors. Twenty micrograms of protein lysate were loaded on a 12% polyacrylamide gel. Use primary antibodies TXNIP (1:1000), REDD1 (1:1000), p-P65 (1:1000), p-IRE1-α (1:1000) and β-actin (1:1000). The image was detected by an enhanced chemiluminescence (ECL) detection system (Millipore, USA). β-actin was used as a loading control. Data were collected and analyzed from 3 independent samples.

2.12. Serum amylase, amylase and LDH secretion measurement

Blood samples were collected, centrifuged at 3000 rpm for 10 minutes, and 50 μL of serum was diluted to 200 μL. Serum lipase, amylase and LDH were measured by an automatic biochemical analyzer (Roche, Mannheim, Germany) according to the product specification.

2.13. Molecular docking studies

In silico investigation was carried out to rationalize the binding mechanism of Drug D with GSDMD (PDB ID: 6N9N). The chemical composition and protein crystal structure of Drug D and GSDMD were imported into Discovery Studio 2019 software. The docking process was done by using the docking optimization CDOCKER tool in the software.

2.14. Statistical analysis

The data and statistical analysis are in line with the recommendations of pharmacological experiment design and analysis [18, 25, 26]. Data are expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) or Student t-test. GraphPad Prism version 5.01 (GraphPad Prism Software Inc., San Diego, CA, USA) was used for statistical analyses and preparation of figures. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 GSDMD in ER of acinar cells is up-regulated in L-Arg-AP

The necrosis of acinar cells, the functional unit of the exocrine pancreas, is an important event in AP [1]. Although both ERS and GSDMD regulate the release of inflammatory cytokines from acinar cells in AP mice [3–5], their effects on the pathogenesis of AP remain unclear. Firstly, this study performed immunofluorescence staining on GSDMD and ER marker protein CALNEXIN [27] to explore the relationship between GSDMD and ER. The results showed that L-Arg significantly up-regulated the accumulation of GSDMD in ER of acinar cells (Fig. 1a).

Then, the ER protein of mice primary acinar cells was extracted. Firstly, the purity of the isolated ER was confirmed using the ER marker protein CALNEXIN (Fig. 1b). Secondly, we explored the level of GSDMD in ER by immunoblotting, and found that L-Arg increased the level of GSDMD in ER of acinar cells obviously (Fig. 1b). We conclude that L-Arg induces the accumulation of GSDMD in ER of acinar cells.

3.2 Inhibition of GSDMD accumulation in ER ameliorates AP with the down-regulation of TXNIP

To further evaluate the effect of GSDMD in ER in AP, we employed AP models (Fig. 2a). The levels of serum amylase (Fig. 2b), lipase (Fig. 2c) and LDH (Fig. 2d) were significantly increased by L-Arg treatment, accompanied by elevation of pancreas IL-1β (Fig. 2e). As illustrated in Fig. 2f and Fig. 2g, L-Arg induced remarkable pathological changes by edema and increasing infiltration of inflammatory cells. Additionally, DSF [28] significantly attenuated pancreatic damage. As we expected, our results also revealed that the expression of GSDMD was significantly increased in ER in pancreas tissues (Fig. 2h) and primary acinar cells (Fig. 2i). Meanwhile, DSF significantly down-regulated the level of GSDMD in ER (Fig. 2h, i).

TXNIP is related to ROS bursts in cells, which meditates ERS [15, 29]. Studies reveal that the accumulation of GSDMD in mitochondria causes ROS bursts [15]. Interestingly, we found L-Arg increased TXNIP expression remarkably (Fig. 2j). Additionally, DSF treatment decreased the expression of TXNIP obviously (Fig. 2j). Therefore, these results demonstrate that the inhibition of GSDMD accumulation in ER ameliorates AP with the down-regulation of TXNIP.

3.3 TXNIP inhibitor (RES) mitigates pancreatic necrosis, systemic inflammation and oxidative stress in L-Arg induced AP, without changes of GSDMD

In order to explore whether TXNIP signal regulated AP, we used RES (an inhibitor of TXNIP) in mice AP (Fig. 3a). The data revealed that serum amylase (Fig. 3b), lipase (Fig. 3c), LDH (Fig. 3d) were decreased notably after RES was administered, accompanied by down-regulation of pancreas IL-1β (Fig. 3e). As illustrated in Fig. 3f and Fig. 3g, DSF significantly attenuated pancreatic damage. There are literatures show that TXNIP regulates the expression of HIF-1α [30]. As shown in Fig. 3i, RES significantly inhibited the expression of TXNIP and HIF-1α. As expected, it did not affect the expression of GSDMD in the ER (Fig. 3h). We conclude that RES mitigates pancreatic necrosis, systemic inflammation and oxidative stress in L-Arg induced AP via TXNIP/HIF-1α signal pathway.

