Low Expression of Mas1 Receptor in Pancreas Related with Acute Pancreatitis

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Research

Keywords: Mas1 receptor, intracellular zymogens activation, acute pancreatitis, colocalization of LC3II with trypsin

Posted Date: October 27th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-96232/v1

Abstract

Background: Acute pancreatitis (AP) continues to be one of the most common causes of hospitalization among all gastrointestinal disease. There is a lack of therapies directed to its molecular pathogenesis. The aim of this study was to investigate the role and major mechanisms of Mas1 receptor in AP.

Methods: AP was induced in C57BL/6 mice by administration of caerulein and lipopolysaccharide. The effects of intervening Mas1 receptor on the severity of AP, trypsinogen activation peptide (TAP), zymogens distribution and autophagy activity were detected in vivo. Then Mas1-lentivirus transfected AR42J cell and incubated with caerulein. TAP, autophagy activity and co-location of trypsin and autophagic were further detected in vitro. Human pancreas tissue from patients with AP and without AP were also used for Mas1 receptor protein and mRNA expression levels.

Results: Mas1 receptor proteins were down-regulated in AP mice pancreas tissue as well as in human patients with AP. Compared with Mas1 receptor inhibited in AP mice, AP mice with Mas1 receptor activated decreased severe pathological changes in pancreata, lower levels of trypsinogen activation concomitant with zymogens basolateral distribution, and autophagy down-regulation. Mas1 receptor knockdown and over-expression further verified those results in vitro and showed Mas1 receptor knockdown in AR42J cells had an increased colocalization of LC3II with trypsin.

Conclusions: Mas1 receptor decreased expresssion in pancreas may promote zymogens premature activation relating to AP.

Background

Acute pancreatitis (AP), an exocrine disorder of the pancreas, continues to be one of the most common causes of hospitalization among all gastrointestinal disease, with a significant morbidity, mortality and economic burden[1, 2]. However, there is a lack of therapies directed to its molecular pathogenesis[3]. Chiari proposed that pancreatitis was an auto-digestive disease more than a century ago[4], and several models of AP had shown the intra-acinar activation of the zymogen (unactivated trypsinogen) to trypsin played an important role in the disease pathogenesis[5, 6].

Inhibiting zymogen activation is one of the strategies in AP treatment. The mechanism of zymogen activation remains unknown. However, there is strong evidence that this pathologically process occurs primarily in cytoplasmic vacuoles of acinar cell basolateral region during the early phase of AP[7, 8] and autophagy is involved in the process[9–11]. Autophagy is an early event in pancreatic acinar cells during AP, and regulating autophagy activity can influence the severity of AP[12]. Most studies suggested that autophagy promoted the development of pancreatitis for increased zymogen activation produced by the rising autophagy or for the decreased recyclable small molecules caused by the blocked autophagic flux[13]. However, the opposite view suggested a concerning autophagy called zymophagy that works as a protective role in AP for reducing intracellular activated zymogen[14]. These significantly contradictory

opinions have led the affections and mechanisms of autophagy in zymogen activation in AP controversial.

Mas1 receptor, a class 1 G protein-coupled receptor (GPCR), is known as an important receptor of agonistangiotensin1-7 (Ang(1–7)) and antagonist D-Ala-7-Ang(1–7) (A779). There were lots of evidences that blockade the intraorgan angiotensin-converting enzyme (ACE)–angiotensin (Ang) II–angiotensin II type 1 receptor (AT_1R) played an important role in relieving AP [15]. While ACE2-Ang(1–7) activated Mas1 receptor has been considered as the principle counter-regulatory axis of ACE–AngII–AT_1R[16]. The Mas1 receptor gene and protein expression were significantly increased is associated with a decrease in amylase level according to our previous study[17]. Subsequently, we demonstrated that the therapeutic role of anti-protease ulinastatin may be closely related to the up-regulation of Mas1 receptor and Ang(1–7)[18]. Moreover, Mas1 receptor antagonist A779 has been found to increase amylase secretion[18]and exacerbate severity of AP in AR42J cells[19]. These findings showed that Mas1 receptor may has an important role as negative regulator for pathologic events in AP. Meanwhile, Mas1 receptor mediates Ang(1–7) also has been found to inhibite autophagy in multiple cells and animals. It could mediate cardioprotection via inhibition autophagy[20], and attenuated pulmonary fibrosis by improving impaired autophagic flux [21].

Thus, Mas1 receptor may play a significant role in intra-acinar zymogen activation modulation in AP protection. However, the exact mechanism remains to be elucidated. This article intends to investigate the impact of Mas1 receptor on intra-acinar zymogen premature activation in vivo and vitro, which is the main critical element on the pathogenesis of early AP, to betters how its mechanism in AP and to further reveal the molecular mechanism of zymogen activation from the perspective of autophagy.

Materials And Methods

Reagents and antibodies

Caerulein (CAE), lipopolysaccharide (LPS), Ang(1-7) (an agonists of Mas1), A779 (an antagonists of Mas1) and medium F-12 K were purchased from Sigma Chemical (Sigma-Aldrich, St. Louis, MO, USA). AR42J cell line was from the American Type Culture Collection (ATCC® CRL1492™; ATCC, Rockville, MD). The lentivirus containing Mas1 receptor shRNA and Mas1 receptor over-expression were purchased from Genechem (Shanghai, China); Antibodies against Mas1 receptor was from Alomone Lab (AAR-013, Jerusalem, Israel). Anti-LC3 was from MBL life science (M186-3, Japan). Anti-Trypsin (sc-137077) from Santa Cruz Biotechnology (Dallas, TX)Anti-VAMP8 (ab76021), anti-Munc18c (ab175238), anti-SNAP23 (ab3340), anti-syntaxin4 (Syn4) (ab184545) and anti-SQSTM1 / p62 (ab56416) were from Abcam (Cambridge, UK). HRP-Conjugated GAPDH Antibody (HRP-60004) was from Protein tech (Rosemont, USA). Goat anti–rabbit and goat anti–mouse secondary antibodies from ZSJQ-BIO (Beijing, China); polyvinylidene fluoride membranes (0.45/0.22μm) and enhanced chemiluminescence from Millipore (Darmstadt, Germany); the Image Lab software was from Bio-Rad (Hercules, Calif); Trizol reagent was

from Sigma-Aldrich; and SYBR Green PCR Master Mix, reverse transcription–polymerase chain reaction (RT-PCR) reagents, and cDNA Synthesis Kit from Applied Biosystems (Thermo Fisher, Waltham, Mass).

Human tissue specimens

A tissue microarray containing 6 clinically annotated pancreatic cancer specimens and corresponding non-cancerous tissues were obtained from Beijing Friendship Hospital from February to June in 2017. All the patients enrolled in this study provided written informed consent.

Animals and pancreatitis induction

Mice including wild-type (WT) C57BL6/J (6–8 weeks, 20–22g, male) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were housed under specific-pathogen-free conditions in individually-ventilated cages with wood shavings as bedding material (4–6 mice per cage), 12 h dark/light cycle at 22°C and free access to water and standard rodent diet. All mice (20–22g, male) were marked with an earmark and a randomized table was used; they were allocated into groups in a completely randomized manner (n = 6 per group). All the experiments involving animals were conducted under the principle for replacement, refinement and reduction (the 3Rs) and according to the legislation on the protection of animals and were approved by the Animal Ethics Committee of Capital medical university. Acute pancreatitis was induced in C57Bl/6 mice by hourly intraperitoneal injections of caerulein (100 mg/kg body weight) up to 8 hours, and one-dose LPS (10mg/kg) at the last injection. Animals were sacrificed 0, 0.5, 2, 6, 12 and 24 hours after the last injection.

