α-lipoic acid inhibits cerulein/resistin-induced expression of interleukin-6 by activating peroxisome proliferator-activated receptor-γ in pancreatic acinar cells

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Abstract. Cerulein-induced pancreatitis resembles human acute pancreatitis in terms of pathological events, such as enzymatic activation and inflammatory cell infiltration in the pancreas. Cerulein is a cholecystokinin analog that increases levels of reactive oxygen species (ROS) and interleukin-6 (IL-6) expression level in pancreatic acinar cells. Serum levels of resistin, which is secreted from adipocytes, are reportedly higher in patients with acute pancreatitis than in healthy individuals. Previously, it was shown that the adipokine resistin can aggravate the cerulein-induced increase in ROS levels and IL-6 expression level in pancreatic acinar cells. Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a key regulator of the transcription and expression of antioxidant enzymes, including heme oxygenase 1 (HO-1) and catalase. α-lipoic acid, a naturally occurring dithiol antioxidant, can prevent cerulein-induced pancreatic damage in rats. In the present study, it was aimed to investigate whether α-lipoic acid can attenuate the cerulein/resistin-induced increase in IL-6 expression and ROS levels via PPAR-γ activation in pancreatic acinar AR42J cells. The anti-inflammatory mechanism of α-lipoic acid was determined using reverse transcription-quantitative PCR, western blot analysis, enzyme-linked immunosorbent assay, immunofluoresc2ence staining and fluorometry. Treatment with cerulein and resistin increased ROS levels and IL-6 expression level, which were inhibited by α-lipoic acid in pancreatic acinar cells. α-lipoic acid increased the nuclear translocation and expression level of PPAR-γ and the expression levels of its target genes: HO-1 and catalase. The PPAR-γ antagonist GW9662 and HO-1 inhibitor zinc protoporphyrin reversed the inhibitory effect of α-lipoic acid on cerulein/resistin-induced increase in ROS and IL-6 levels. In conclusion, α-lipoic acid inhibits the cerulein/resistin-induced increase in ROS production and IL-6 expression levels by activating PPAR-γ and inducing the expression of HO-1 and catalase in pancreatic acinar cells.

Introduction

Acute pancreatitis is a detrimental disease characterized by local and systemic inflammatory symptoms (1). Inflammatory disorders with increased levels of proinflammatory mediators, including interleukin-6 (IL-6), are a critical feature of acute pancreatitis, which is associated with the premature activation of zymogens, such as trypsinogen and chymotrypsinogen, within pancreatic acinar cells (2,3). Oxidative stress and obesity are risk factors for poor outcomes in acute pancreatitis. Obese patients with acute pancreatitis exhibit higher inflammatory responses than non-obese patients (4,5). Despite accumulating evidence regarding the pathogenesis of acute pancreatitis, unresolved questions concerning the relationship among obesity, adipohormones, and the disease remain unaddressed.

The cholecystokinin (CCK) analog cerulein can induce symptoms similar to those of human acute pancreatitis. Treatment with high amounts of cerulein results in dysregulated digestive enzyme production, cytoplasmic vacuolization, edema formation and inflammatory cell infiltration into the pancreas (6,7). Therefore, cerulein-induced acute pancreatitis is widely employed to investigate the pathological mechanisms underlying acute pancreatitis.

Resistin is a cysteine-rich adipocytokine secreted by adipocytes and macrophages (8). Serum resistin levels increase with obesity (9). Bokarewa *et al* (10) have revealed that resistin upregulates proinflammatory mediators such as tumor necrosis factor-α (TNF-α) and IL-6 in human peripheral blood mononuclear cells and mice joints under arthritic conditions. Resistin can increase TNF-α in macrophages (11) and activate nuclear factor-κB (NF-κB) to induce IL-6 expression in pancreatic acinar cells (12). These studies suggested that resistin is associated with major local and systemic inflammatory responses. Jiang and Wang (13) have demonstrated that resistin aggravates TNF-α and IL-6 expression

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in cerulein-treated pancreatic acinar cells. Previously, it was demonstrated that resistin, which binds to toll-like receptor 4, can amplify the effects of cerulein, that binds to CCK receptor, for IL-6 expression via NADPH oxidase-mediated reactive oxygen species (ROS) production in pancreatic acinar cells (14).