3.4 Drug D, targeting GSDMD, protects against L-Arg-induced necrosis of primary acinar cells

Preliminary researches in this laboratory found that Diosgenin derivatives had anti-inflammatory and anti-oxidative stress effects [17, 18]. Here we tried to explore whether Drug D (one of Diosgenin derivatives) acted as a GSDMD antagonist to protect AP. Results revealed that Drug D potentially interacted with GSDMD (LEU 23, LYS 52 and ILE 467) (Fig. 4a, b), indicating that Drug D is a GSDMD antagonist. Next, we detected whether Drug D decreased the necrosis of acinar cells, and found that Drug D protected acinar cells from L-Arg damage obviously (Fig. 4c, d). Collectively, Drug D, targeting GSDMD, protects against L-Arg-induced necrosis of acinar cells in vitro.

3.5 Drug D moderates pancreatic necrosis and systemic inflammation with the GSDMD down-accumulation of ER in AP

Then, we further detected whether Drug D protected AP by targeting GSDMD in ER in this study. Here, we evaluated the effects of Drug D on pancreas injury (Fig. 5a). Serum amylase (Fig. 5b), lipase (Fig. 5c) and LDH (Fig. 5d) were decreased dose-dependently after treatment with Drug D, accompanied by the decreased pancreas IL-1β (Fig. 5e). Supportively, the typical pathological changes such as pancreatic tissue oedema, inflammatory cell infiltration and acinar cell necrosis were also alleviated by Drug D (Fig.

5f, g). Besides, the level of p-IRE1α (a marker of ERS) in pancreas tissues was down-regulated by Drug D in a dose-dependent manner (Fig. 5h), which further confirmed that Drug D alleviated ERS. In our study, we also found that the accumulation of GSDMD in ER was mitigated by Drug D obviously (Fig. 5i, j). Therefore, these data demonstrate that Drug D protects AP by regulating the accumulation of GSDMD in ER

3.6 Drug D alleviates TXNIP up-regulation and oxidative stress in AP

On the basis of our results, we here speculated that Drug D regulated the severity of AP through TXNIP up-regulation and oxidative stress. Previous studies report that HIF-1α regulates the expression of REDD1, and REDD1 is involved in the balance of oxidative stress [30–32]. We found that Drug D down-regulated the expression of TXNIP (Fig. 6a, c), HIF-1α (Fig. 6b, c) and REDD1 (Fig. 6c). Additionally, we also found that Drug D decreased the level of MDA (Fig. 6d) and increased the levels of GSH (Fig. 6e), CAT (Fig. 6f) and GPX (Fig. 6g) in a concentration-dependent manner. Collectively, these data indicate that Drug D alleviates TXNIP up-regulation and oxidative stress in AP.

3.7 Knockdown of GSDMD reduces Drug D-inhibited pancreatic necrosis, systemic inflammation and oxidative stress in pancreas tissues

Our results showed that Drug D ameliorated AP through GSDMD/TXNIP/HIF-1α signaling. Then, we further evaluated the effect of $GSDMD^{-/-}$ on AP (Fig. 7a, b). As we expected, $GSDMD^{-/-}$ mice reversed pancreatic tissue oedema, inflammatory cell infiltration and acinar cell necrosis (Fig. 7c). Additionally, we also found that $GSDMD^{-/-}$ mice down-regulated TXNIP/HIF-1α signaling, as shown by the decreased TXNIP (Fig. 7d), HIF-1α (Fig. 7e), p-IRE1α (Fig. 7f), REDD1(Fig. 7g) and NF-kB (Fig. 7g). However, the signaling did not further decrease after Drug D treatment. Conclusively, Knockdown of GSDMD reduces Drug D-inhibited pancreatic necrosis, systemic inflammation and oxidative stress in pancreas tissues.

4. Discussions

AP is an unpredictable and potentially lethal disease [29]. In this study we demonstrated, for the first time, GSDMD was overexpressed in ER of acinar cells, which exacerbated AP by TXNIP/HIF-1α pathway. Our experimental results also confirmed that Drug D, acted as a GSDMD antagonist, efficiently decreased ERS by decreasing the accumulation of GSDMD in ER through TXNIP/HIF-1α pathway in AP.

Previous studies report that GSDMD is increased in AP, resulting in the release of proinflammatory cytokines (IL-1β) and then amplifies the local or systemic inflammatory effects [3, 4]. Blocking the cascaded amplification of the inflammation improves the outcomes of AP [3, 4]. In our study, we also found that GSDMD was significantly up-regulated, which is supported by Gao *et al* [3]. Of particular

interest, we detected the relationship between GSDMD and ER, and found for the first time that GSDMD was accumulated in ER of acinar cells in AP. This observation might be explained by the fact that ER membranes contain cardiolipins, and Gasdermin-N domain of GSDMD show strong preference for binding to cardiolipin [15]. Together, the accumulation of GSDMD in ER of acinar cells regulates the severity of AP.