AR42J cell culture and transfection

AR42J cells were cultured in F-12 K medium supplemented with 20% fetal bovine serum at 37 °C with a humidified atmosphere containing 5% CO2. The lentivirus containing Mas1 receptor knockdown and over-express were purchased from Genechem (Shanghai, China); The cells were plated in six-well plates and transfection was conducted at 70–80% confluence. Transfection of AR42J cells with lentivirus according to the manufacturer's instructions. The sequences of the Mas1 receptor gene are GENE_ID (25153) from Genbank (XM_006227860). The Non-Targeting lentivirus Pool was used as controls. Since successfully transfected, the AR42J cells expressed green fluorescent protein, and the transfection efficiencies of Mas1 receptor lentivirus were observed using an Leica DMI3000B fluorescent microscope. Forty-eight hours after transfection, the cells were used for experiments. The effectiveness of lentivirus in changing Mas1 receptor expression was evaluated by RT-PCR and western blot.

Tissue handling

Pancreas was snap frozen in liquid nitrogen and stored at -80 °C for measurement. For histology, tissue was fixed in 4% formalin for paraffin embedding or embedded in Tissue Tec (OCT, Sakura, Los Angeles, CA) for cryo sections. Collected blood samples were centrifuged and serum was stored at -80°C.

Histological examination

For light microscopy, fresh specimens of murine pancreas were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The tissues were embedded in paraffin, and 5 mm sections were processed for hematoxylin and eosin (H&E) staining by standard procedures. Multiple randomly chosen microscopic fields from at least three mice in each group were examined and scored by two pathologists in a blind manner based on the presence of vacuolization, interstitial edema, interstitial inflammation, the number of acinar cell necroses, as previously described. The scoring assessment was performed on a scale of 0–3 (0 being normal and 3 being severe) on each parameter mentioned above, and the sum of the scores were used to evaluate the severity of acute pancreatitis.

Biochemical assay

Serum amylase, lipase, trypsinogen-activation peptide (TAP, Mouse, Rat) and Ang(1-7) was determined by means of acommercially available kit (Blue Gene, Shanghai, China), and expressed as units per liter (U/l). Assays were performed in duplicate using the Luminex 100 System (Austin, TX, USA).

Electron microscopy evaluation of cultured cells and mice pancreatic tissues

Acinar cell ultrastructure was examined by electron microscopy (EM). Cultured cells and pancreatic tissues from AP mice were fixed with 2.5% glutaraldehyde one hour and then in 0.1 M cacodylate buffer (pH 7.4) overnight post fixed in 1% OsO4 in the same buffer, enbloc stained with 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in Poly/Bed 812 (Polysciences). Ultrathin sections were cut on Leica EM UC7 ultramicrotome, stained with lead citrate and examined in a HITACHIH7650 electron microscope at 60kV.

Immunofluorescence analysis

Pancreas tissues or AR42J cell were fixed in suspension with 4% paraformaldehyde (methanol free) for 30 minutes at room temperature and then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. Non-specific binding was blocked with 5% rabbit or 5% goat serum. Then, tissues or cells were stained with primary antibodies against Mas1 (1:500; Alomone Lab, AAR-013), LC3B (1:500; MBL, M186-3) and Trypsin (1:200, Santa Cruz, sc-137077). The cells were then labeled with Alexa Fluor 488 (green) and Alexa Fluor 633 (far red) econjugated secondary antibodies (Thermo Fisher Scientific), and Nuclei were stained with 4’6’-diamidino-2-phenylindol (DAPI, 1:10,000; Sigma-Aldrich, D8417) for 5 min. Images were acquired with a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY) .

Nucleus isolation

Fresh intact mice pancreas were isolated and purified from mice killed by decapitation. All isolation procedures were performed on ice or at 4 °C. Pancreas was rinsed with ice-cold solation buffer A containing 320 mM sucrose, 3 mM $MgCl_2$, and 20 mM Tris, pH 7.4. Pancreas pieces were homogenized in buffer A using a glass homogenizer. The homogenates were centrifuged for 15 min at 1000×g at 4 °C. The supernatants were removed, and the pellets were resuspended in 4 ml of buffer B (2.2 M sucrose, 1

mM $MgCl_2$, and 10 mM Tris, pH 7.4) and differentially centrifuged for 60 min at 60,000×g (Beckman XL-90 ultracentrifuge, Brea, CA, USA) using a swing out rotor at 4 °C. After centrifugation, the pellet containing the isolated nuclei was resuspended and washed by centrifugation in 2 ml of buffer A. Protein content in isolated nuclei was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Fremont, CA, USA). For WB analysis, isolated nuclei were processed using the Nuclear Extract Kit (Active Motif, CA, USA) to eliminate the DNA and conserve only the nuclear proteins.

Reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from acinar cells using TRIzol (Invitrogen), followed by reverse transcription with a DNA reverse transcription system (Invitrogen). And Quantitative RT-PCR was performed using the SYBR Green PCR Master Mix and RT-PCR reagents and the Prism 7500 Sequence Detection System. Polymerase chain reaction was carried out for 40 cycles (20 seconds at5 0 °C, 10 minutes at 95 °C, and 40 cycles of 15 seconds at 95 °C, and 1 minute at 60 °C). The primer sequences were as follows Table1. GAPDH (B661104-0001; B661304-0001; B661204-0001; Sangon Biotech (Shanghai) Co., Ltd.) was included in each reaction as an internal standard, and relative quantitative gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method.

Western blot analysis

For western blot analyses, portions of frozen pancreas tissue and AR42J cells were rapidly homogenized in liquid nitrogen. Total protein and nuclear protein were extracted separately using the total Protein Extraction Kit (Sigma) according to the manufacturer's instructions. The concentrations of protein were determined using the BCA method (Pierce, Rockford, IL, USA). Each 30 μg aliquot of total protein or nuclear protein was loaded in a 10% or 12% sodiumdodecyl sulfate-polyacrylamide gel electrophoresis gel, and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After complete protein transfer, the membranes were blocked with 5% milk powder solution for 2 hat room temperature and incubated at 4 °C overnight with rabbit monoclonal anti-Mas1, anti-VAMP8, anti-SNAP23, anti-SYN4, anti-Trypsin, anti-HRP-Conjugated GAPDH and mice monoclonalanti-SQSTM1/p62, anti-LC3 antibody subunit diluted at a 1:1000 dilution in 5% milk powder solution. The following day, membranes were washed twice and incubated for 1 hour at 25 °C with anti-rabbit or anti-mice immunoglobulin G horseradishperoxidase-conjugated secondary antibody (1:5000). After 3 washes with Tris-HCl buffer salt solution + Tween later, enhanced chemiluminescence was used to detect immune reactive bands. Densitometric analysis of bands was performed using Image Lab software(170-8195) from Bio-Rad (Hercules, Calif), and data were expressed in arbitrary units.

Study Approval

All the experiments involving animals were conducted under the principle for replacement, refinement and reduction (the 3Rs) and according to the legislation on the protection of animals and were approved by the Animal Ethics Committee of Capital medical university (AEEI-2018-074). All the patients enrolled in this study provided written informed consent and have been approved by the appropriate ethics

committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Statistical analysis

Data were presented as the means ± SD. Statistical analyses were done using SPSS V.16.0 for Windows (IBM) or GraphPad Prism (GraphPad Software, San Diego, California, USA). The student's t-testor one-way ANOVA was used for comparison between groups. *P* values <0.05 were considered to be statistically significant.