In a clinical study, serum levels of resistin, IL-8 and C-reactive protein, which are inflammatory indices, were higher in 32 patients with acute pancreatitis than in 30 healthy individuals (15). Kibar *et al* (16) have examined the relationship between the severity of acute pancreatitis and serum resistin levels in 59 patients with acute pancreatitis. C-reactive protein and resistin levels were measured, along with other blood parameters. Patients were divided into two groups: mild and moderate/severe acute pancreatitis. It was found that the level of serum resistin was an improved inflammatory marker than that of C-reactive protein for determining the severity of acute pancreatitis in humans. Similarly, Ściskalska *et al* (17) identified that the plasma levels of resistin were 2-fold higher in 35 patients with acute pancreatitis than those in 95 healthy individuals. Plasma levels of advanced oxidation protein product (AOPP), mainly formed from oxidized albumin (a predominant antioxidant in plasma), were higher in patients with acute pancreatitis than in healthy individuals. Accordingly, these findings suggested that increased resistin and decreased albumin levels in plasma induce prooxidative effects of resistin, resulting in enhanced levels of AOPP in patients with acute pancreatitis. Taken together, increased resistin levels may aggravate the severity of acute pancreatitis. Accordingly, overproduction of obesity-associated resistin may aggravate the severity of acute pancreatitis.

α-lipoic acid is an endogenous 6,8-dithiol-octanoic acid, which is naturally synthesized in small amounts in humans (18). During fatty acid synthesis, α-lipoic acid is synthesized in the mitochondria from octanoic acid, which binds to the acyl-carrier protein (19). In food sources, α-lipoic acid is present in the form of lipoyllysine, a lipoic acid covalently bound to lysine in proteins. Lipoyllysine-rich animal tissues include the heart, kidneys, and liver, while broccoli and spinach are vegetables enriched in lipoyllysine (20). Consumption of lipoyllysine-rich food does not increase plasma levels of free lipoic acid (21,22). However, supplementation with free α-lipoic acid (50 to 600 mg, as dose 1,000 times greater than the dietary content) increases plasma levels of free α-lipoic acid (23). Typically, plasma concentrations of α-lipoic acid peak within ≤1 h, as it is rapidly metabolized and excreted after oral ingestion (24-26).

α-lipoic acid reportedly functions as a powerful antioxidant, distinguished by its remarkable biological activities, such as scavenging of reactive oxygen and nitrogen species, regeneration of other antioxidants, metal ion chelation and activation of antioxidant signaling pathways (21,27-29). Hence, there is a surge of interest in the pharmacological properties of α-lipoic acid, with an increasing number of studies confirming its therapeutic effect in several diseases, including diabetes, atherosclerosis, neurodegeneration and acquired immune deficiency syndrome (30). In an acute pancreatitis model, intraperitoneal injection of α-lipoic acid reduces the ratio of pancreatic weight/body weight and serum levels of amylase, lipase and cytokines (IL-1β, IL-6, TNF-α) in rats subcutaneously injected with CCK (31). These studies demonstrated the potentially protective effect of α-lipoic acid against acute pancreatitis. However, whether α-lipoic acid

can ameliorate acute pancreatitis by reducing the production of proinflammatory cytokines via the antioxidant signaling pathway remains unclear.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that represent the ligand-activated nuclear receptors family, a member of the steroid receptor super-family (32,33). All subtypes of the PPAR family (PPAR-α, -γ, and -β/δ) activate their target genes by forming heterodimers with the retinoid X receptor and binding to PPAR response elements (PPREs) of those genes (34). Each PPAR subtype displays various biological functions. For example, PPAR-γ regulates lipid metabolism and adipocyte differentiation. PPAR-γ plays a pivotal role in regulating inflammation (35-37). Previously, it was revealed that the PPAR-γ ligand troglitazone reduces IL-6 expression level by inhibiting Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling in pancreatic acinar cells (38). Furthermore, PPAR-γ acts as a transcription factor for heme oxygenase 1 (HO-1) and catalase by binding to PPREs in the target gene promoters. HO-1 is a rate-limiting enzyme that protects cells against oxidative stress (39,40). Krönke *et al* (41) have indicated that PPAR-γ, upon ligand binding, is translocated to the nucleus, where it binds to the HO-1 promoter to induce HO-1 expression. HO-1 supports the protective role of PPAR-γ activation against various stressors (42,43). Catalase mediates the protective effects of PPAR-γ ligands against oxidative damage (44). Based on these studies, PPAR-γ is a crucial regulator of redox signaling and exerts a protective effect via the transcriptional activation of antioxidant genes.