ERS is caused by persistent external damage factors, which is an initiating factor for AP [5]. However, it is unclear whether the ERS is associated with GSDMD. In consistency with these studies, our study also found that ERS was obviously up-regulated in AP. In addition, we detected the reasons about the ERS, and found for the first time that ERS was induced by the GSDMD accumulation in the ER. Therefore, inhibiting the accumulation of GSDMD in the ER is crucial to protect AP.

TXNIP is a regulator of oxidative stress [14], has been studied in many diseases such as pancreatic cancer [11, 12], but how ERS regulates oxidative stress in AP is still unknown. A previous study demonstrates that TXNIP is regulated by p-IRE1α in hepatocyte [33]. Similarly, we also found that TXNIP was up-regulated by ERS. Meanwhile, we used DSF (an inhibitor of GSDMD) to further detect the relationship between ERS and TXNIP. In our study, we found that DSF down-regulated TXNIP obviously, indicating that the accumulation of GSDMD in ER regulates oxidative stress in AP.

We preliminary studies found that diosgenin and its derivatives had anti-inflammatory and anti-oxidative stress effects [16, 17]. The effects and mechanisms of Drug D in AP are still unknown. In this present study, it was found that Drug D, could bind at allosteric pocket of GSDMD through molecular docking, suggesting that Drug D might be a GSDMD antagonist. Based on the fact that genetic inactivation ($GSDMD^{-/-}$) and specific inhibition (DSF) of GSDMD significantly reduce pancreatic necrosis and systemic inflammation in AP [3, 4]. As expected, we found that $GSDMD^{-/-}$ mice improved AP obviously. However, Drug D did not further improve AP, indicating that Drug D protected AP via targeting GSDMD. Taken together, Drug D is expected to be a potential effective therapeutic strategy for the development and transformation of new drugs. The limitations of our study are as follows: This study was performed in mice; further studies are needed in humans.

In summary, our results show that GSDMD is accumulated in ER of acinar cells, which aggravates AP in local pancreatic symptoms and systemic inflammation. Drug D, as an inhibitor of GSDMD, improves AP by regulating the GSDMD/TXNIP/HIF-1α pathway. Our research provides new methods for clinical treatment of AP.

Declarations

Ethics approval

All animal experiments were performed in accordance with the principle for replacement, refinement, and reduction (the 3Rs) and were approved by the Animal Ethics Committee of Sichuan University (20211363A, Chengdu, China).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Cuicui Zhang], [Hai Niu], [Chengyu Wan], [Xiuxian Yu], [Guang Xin], [Yuda Zhu], [Zeliang Wei], [Fan Li], [Yilan Wang], [Kun Zhang], [Shiyi Li], [Yuman Dong], [Yangying Li] and [Wen Huang]. The first draft of the manuscript was written by [Cuicui Zhang] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