Results

Human Pancreatic Exocrine Injury Depletes Mas1 Receptor Protein Level

The expression of Mas1 receptor proteins in human pancreatic cancer (PC) tissue are abnormal, we performed immunofluorescence analysis, Western blot and RT-PCR analysis in human tissue respectively. The tissues, which contained 6 cases of pancreatic cancer tissues and 6 cases of normal pancreas tissues, one of them was a patient with pancreatic cancer complicated with AP (PC+AP) (HE staining, Figure1, A), were immunofluorescence analysis of Mas1 receptor protein (Figure1, C). The expression rate of Mas1 receptor protein in pancreatic acinar cells is at a lower level in the cell cytoplasm. Compared with normal pancreas tissues, weak immunofluorescence staining for Mas1 receptor was observed in pancreatic cancer tissues (Figure1, C) and Mas1 receptor protein expression was significantly decreased (Figure1, D), whereas the mRNA of Mas1 receptor expression was up-regulated (Figure1, B) in the pancreatic cancer tissue. These results provide strong evidence supporting that Mas1 receptor protein is depleted under pancreatic exocrine injury.

AP Injury Peaked After 12 h of CAE+LPS Administration and Mediated the Down-regulation of Mas1Receptor protein

As shown in Figure 2A and 2B, after administration of CAE+LPS, the mice pancreatic tissues presented with acute inflammatory injury, which manifested as edema, bleeding, inflammatory cell infiltration, and acinar cell necrosis compared to control mice. More specifically, from 0.5 h to 12 h, and at 24 h, the pancreatic pathological changes showed significant improvement. This histological analysis revealed that AP is a self-resolving process during CAE+LPS induced pancreatitis. Serum amylase, lipase and trypsinogen activation peptide (TAP) serum levels (Figure2, C) are common biochemical markers of AP in mice, and they increased gradually at 0.5 h after AP onset, and then dropped at 12 h, as shown in Figure2, C(a) and (b). The levels of TAP in AP serum went up from 0.5 h to 12 h and then dropped down (Figure2, C(c)) in the development of AP, suggesting the model of AP was successfully established between 6 h and 12 h.

The Mas1 receptor protein level was reduced more obviously in AP tissues compared to control mice (Figure2, D). We noticed that Mas1 receptor protein expression, which was decreased after AP onset,

finally significantly descended at 6 h and 12 h (Figure2, D). The dynamic changes of Mas1 receptor protein was consistent with pancreatic pathological damage, trypsin secretion and activation serum levels. In addition, we analyzed the correlation between Mas1 receptor expression and pancreatic pathological injury, pancreatic trypsin secretion and activation, the correlation indexes were -0.5933, -0.3821 and -0.4950 respectively, which were statistically significant negative correlation (P<0.05). Therefore, we hypothesized that the Mas1 receptor may be involved in the pathogenesis of AP and play a vital role in trypsin secretion and activation in AP. Because the expression of Mas1 receptor decreased most significantly at 6 h to 12 h compared with the control group and the pathological score was highest at 12 h, the 12 h point was chosen for the following study.

Mas1 Receptor Signal Activation Reduces Pancreatic Histological Damage in Experimental AP

To better understand Mas1 receptor's role in AP, we used Mas1 receptor agonists Ang(1-7) and antagonist A779 to intervene CAE+LPS induced AP in the present study. Immunofluorescence assay reveals that Ang(1-7) and A779 were able to successfully activate or restrain Mas1 receptor protein expression during experimental AP (Figure3, A). Additionly, we investigated the possible presence of Mas1 receptor at cytoplasmic and nuclear levels using the nuclear marker DAPI. The nuclear presence of Mas1 receptor was mainly observed at the nuclear membrane level with a co-localization in the pancreas, (Figure3, A). These results are consistent with previous study that the expression of Mas1 receptor protein mainly expressed in the nucleus of sever AP acinar cell[17]. Then we measured the protein level and distribution in isolated nuclei and cytoplasm, and observed that Ang(1-7) and A779 can change the expression of total Mas1 receptor and mainly changes in the nucleus (Figure3, B). These results suggest that Mas1 receptor activation and related signaling pathways did not mainly take place in pancreatic acinar cell membrane but in an intracrine (intracellular/nuclear) pathway.

Pancreatic tissue with histological examination (H&E) staining of mice was shown in Figure3, C. After 12 h post-disease induction, the pancreatic tissue of mice showed the typical pancreatitis performance. In contrast to Mas1 receptor restrain of A779 and AP groups, Mas1 receptor activation with Ang(1-7) alleviated AP as shown by reducing pancreas edema, inflammation and histopathology score levels (figure3, D). These results suggest that Mas1 receptor activation provides a protection against AP.

Mas1 Receptor Activation Inhibits Experimental AP Induced Zymogen Basolateral Distribution in Acinar Cell

The exocrine pancreas is composed of polarized pyramidal acinar cells. As shown in Figure4, A(a), electron microscopy revealed that the pancreas tissue of control group, the basolateral region contains the nucleus and the apical region which ZGs dock and is ready to release their contents to the outside; ZGs have a wide range of sizes and fill a greater portion of the cytoplasm but not seen in the basolateral portions of the cell. However, ZGs distribute away from the apical pole and agglomerate near basolateral plasma membrane (PM) during CAE+LPS induced AP in Figure4, A(a, b, c); The size and number of ZGs per cell profile increases on each groups (Figure4, A(a, b, c)). Mas1 receptor agonist Ang(1-7) improves

the size and number of ZGs (figure4, A) and reduces ZGs basolateral distribution (figure4, B) and serum amylase levels (figure4, C). However, inhibition of Mas1 receptor activation by A779 did not significantly aggravate ZGs basolateral distribution and the serum amylase levels.

Physiologic regulated ZGs apical exocytosis and pathologic basolateral exocytosis in pancreatitis. These molecular of syntaxin4 (Syn4), SNAP23, VAMP8 and Munc18c mediated ZGs basolateral exocytosis. To further confirm whether inhibiting Mas1 receptor could sharpen ZGs basolateral secretion to balance its basolateral distribution during AP, we found that the mRNA and protein of VAMP8, Munc18c and SNAP23 are all changed during AP except Syn4 (figure4, D, E) as expected. But Mas1 receptor antiagonist A779 intervention did not yet change the basolateral exocytosis related protein and gene expressions (figure4, D, E). These results suggest that Ang(1-7) actives Mas1 receptor improving zymogen basolateral distribution not by ZGs basolateral exocytosis, Mas1 receptor antagonists A779 had similar results. These results suggest that Mas1 receptor activation improves zymogen basolateral distribution providing a protection against AP may not through improving ZGs basolateral exocytosis.