In the present study, it was aimed to determine whether α-lipoic acid inhibits the cerulein/resistin-induced increase in ROS levels and IL-6 expression level by activating PPAR-γ and upregulating its target genes HO-1 and catalase in pancreatic acinar AR42J cells.

Materials and methods

Materials. Dichlorofluorescein diacetate (DCF-DA; cat. no. D399), resistin, cerulein, α-lipoic acid and PPAR-γ antagonist GW9662 were purchased from Sigma-Aldrich; Merck KGaA. HO-1 inhibitor protoporphyrin (ZnPP; cat. no. sc-691550) was purchased from Santa Cruz Biotechnology, Inc. Resistin was dissolved in distilled water to (final concentration: 100 µg/ml). Cerulein was dissolved in phosphate-buffered saline containing 0.1% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.; final concentration 10^{-4} M). α-lipoic acid was dissolved in 0.5 M ethanol as a solvent (final concentration, 250 mM). The PPAR-γ antagonist GW9662 (Sigma-Aldrich; Merck KGaA) and the HO-1 inhibitor ZnPP were both dissolved in dimethyl sulfoxide (DMSO; final concentration, 100 and 1 mM, respectively). All products were stored at -20˚C until use. Cells incubated with vehicle alone (<0.1%) served as the control.

Cell line and culture conditions. Rat pancreatic acinar AR42J cells (pancreatoma, cat. no. CRL-1492) were obtained from the American Type Culture Collection and cultured as previously described (14).

Experimental protocol. First, to determine whether α-lipoic acid activates PPAR-γ, cells were treated with α-lipoic acid

(5 μM) alone for 1, 2, and 3 h, and protein levels of PPAR-γ and its target genes catalase and HO-1 were assessed. To investigate the inhibitory effect of α-lipoic acid on cerulein/resistin-induced alterations, cells were pretreated with α-lipoic acid (2 or 5 μM) for 2 h. Then the cells were pre-stimulated with resistin (2 ng/ml) for 30 min prior to the addition of cerulein (10^{-8} M). Cells were stimulated with cerulein/resistin for 45 min (for determination of intracellular ROS, protein levels of PPAR-γ, catalase and HO-1, and immunocytochemistry of PPAR-γ and catalase activity), 4 h (for IL-6 mRNA expression), and 24 h (for IL-6 protein levels). To determine the role of PPAR-γ or HO-1 in mediating the protective effect of α-lipoic acid on cerulein/resistin-induced changes, cells were pretreated with the PPAR-γ antagonist GW9662 (10 μM) or the HO-1 inhibitor ZnPP (1 μM) with or without α-lipoic acid. After 2 h, the cells were stimulated with cerulein/resistin. For each experiment, the amount of a vehicle was less than 0.1%. A control experiment, in which the vehicle alone was added, was performed simultaneously. Pretreatment time, α-lipoic acid concentration and incubation time of cerulein/resistin for determining ROS and IL-6 protein levels were adapted from our previous studies (14,45).

Determination of intracellular ROS levels. To measure intracellular ROS levels, cells (2x10^5 cells/well) in six-well plates were treated with α-lipoic acid for 2 h and stimulated with cerulein/resistin for 45 min at 37˚C. Intracellular ROS levels were determined by assessing the intensity of DCF-DA as previously described (14).