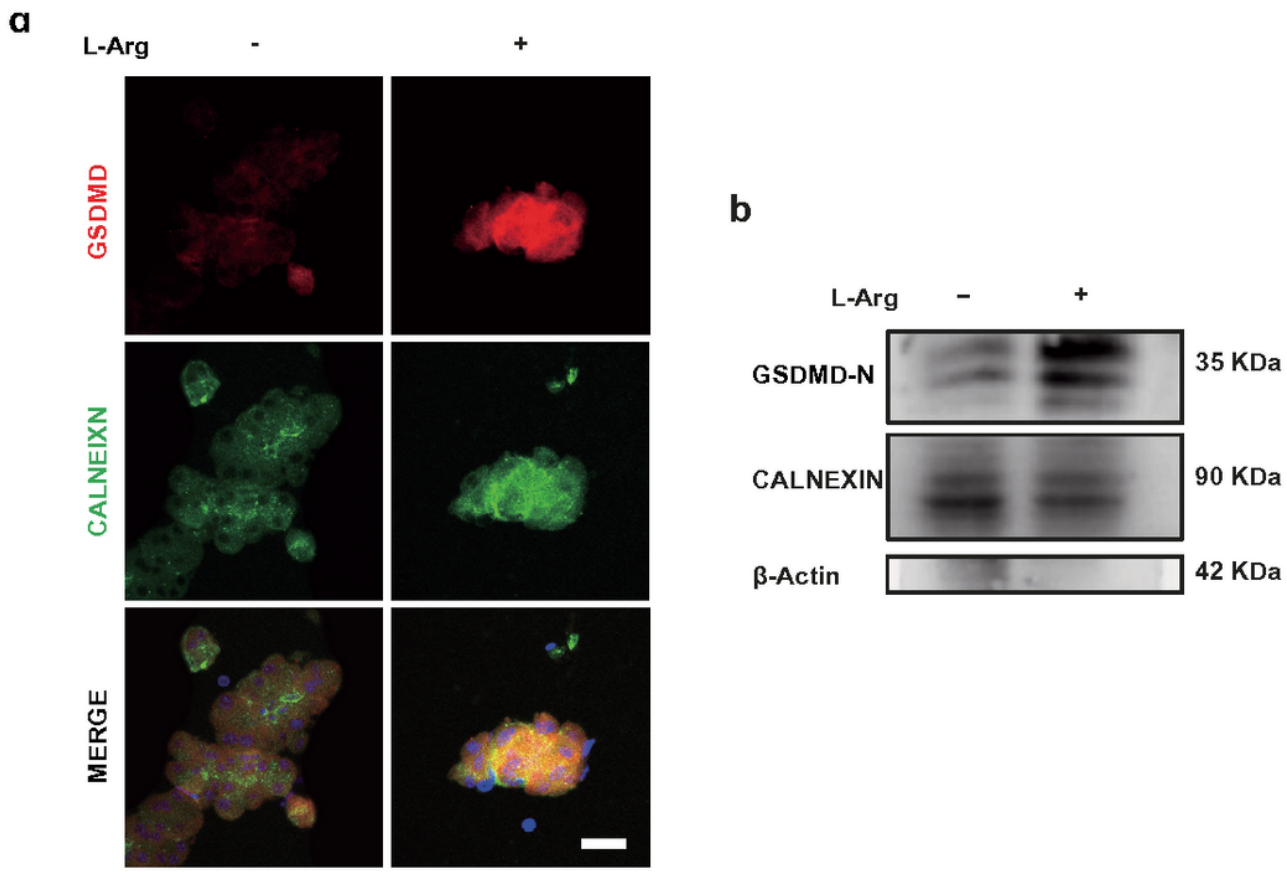

Figure 1

L-Arg induces the accumulation of GSDMD in ER of acinar cells

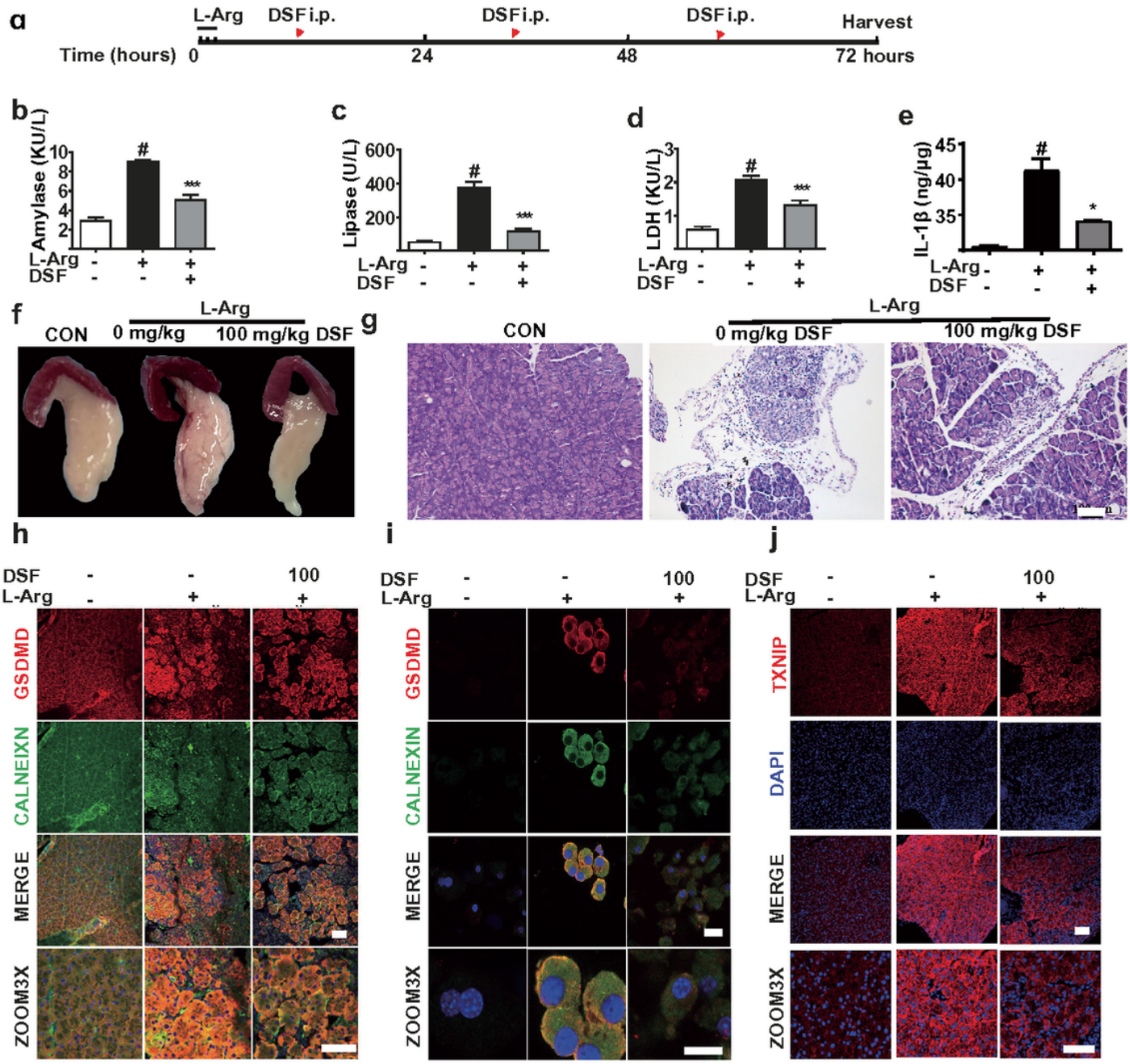

Figure 2

Inhibition of GSDMD accumulation in ER ameliorates AP with the down-regulation of TXNIP