Mas1 ReceptorActivation Inhibits Zymogen Abnormal Activation and Autophagy Activity in Experimental AP

As shown in Figure 5, electron microscopy revealed that the pancreas tissue section from the untreated pancreas acini showed typical acinar cell ultrastructure and acinar lumen, with nuclei (N) in basal position, endoplasmic reticulum (ER) network surrounding the nucleus, and abundant zymogen granules (ZG). The mice that underwent CAE+LPS induced AP had extensive formation of autophagic vacuoles (AVs) containing partially degraded intracellular cargo, especially the zymogen particles (named Zymophagy) (Figure5, A). Microtubule-associated protein 1 light chain 3 (LC3)II and polyubiquitin-binding protein P62/SQSTM1 (P62) are two markers of autophagy. LC3, the mammalian paralog of yeast ATG8, is necessary for phagophore closure. During this process, its cytosolic form (LC3I) was lipidated and became LC3II, which specifically was translocated to the autophagosome membrane[10]. As LC3II is almost exclusively associated with the autophagosome's membranes and presents on early autolysosome's membrane until being degraded, it is commonly used to monitor autophagy[22]. In AP pancreatic tissues, the expression levels of LC3II were increased by immunofluorescence assay and western blotting analyses (Figure5, B, D). These results suggest that autophagy level was up regulated in AP. Accumulation of autophagosomes could be explained by increased formation or defective fusion with lysosomes, whereas accumulation of autolysosomes indicates defective lysosomal degradation. The P62 is specifically degraded by autophagy and is kept at a low level in normal conditions. Thus, excessive cell vacuolization and accumulation of P62 (especially together with LC3II) are markers of impaired autophagy. In the AP group, the expression levels of P62 were increased by western blotting analyses (Figure5, D). These changes of P62 and LC3II demonstrate that there are inducing impaired autophagy in AP. Ang(1-7) treatment reduced the levels of autophagy marker LC3II and P62 accumulation, also decreased the serum TAP levels (Figure5, C). However, A779 intervention reversed these changes and increased trypsin activation (TAP level) (Figure5, C). These results suggest that Mas1

receptor activation remission impaired autophagy, mostly decreased autolysosomes accumulation, which active zymogen leading to pancreas acinar cell impaired.

Mas1 Receptor Knockdown Aggravates Zymogen Activation by Increasing Impaired Zymophagy in CAE Induced Pancreatic Acinar Cells

We used lenti-virus intervene AR42J acini cells and establish Mas1 receptor over-expression (KO) and knockdown (KD) model. Then we applied well-established AP CAE (10 nM, 12 h) that simulate AR42J acini cells. After evaluated the formation of AVs under electron microscopy, we found an increased accumulation of AVs (containing ZG, zymophagy) along with depletion of Mas1 receptor in AR42J exposed to caerulein treatments (Figure6, C). Similar to the mice pancreas results (Figure 3), Mas1-KD acini showed more LC3II-GFP punctae, with an increase after caerulein stimulation in negative control condition (Figure5, A). Consistently, Mas1-KD acini showed more elevated LC3II/LC3I conversion and p62 accumulation under negative control condition (with CAE) (Figure5, B). Mas1 receptor deletion therefore increases AVs (zymophagy) and autolysosome accumulation, which amplifies increased the trypsin activation and caerulein-induced impairment in autolysosome clearance.

Intra-acinar premature zymogen activation, a key event causing pancreatitis[23, 24] occurs primarily in cytoplasmic vacuoles[7] which were recently linked to perturbed autophagy[25](containing ZG, zymophagy) and identified to occur in early autolysosomes. Because Mas1-KD acini accumulated more AVs (containing ZG), it led us to further investigate whether zymophagy is the cellular location of zymogen activation using the marker trypsin against LC3II-GFP–positive vacuoles. As expected, Mas1-KD acini showed trypsin occurred predominantly within these AVs after caerulein stimulation (Figure5, A). In Mas1-KD acini stimulated with caerulein, there was an increase in LC3II/trypsin-positive vacuoles (Figure5, A), consistent with higher intracellular trypsin activity (Figure5, D, E). We did not observe any significant difference in trypsin activity in control (no CAE) cells where autophagy remained functional. Tounequivocally show that excessive AVs formation and intracellular trypsinogen activation were functional consequences of Mas1 receptor deletion, we performed Mas1 receptor rescue expression. Mas1-KO acini reduced LC3I to LC3II conversion (Figure5, B(b)) and also reduced intracellular zymogen production and activation (Figure5, D, E) to levels similar to negative-GFP-transduced WT acini after caerulein stimulation. These results together indicate that Mas1 receptor deletion in acinar cell, is mechanistically linked to the severity of AP.

Discussion

The pathogenesis of early AP is mainly considered to the zymogen (trypsinogen) turns into trypsin, causing the auto-digestion of the pancreas. Prevention strategies for AP are basically formulated against zymogen activation. Currently, one focus of the research in the etiology of AP is the effect of autophagy flux barrier on zymogen activation. Increased activity of autophagy has previously been believed to aggravate the severity of AP. Our data are likely to what emodin did with reduction of AP by relieving autophagy response[26],as well as the ameliorates of AP observed in Mas1 receptor agonist Ang(1–7)

mice by inducting the expression of LC3II/I and P62 that connect to autophagosome increased and then prevent autolysosome function. Autophagy has long been known to be closely related to trypsinogen activation in early studies. The current data implied the activation of trypsinogen, which is similar to that observed in Atg5 knockout mice[25], which deemed that autophagy delivered trypsinogen to the lysosome and subsequently promoted activation of trypsinogen to trypsin, as well as the double-membrane structures found in pancreatic acinar cells in AP with incompletely digested cell contents and activated trypsin. Thus, it is very urgent to find effective therapeutic strategies for restraining autophagy.

Mas1 receptor exerts protective effects in the brain, heart, vessels, and kidney, and its tissue distribution is ubiquitous[27]. Its up-regulate expression could inhibite autophagy activity in multiple cells and animals. In our previous study[18], we showed that intervention Ang(1–7) could activate Mas1 receptor and decrease CAE-mediated pathological damage, while A779 could inhibit this signalization, although the underlying mechanism remains unclear. Mas1 receptor plays a key role in the inhibition of pathological damage in AP. Ang(1–7) binding to the Mas1 receptor can reduce serum amylase levels and the secretion of pro-inflammatory cytokine by suppressing the PI3K/AKT signaling pathway[19]. In addition, we showed that After 48 h post-disease induction, the expression of Mas1 receptor protein significantly increased, and was mainly expressed in the nucleus of cells[17]. Mas1 receptor, as a GPCR, can be desensitized and internalized to produce certain biological effects. Based on these results, we concluded that Mas1 could play a key role in treating AP. In the present study, we investigated the mechanism underlying the effect of Mas1 receptor on promoting the recovery of AP, and the results showed that Mas1 receptor up-regulated mainly concentrated in the nucleus and restrained zymogen premature activation by inhibiting autophagy activity. Zymogen premature activation is involved in the pathologic process of AP, and inhibiting autophagy activity serve as a good method for the treatment of AP. Many studies have revealed that autophagy could promote pancreatic zymogen premature activation[28,29]. However, few studies demonstrated that changing Mas1 receptor alleviates AP damage by affecting autophagy activity. In the present study, we demonstrated that Mas1 receptor plays a role as a protection factor for AP by suppressing autophagy (zymophagy) and autolysosome accumulation.

Thereful, we first explored that CAE (along or combined with LPS) stimulation caused an obvious decline in pancreatic cellular expression of Mas1 receptor, and the expression of Mas1 receptor in experimental AP is negatively correlated with pathological score and trypsin activation levels between 0 and 24 h. Meanwhile, it was found that the expression of Mas1 receptor was negatively correlated with the secretion and activation of trypsin, and Mas1 receptor had intracellular signal transduction, which was consistent with our previous results[17]. Then we explored the possible mechanism using Ang(1–7)/A779 interfering with the function of Mas1 receptor. The results showed that Ang(1–7) inhibited trypsinogen activation more effectively. The expression levels of serum amylase, lipase and TAP in A779 were also the highest and the lowest in Ang(1–7). At the same time,the expression levels of LC3II/I, P62 in A779 were also the highest and the lowest in Ang(1–7). These results showed that trypsinogen activation was regulated by Mas1 receptor function changing for AP.