Reverse transcription-quantitative (RT-q) PCR analysis. mRNA expression of IL-6 was assessed using RT-qPCR, as previously described (14). Total RNA was isolated using the TRI reagent (RNA/DNA/Protein isolation reagent, Molecular Research Center, Inc.). Total RNA (2 μg) was used for cDNA synthesis, and 100 Units MuLV reverse transcriptase (Promega Corporation), 0.23 μl random hexamers (500 pg/ml; Promega Corporation), 1.25 μl dNTPs (10 mM), 0.63 μl RNasin (40 U/ml) and 5 ml 5X reaction buffer [containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl$_2$ and 10 mM DTT] were added to the reaction. The 25-μl cDNA synthesis reaction mixture was incubated at 23˚C for 10 min, 37˚C for 60 min and 95˚C for 5 min. cDNA was used for qPCR with specific primers for IL-6 and β-actin. Sequences of the IL-6 (accession number M26745) primers used to produce the desired 242-bp PCR product were: forward, 5'-GCCCTT CAGGAACAGCTATGA-3' and reverse, 5'-TGTCAACAA CATCAGTCCCAAGA-3'. Sequences of the β-actin (accession number XM_032887061.1) primers used to produce the desired 353-bp PCR product were: forward, 5'-ACCAACTGGGAC GATATGGAG-3' and reverse, 5'-GTCAGGATCTTCATG AGGTAGTC-3'. cDNA was added in a SYBR Green Realtime PCR Master Mix (Toyobo Life Science) containing 10 pg/ml forward and reverse primers for IL-6 and β-actin. For PCR amplification, the cDNA was amplified using the following thermocycling conditions: 45 repeat cycles of denaturation at 95˚C for 30 sec, annealing at 53˚C for 30 sec and extension at 72˚C for 30 sec. During the first cycle, the denaturation step at 95˚C was extended to 3 min. Amplification specificity was validated by melting curve analysis generated at the end of

each reaction. All genes presented a single peak in the melting curve, indicating the absence of primer-dimer formation during the reaction and the specificity of the amplification. Relative changes in gene expression between untreated cells and treated cells were determined using the 2$^{-\Delta\Delta Cq}$ method (46). Levels of the target transcript were normalized to β-actin endogenous control and were constantly expressed in the group.

Preparation of whole-cell extracts and nuclear extracts. Whole cell extracts, cytosolic extracts and nuclear extracts were prepared as previously described (47). Cells were harvested using trypsin-ethylenediaminetetraacetic acid (EDTA), followed by centrifugation at 1,000 x g for 5 min at 4˚C. The pellets were resuspended in lysis buffer containing 10 mM Tris (pH 7.4), 150 μM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1 tablet/50 ml complete protease inhibitor (Roche Diagnostics GmbH). The cells were then lysed by drawing the cells through a 1-ml syringe with several rapid strokes. The lysate was incubated on ice for 30 min and then centrifuged at 13,000 x g for 15 min at 4˚C. The supernatants were collected as whole-cell extracts. Cytosolic and nuclear extracts were prepared using a NE-PER® nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, the cells were suspended in cytoplasmic extraction reagent containing protease inhibitor and vortexed for 15 sec, followed by centrifugation at 13,000 x g for 10 min at 4˚C. The supernatant was used as the cytosolic extract. The nuclear pellets were resuspended in nuclear extraction reagent, vortexed, and centrifuged at 13,000 x g for 10 min at 4˚C. The supernatants were collected and used as nuclear extracts. The specificity of the nuclear extract was confirmed by the presence of lamin B1 in the nuclear fraction. Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Inc.). A standard curve was developed using a series of bovine serum albumin (Sigma-Aldrich; Merck KGaA). The specificities of the nuclear and cytosolic extracts were confirmed by the predominant presence of lamin B1 in the nuclear extracts and aldolase A in cytosolic extracts, respectively.

Western blotting. Whole-cell extracts (10-40 μg protein/lane) were loaded onto 8-12% sodium dodecyl sulfate-polyacrylamide gels and separated by electrophoresis, and then transferred onto nitrocellulose membranes (Amersham, Inc.) by electroblotting and stained with Ponceau S for 5 min at 20-25˚C. The membranes were blocked with 3% non-fat dry milk in Tris-buffered saline and 0.2% Tween-20 (TBS-T) for 1 h at 20-25˚C and then incubated with antibodies against PPAR-γ (cat. no. sc-7273; dilution 1/1,000; Santa Cruz Biotechnology, Inc.), catalase (cat. no. ab16731; dilution 1/1,000; Abcam), HO-1 (cat. no. ADI-SPA-895; dilution 1/1,000; Enzo Life Science, Inc.), lamin B1 (cat. no. ab16048; dilution 1/500; Abcam), aldolase A (cat. no. sc-390733; dilution 1/1,000; Santa Cruz Biotechnology, Inc.) and actin (sc-1615; dilution 1/2,000; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. This was followed by incubation with secondary antibodies [anti-goat (cat. no. sc-2354; dilution 1/3,000), anti-mouse (cat. no. sc-2005; dilution 1/3,000) or anti-rabbit (cat. no. sc-2357; dilution 1/3,000) conjugated to horseradish peroxidase; Santa Cruz Biotechnology, Inc.] for 2 h at 20-25˚C. Proteins were