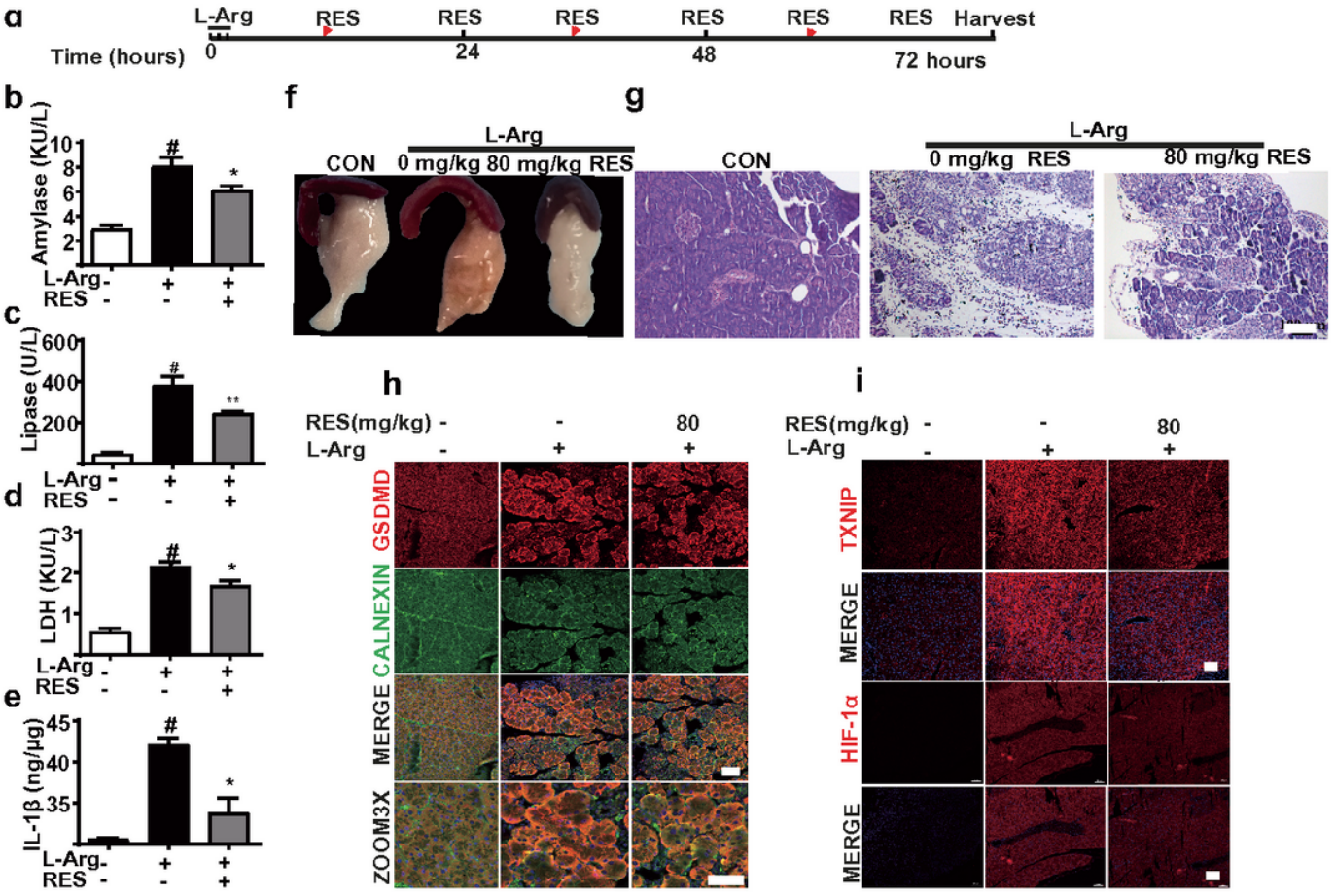

Figure 3

TXNIP inhibitor (RES) mitigates pancreatic necrosis, systemic inflammation and oxidative stress in L-Arg induced AP, without changes of GSDMD