ZGs basolateral exocytosis is an important factor to aggravate AP and the vital mechanism is mediated by interactions between the four proteins (Syn4, SNAP23, VAMP8 and Munc18c), which expression changes affect the basolateral exocytosis[30–33]. Thereful, we further demonstrated that in CAE-induced pancreatic acinar cells, these three proteins (SNAP23, VAMP8 and Munc18c) expression changes except Syn4, and mRNA expression also shows corresponding changes. Furthermore, from the electron microscope results, we could see that ZGs are dramatically increased and accumulated basolateral plasma membrane in CAE-induced pancreatic acinar cells. These results suggest ZGs basolateral distribution and secretion abnormal in AP. Nevertheless, changed Mas1 receptor function directly effects ZGs basolateral distribution, but not on the ZGs basolateral exocytosis and the expression of related proteins and mRNA. These results suggest us that the effect of Mas1 receptor on AP may improve ZGs basolateral distribution not by basolateral secretion. There may be another regulatory mechanism that affects ZGs basolateral distribution in AP, laiding a foundation for our follow-up studies.

To further reveal the mechanism underlying the effect of Mas1 receptor on inhibiting trypsinogen activation, we using genetic engineering methods and by constructing Mas1 receptor over-express/knock down in AR42J cell. The results showed that over-expression of Mas1 receptor inhibited activity of TAP as well as the expression of trypsin and autophagy related proteins LC3II/I and P62. Furthermore, Mas1 receptor over-expression inhibited AVs accumulation and co-localization of AVs and activated trypsin (cntaining ZG, zymophagy) in AR42J cells. However, Mas1 receptor depleted in AR42J cells conversely promoted co-localization of AVs with activated trypsin, TAP levels and restored impaired zymophagy. These data demonstrated that Mas1 receptor plays as a critical factor that modulating the susceptibility to CAE-induced trypsinogen activation by inhibiting impaired zymophagy in AR42J cells in vitro. In support of our conclusion, we have also verified that the expression of Mas1 receptor protein is reduced when pancreatic exocrine injury occurs in human pancreatic cancer tissues, although the mRNA was elevated. This situation may be due to the degradation by hydrolase or Mas1 receptor proteins synthesis of obstacles.

In summary, this study demonstrated that the drastic decrease of Mas1 receptor in response to CAE (or combined with LPS) is a vital pathological change for the induction of early AP, which in turn triggers ZGs basolateral distribution and trypsinogen abnormal activation. Restoring impaired zymophagy could alleviate zymogens abnormal activation. The results strongly suggest that Mas1 receptor may provide a promising therapeutic target to ameliorate trypsinogen abnormal activation-induced pancreas damage.

Declarations

Acknowledgments

The authors would like to acknowledge the department of general surgery, Capital Medical University, Beijing Friendship Hospital for kindly providing human pancreatic cancer tissue and normal pancreatic tissue. The studies were supported by a grant from 2016 National Natural Science Foundation (no. 81571933) to Dr. CY and a grant from Beijing Municipal Education Commission (no.KM20191025007)

Author Contributions

Xiaozheng Yu, Chunyun Li and Tianyu Cui were involved in conducting experiments, acquiring and analyzing data. Ruixia Liu conducted pathological examination of acute pancreatitis. Chenghong Yin conceived the project, designed the research studies, analyzed and interpreted the data and wrote the manuscript, along with Xiaozheng Yu and Ruixia Liu.

Conflict and Interest

All authors (Xiaozheng Yu, Chunyun Li, Tianyu Cui, Tianhe Li, Ruixia Liu and Chenghong Yin) have declared that no conflict of interest exists.

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Table

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Figures

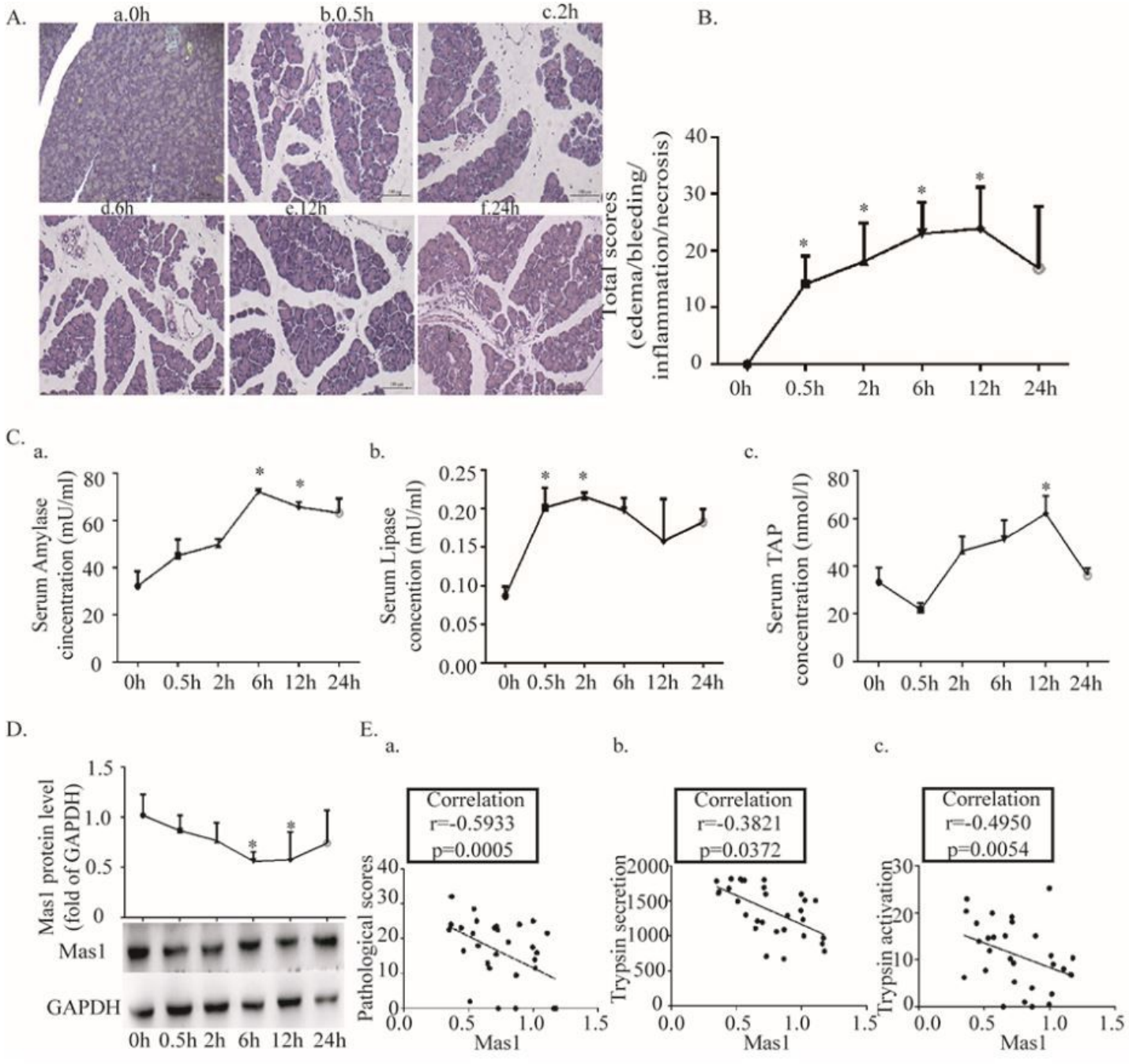

Figure 1

Human pancreatic exocrine injury depletes Mas1 receptor protein level (A) Representative HE staining of control pancreas (a) and pancreatic cancer (b) tissues (n=6 each group). (C) immunofluorescence shows the expression of Mas1 receptor in human pancreatic acinar cells and (B.D) the expression of Mas1 receptor protein and mRNA was decreased in pancreatic cancer pancreatic tissue with exocrine injury compared with normal human pancreatic tissue.