visualized using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Inc.) and an EZ-Capture ST imaging system (ATTO Corporation).

The intensity of each protein band was densitometrically quantified using the ImageJ software version 1.47 (National Institutes of Health). Densitometry data represent the mean ± standard error (SE) from three immunoblots and are shown as the relative density of the protein bands normalized to the loading control actin level. The ratio of the control group (cells without cerulein/resistin stimulation and without α-lipoic acid treatment) was set at 1.

Immunofluorescence staining. To measure the nuclear translocation of PPAR-γ, cells on coverslips placed in six-well plates were pretreated with α-lipoic acid for 2 h and then stimulated with cerulein/resistin for 45 min. Immunofluorescence staining was performed as previously described (48). Briefly, cells were fixed with 4% formaldehyde for 10 min at 20-25°C, permeabilized with 0.2% Triton X-100 for 10 min at 20-25°C, blocked with blocking buffer containing 1% BSA and 0.1% gelatin for 1 h at 20-25°C, and then incubated with the primary antibody against PPAR-γ (dilution 1/200) for 1 h at 20-25°C. After washing, the cells were incubated with donkey anti-mouse IgG-fluorescein isothiocyanate (FITC) (cat. no. sc-2099; dilution 1/200; Santa Cruz Biotechnology, Inc.) for 1 h at 20-25°C. The cells were then washed and covered with the antifade medium Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) for 30 min. Cells stained with FITC were examined using a laser scanning confocal microscope (Zeiss LSM 900; Carl Zeiss AG) and then images were captured. For each coverslip, six fields were measured. Results were obtained from four independent measurements (n=4 for each group). The intensity ratio of green (PPAR-γ) to blue (DAPI) was assessed using ImageJ v.5.0 (National Institutes of Health).

Enzyme-linked immunosorbent assay (ELISA). Briefly, cells ($2x10^5$ cells/well) were seeded in six-well plates. Then, cells were pretreated with or without α-lipoic acid for 4 h and then stimulated with cerulein/resistin for 24 h. IL-6 levels in the medium were determined using an ELISA kit (cat no. #BMS625; Invitrogen; Thermo Fisher Scientific, Inc.).

Determination of catalase activities. Catalase activity was measured using a catalase assay kit according to the manufacturer's instructions (cat. no. ab83464; Abcam). Changes in H_2O_2 levels in whole-cell extracts were measured by fluorometry (excitation and emission at 535 and 587 nm) and used to calculate catalase activities defined in units/mg protein.

Statistical analysis. Data values are expressed as the mean ± standard error (n=12 for each group). Statistical analysis was performed using one-way ANOVA followed by individual comparisons with Tukey's post-hoc test. Data analysis was performed using the SPSS software version 22.0 (IBM Corp.). P≤0.05 was considered to indicate a statistically significant difference.

Results

α-lipoic acid increases the expression levels of PPAR-γ and its target genes HO-1 and catalase in AR42J cells. First, it was
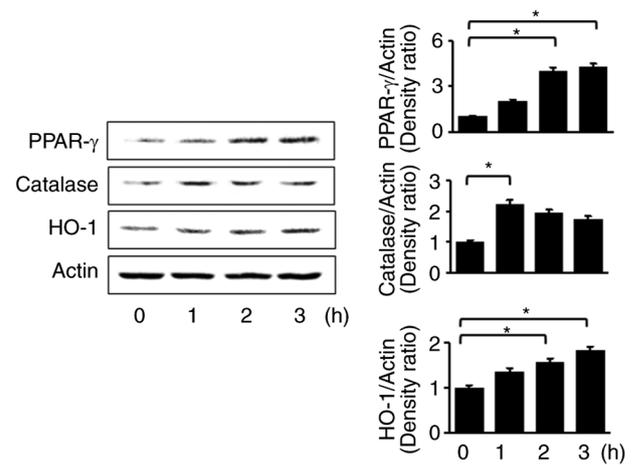