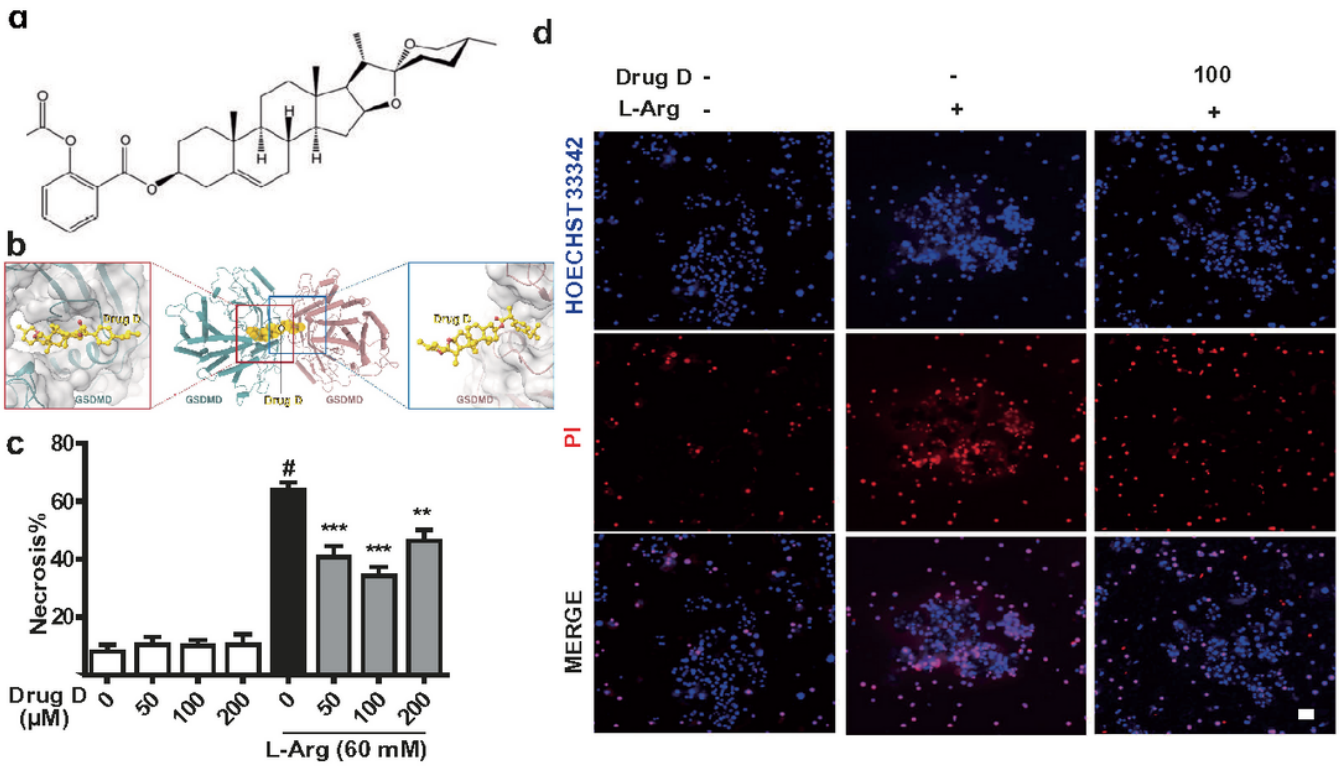

Figure 4

Drug D, targeting GSDMD, protects against L-Arg-induced necrotic cell death pathway activation in isolated primary mice acinar cells