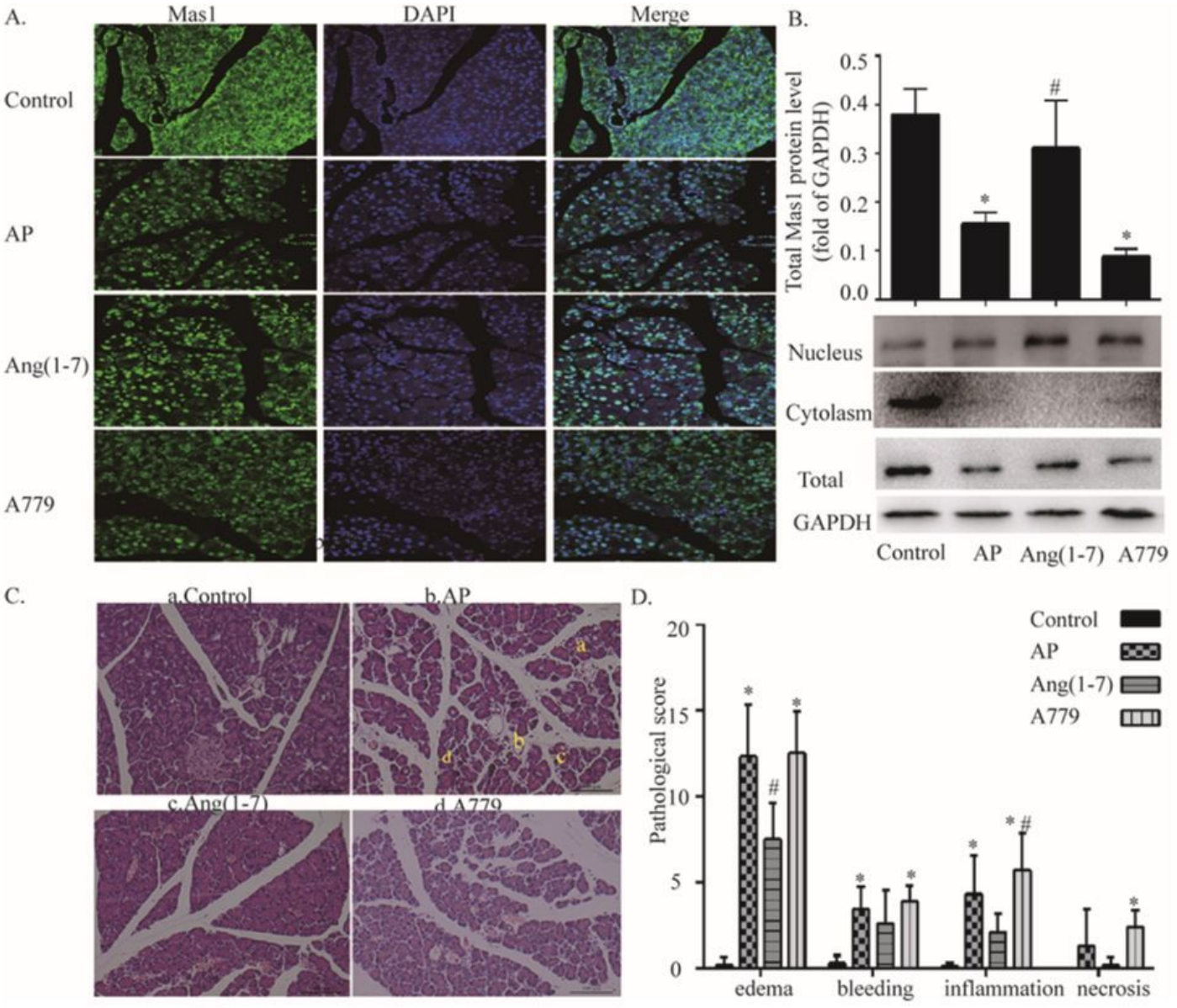

Figure 2

AP injury peaked after 12 h of CAE+LPS administration and mediated the down-regulation of Mas1 receptor protein (A, B) Representative HE staining and histological scores of pancreas; (C) Serum levels of amylase, lipase and TAP; (D) The dynamic changes of Mas1 receptor protein levels in the pancreatic tissues of CAE+LPS induced AP were analyzed by western blotting, GAPDH were used as a control for protein loading. (E) The correlation of Mas1 receptor protein with histological scores, serum amylase level and TAP level in pancreatic tissues. n=6 each group.