Figure 1. Impact of α-lipoic acid on the expression of PPAR-γ, HO-1 and catalase in unstimulated AR42J cells. Cells were treated with 5 μM α-lipoic acid for the indicated time periods. Protein levels of PPAR-γ, catalase and HO-1 in whole-cell extracts were determined by western blot analysis. Actin was used as the loading control (left panel). The densitometry data represent the means ± standard error from three immunoblots and are shown as the relative density of the protein band normalized to actin level (right panel). *P<0.05. HO-1, heme oxygenase-1; PPAR-γ, peroxisome proliferator-activated receptor-γ.

determined whether α-lipoic acid induces the expression of PPAR-γ and its target genes HO-1 and catalase in AR42J cells by measuring protein levels of PPAR-γ, HO-1 and catalase using western blot analysis. As revealed in Fig. 1, α-lipoic acid increased the protein levels of PPAR-γ, HO-1 and catalase. Levels of both PPAR-γ and HO-1 steadily increased until 3 h, while catalase levels increased at 1 h and decreased during 3 h of incubation. Overall, these results suggested that α-lipoic acid induces the expression of HO-1 and catalase, possibly by activating PPAR-γ in AR42J cells. Further study is necessary to determine whether α-lipoic acid induces mRNA expression of PPAR-γ, HO-1 and catalase in AR42J cells.

α-lipoic acid increases nuclear translocation and expression levels of PPAR-γ and its target genes HO-1 and catalase in cerulein/resistin-stimulated AR42J cells. It was determined whether cerulein/resistin decreased levels of PPAR-γ, HO-1 and catalase and whether α-lipoic acid could inhibit these alterations. Protein levels of PPAR-γ, HO-1 and catalase were determined by western blot analysis in cerulein/resistin-stimulated cells. It was revealed that cerulein/resistin decreased protein levels of PPAR-γ and its target genes, HO-1 and catalase, in AR42J cells (Fig. 2A). Treatment with α-lipoic acid reversed the cerulein/resistin-induced reduction in PPAR-γ, HO-1 and catalase in a dose-dependent manner. α-lipoic acid restored catalase activity, which was decreased by cerulein/resistin stimulation (Fig. 2B).