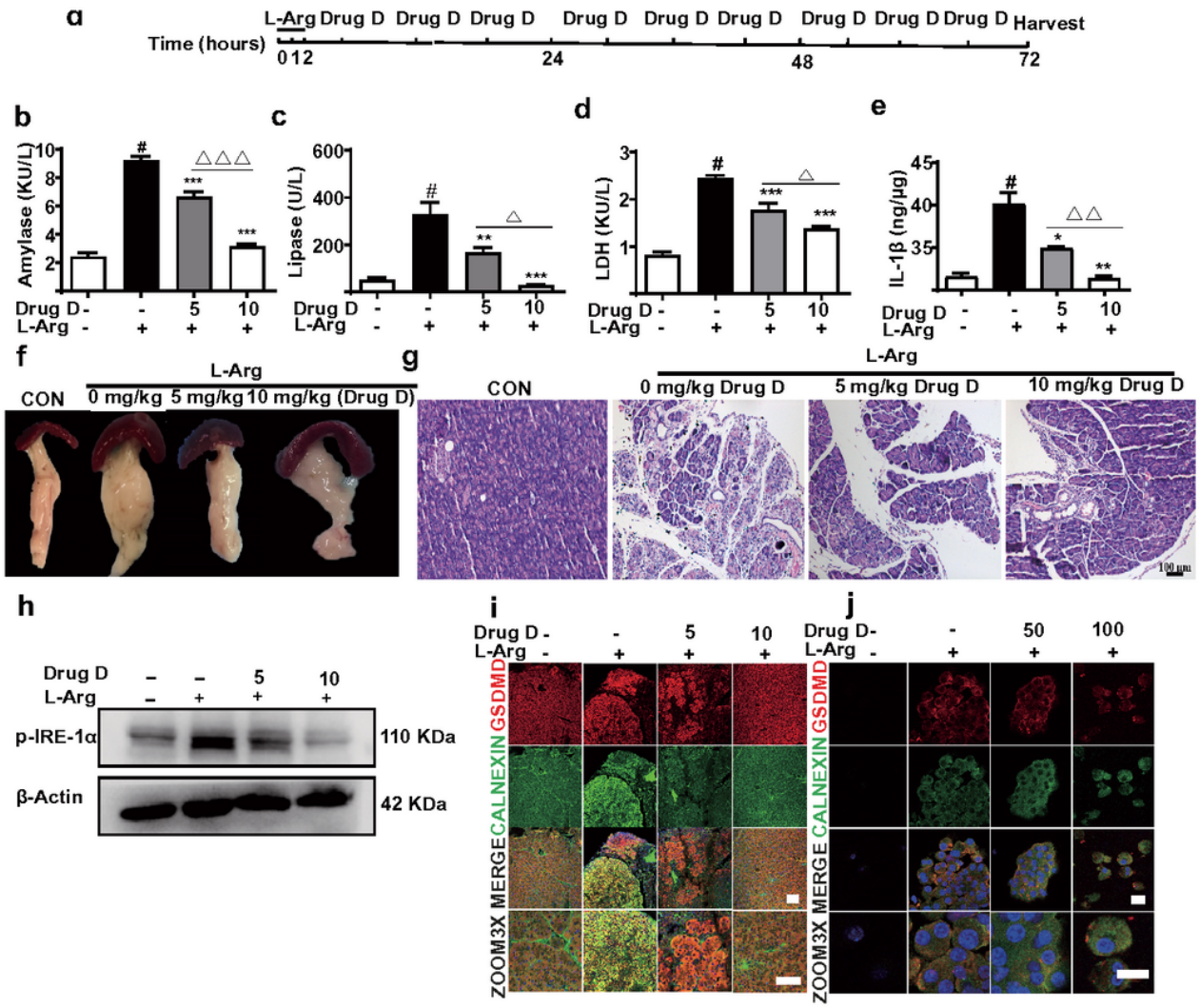

Figure 5

Drug D moderates pancreatic necrosis and systemic inflammation with GSDMD down-accumulation of ER in AP