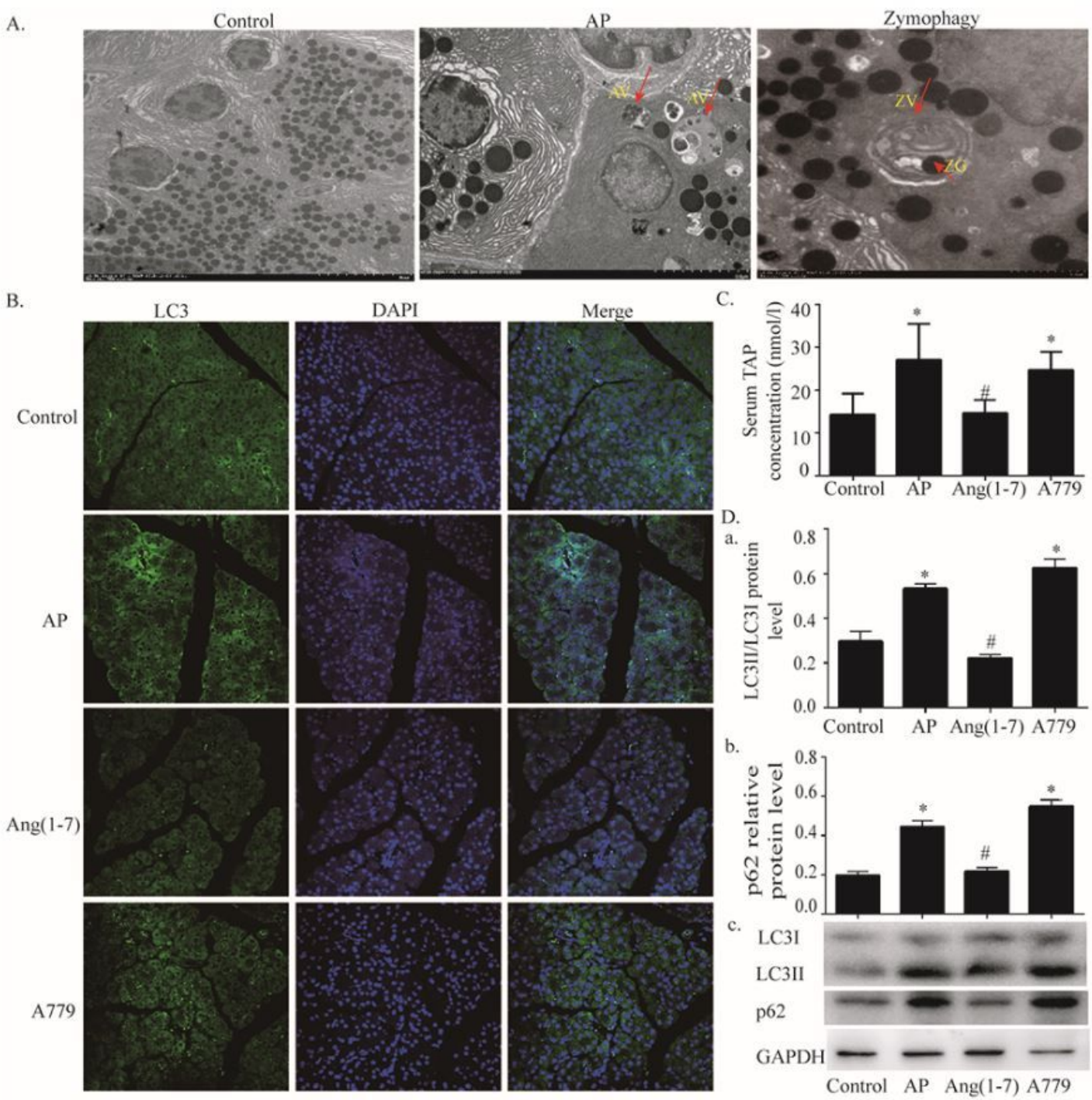

Figure 3

Mas1 receptor activation reduces pancreatic histological damage in experimental AP (A) Representative immunofluorescence images for Mas1 receptor protein in the pancreatic tissues. (B) Protein levels of Mas1 receptor respectively in nuclear□cytoplasm and total cells in the pancreatic tissues were analyzed by western blotting. GAPDH were used as a control for protein loading; (C) Representative HE staining of pancreas and (D) Histological scores of pancreas. *P< 0.05 versus AP group. n =6 each group.

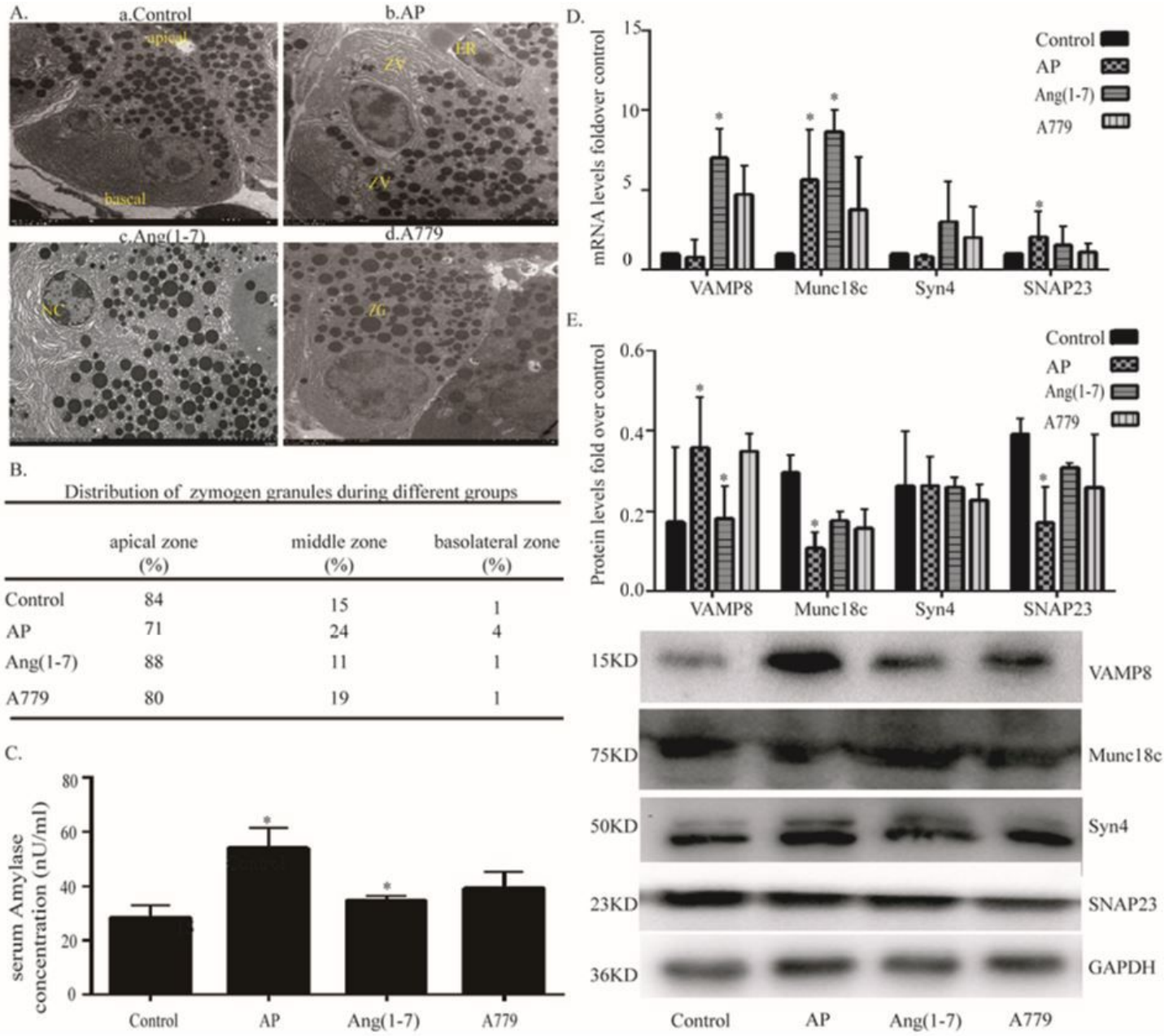

Figure 4

Mas1 receptor activation inhibits experimental AP induced zymogen basolateral distribution in acinar cell (A, B) Transmission electron microscopy images depicte the distribution of granule enzyme. (each from 5 independent experiments) of the fusion events at the apical and basolateral areas, and when combined as total. Apical and basolateral areas were divided by drawing 2 concentric circles centered on the apical lumen of each image where inner circle encompasses two-thirds of the area of the outer circle, and designated as “apical area”. Fusion events outside the apical area are considered “basolateral”. (C) Change in serum activity of amylase. (D, E) The expression of basolateral exocytosis related protein and gene expressions (Syn4/SNAP23/VAMP8/Munc18c).