Using immunofluorescence staining, it was next examined whether cerulein/resistin decreased nuclear levels of PPAR-γ and whether α-lipoic acid reversed the nuclear level of PPAR-γ in cerulein/resistin-stimulated cells (Fig. 3A). PPAR-γ is localized in the cytosol of unstimulated cells, with minimal to no expression detected in the nuclei. Cerulein/resistin treatment decreased the nuclear level of PPAR-γ, which was increased by α-lipoic acid. Additionally,
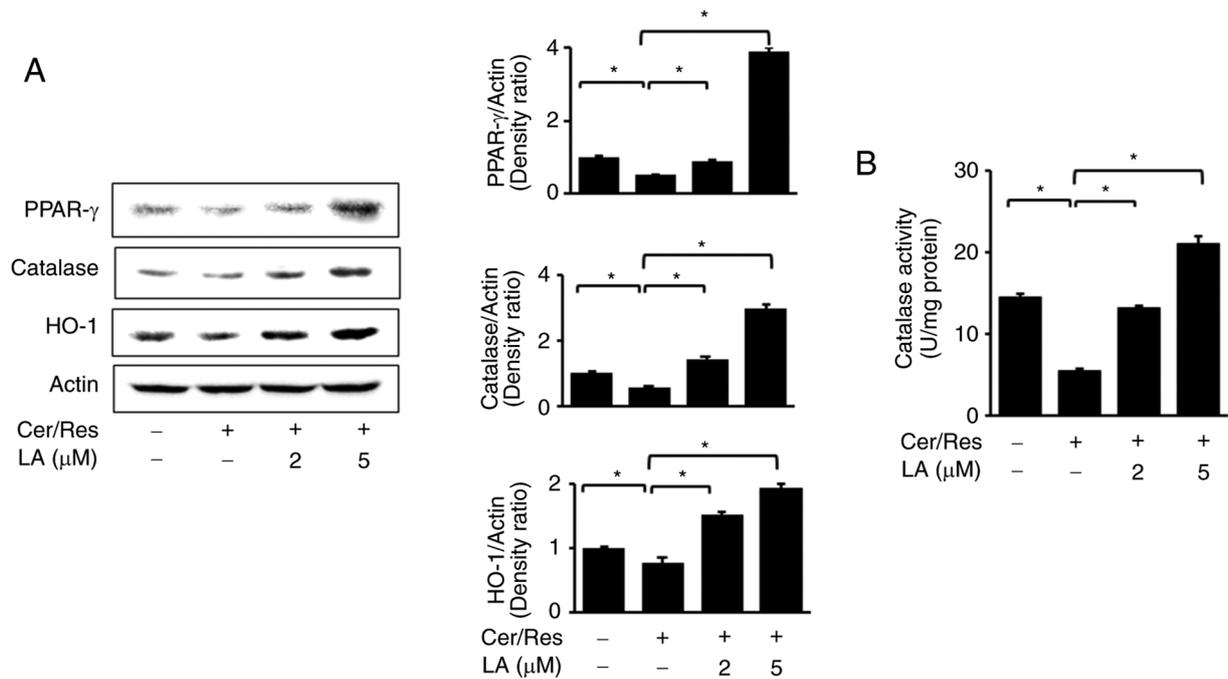
Figure 2. Impact of α-lipoic acid on expression of PPAR-γ and its target genes catalase and HO-1 and catalase activity in cerulein/resistin-stimulated AR42J cells. Cells were pretreated with the indicated concentrations of LA for 2 h and then stimulated with Cer and Res for 45 min. (A) Protein levels of PPAR-γ, catalase and HO-1 in whole-cell extracts were determined by western blot analysis. Actin was used as the loading control (left panel). Densitometry data are presented as the means ± SE from three immunoblots and are shown as the relative density of protein band normalized to actin level (right panel). (B) Catalase activity was determined using a catalase assay kit. Data are expressed as the mean ± SE (n=12 for each group). *P<0.05. PPAR-γ, peroxisome proliferator-activated receptor-γ; HO-1, heme oxygenase-1; LA, α-lipoic acid; Cer, cerulein; Res, resistin; SE, standard error.
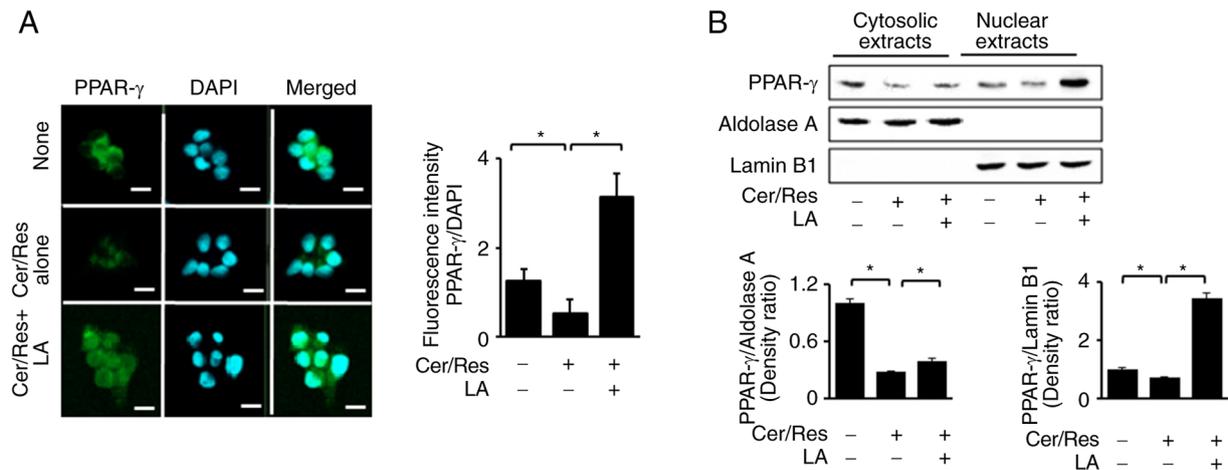