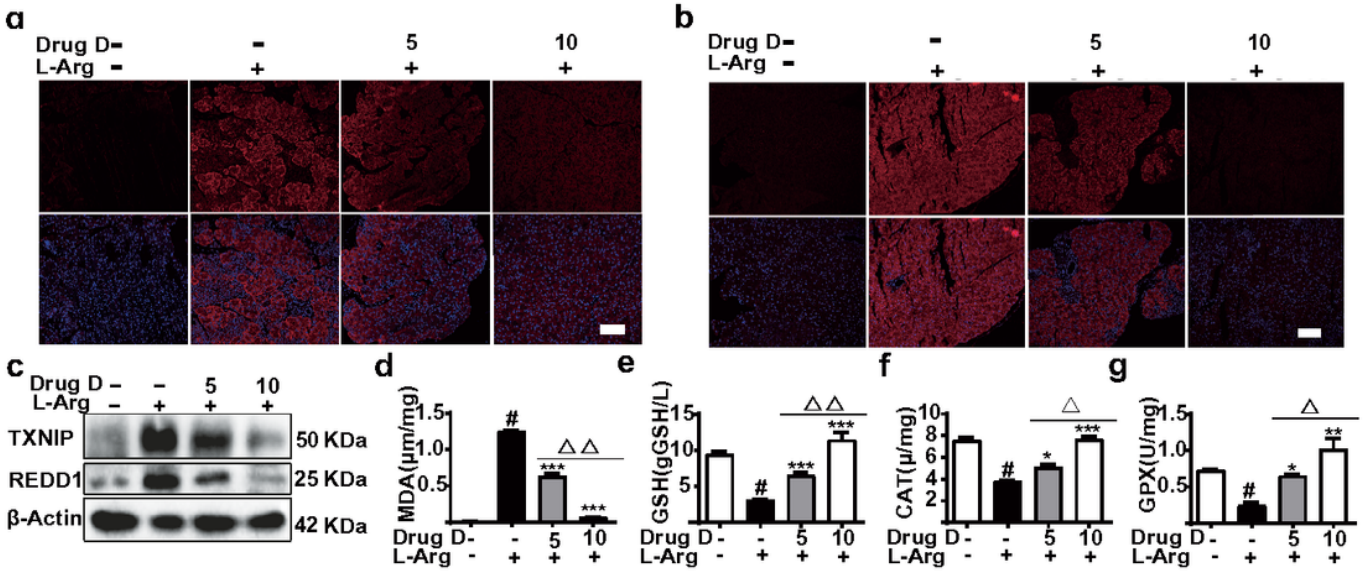

Figure 6

Drug D alleviates TXNIP up-regulation and oxidative stress in AP

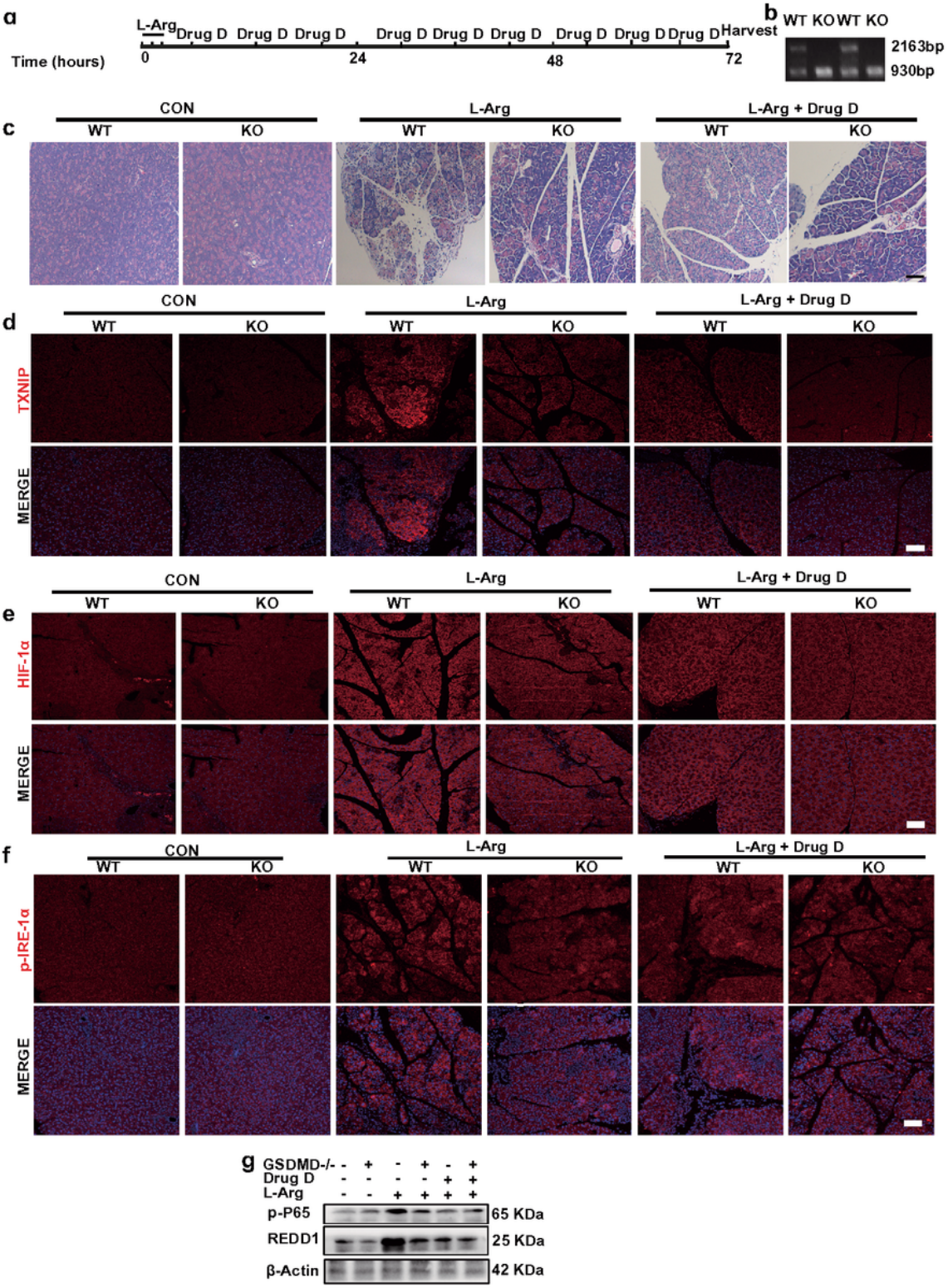

Figure 7

Knockdown of GSDMD reduces pancreatic necrosis, systemic inflammation and oxidative stress in AP