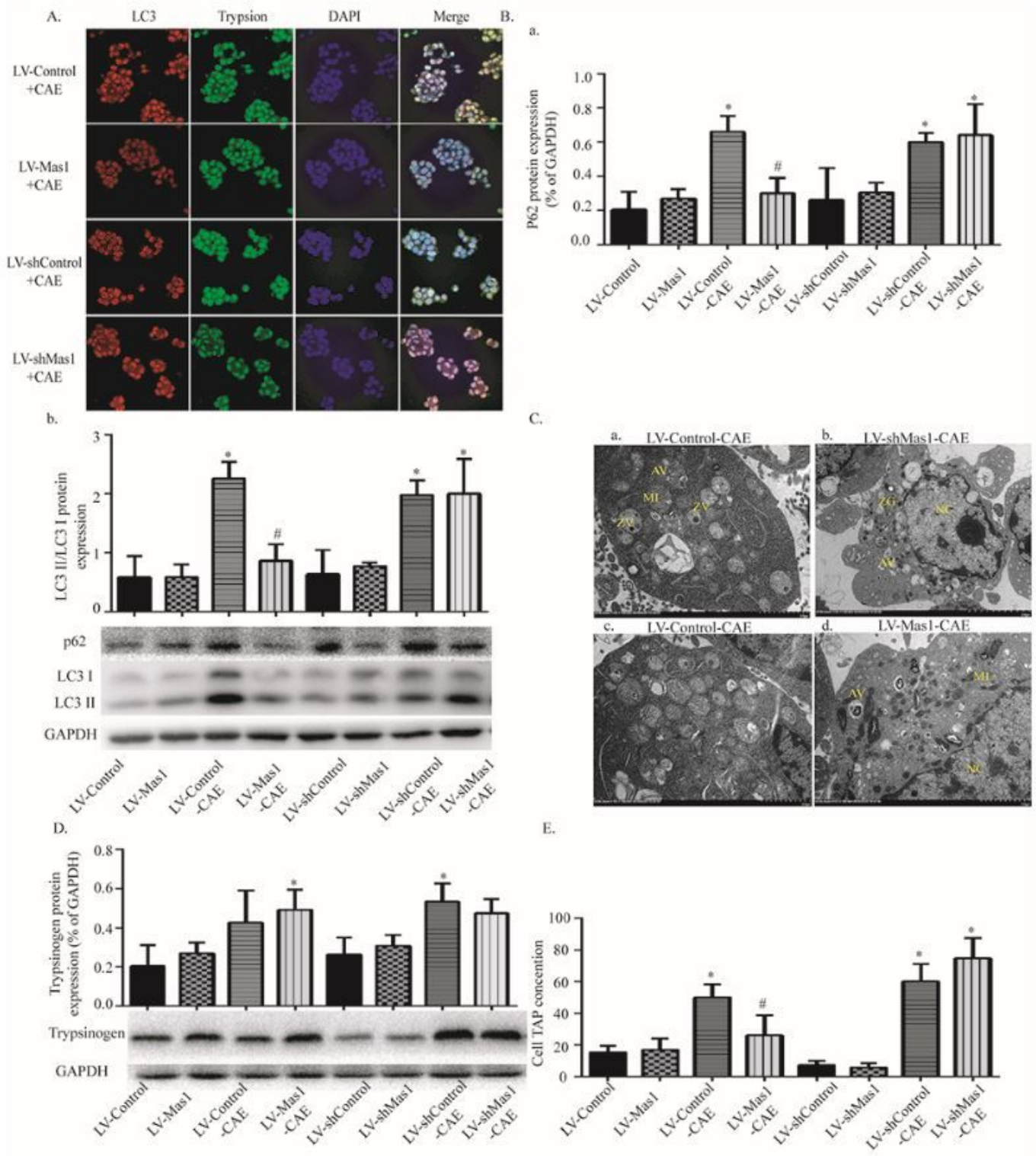

Figure 5

Mas1 receptor activation inhibits zymogen abnormal activation and autophagy activity in experimental AP (A) Transmission electron microscopy images depicting accumulation of distinct AVs, including Zymophagy (Ac). (B) Representative immunofluorescence images for LC3B in the pancreatic tissues and (D) the autophagy markers expression of LC3 II/I and p62 proteins analyzed by western blotting, GAPDH

were used as a control for protein loading. (C) Trypsin activation levels TAP under Mas1 receptor function changes in AP (ELISA).

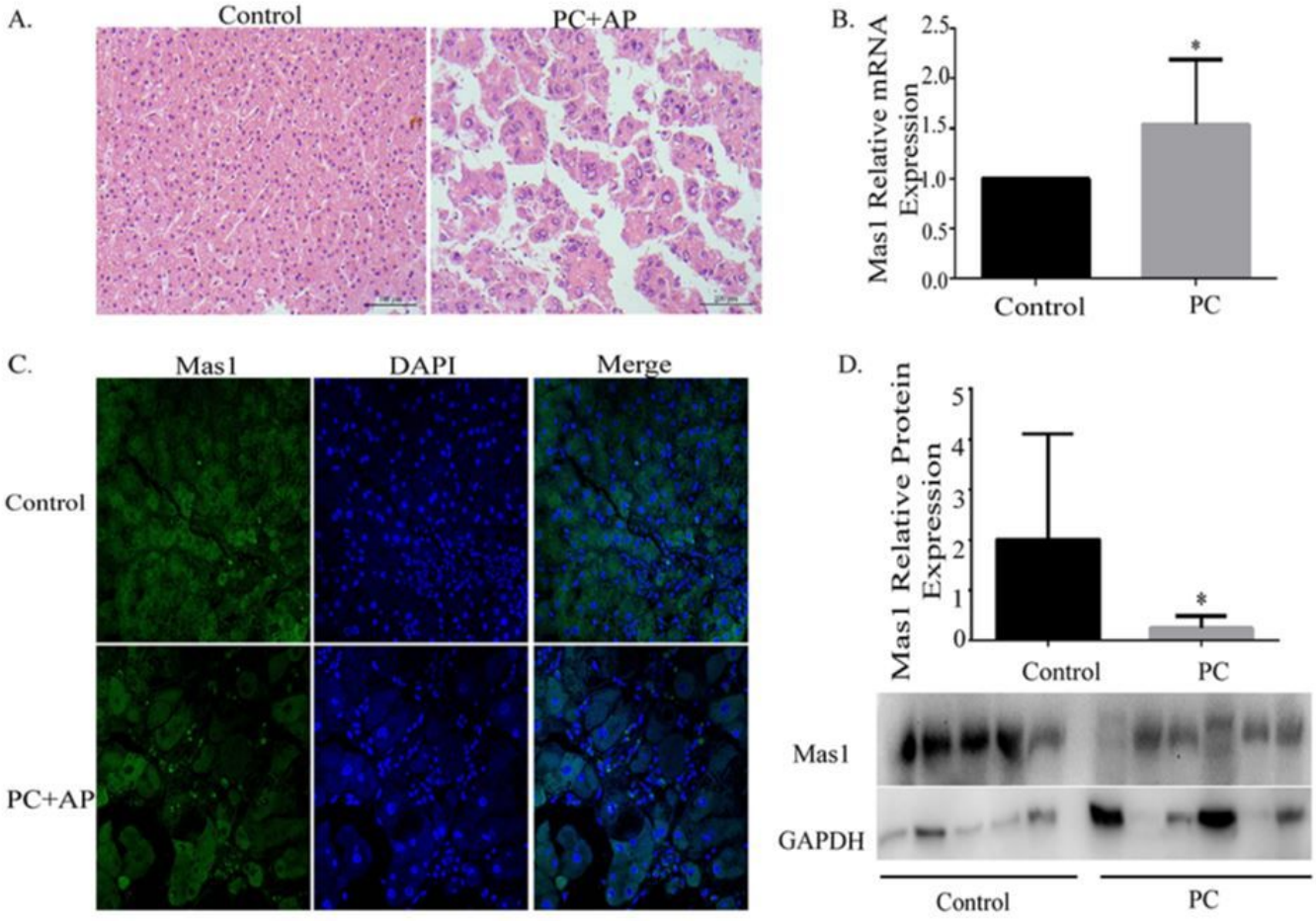

Figure 6

Mas1 receptor knockdown aggravate zymogen activation by increasing impaired zymophagy in caerulein induced pancreatic acinar cells Lentivirus intervene AR42J acini cells and successfully establish Mas1 receptor over-expression and knockdown model. (A) Confocal immunofluorescence images of selected areas showing (B.d) avid formation of LC3II and Trypsin colocalization (Zymophagy) and (B) improve impaired zymophagy related proteins expression of LC3II/I and P62. (C) Transmission electron microscopy images reveal (C.d) attenuate pancreatic acinar cell injury induced by caerulein and reduced the accumulation of AVs. (D.E) Mas1 receptor deletion aggravates trypsin expression and TAP levels in pancreatic acinar cell (western blotting and ELISA).