Figure 3. Impact of LA on nuclear translocation of PPAR-γ in cerulein/resistin-stimulated AR42J cells. Cells were pretreated with 5 µM LA for 2 h and then stimulated with Cer and Res for 45 min. (A) Immunofluorescence staining was performed to determine the levels of PPAR-γ in the cytosolic and nuclear extracts. PPAR-γ was visualized using fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (left panel) with DAPI counterstaining (middle panel) of the same field. Scale bars, 15 µm. For each coverslip, six fields were measured. Results were obtained from four independent measurements (n=4 for each group). The intensity ratio of green (PPAR-γ) to blue (DAPI) was assessed using ImageJ v.5.0 software. (B) Levels of PPAR-γ in cytosolic or nuclear extracts were determined by western blot analysis. Aldolase A is used as a marker of cytosolic marker, while lamin B1 was used as a nuclear marker (upper panel). The densitometry data represent the mean ± standard error from three immunoblots and are shown as the relative density of protein band normalized to aldolase A or lamin B1 levels (lower panel). *P<0.05. LA, α-lipoic acid; PPAR-γ, peroxisome proliferator-activated receptor-γ; Cer, cerulein; Res, resistin; DAPI, 4',6-diamidino-2-phenylindole.

cerulein/resistin stimulation reduced the nuclear and cytosolic levels of PPAR-γ, which was prevented by α-lipoic acid treatment (Fig. 3B). The indices of cytosolic and nuclear extracts, aldolase A and lamin B1, were not changed by treatment of cerulein/resistin with or without α-lipoic acid in AR42J cells.

α-lipoic acid inhibits cerulein/resistin-induced increases in intracellular ROS levels and IL-6 expression levels in AR42J cells. Next, the effect of α-lipoic acid on the cerulein/resistin-induced increase in ROS levels and IL-6 expression level in AR42J cells was investigated. Cells were stimulated with cerulein and resistin for 45 min (for intracellular ROS
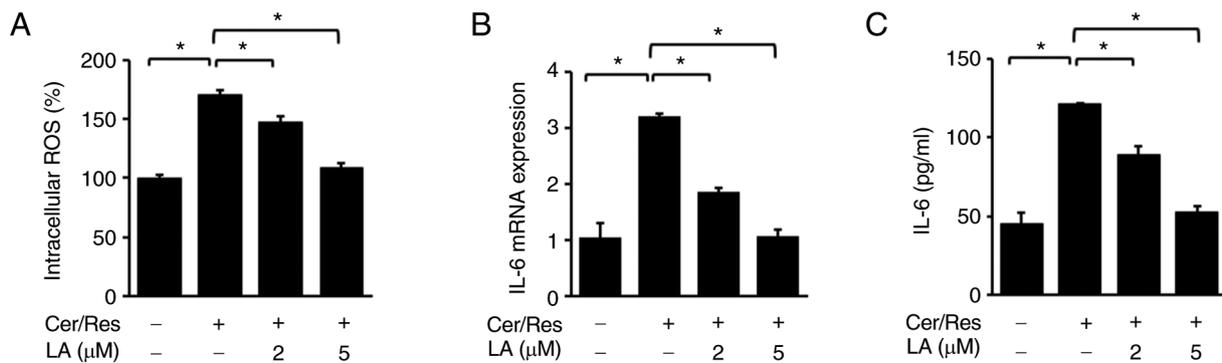
Figure 4. Effect of LA on the levels of intracellular ROS and IL-6 expression in cerulein/resistin-stimulated AR42J cells. Cells were pretreated with LA for 2 h and stimulated with Cer and Res for (A) 45 min, (B) 4 h, or (C) 24 h. (A) Intracellular ROS levels were measured using dichlorofluorescein diacetate. ROS levels are expressed as the relative increase. The value for ROS levels in unstimulated cells was set at 100%. (B) The mRNA expression of IL-6 was assessed by reverse transcription-quantitative PCR analysis. (B) The protein level of IL-6 in the cultured media was determined by ELISA. Data are expressed as the mean ± standard error (n=12 for each group). *P<0.05. LA, α-lipoic acid; ROS, reactive oxygen species; IL-6, intereukin-6; Cer, cerulein; Res, resistin.

levels, Fig. 4A), 4 h (for IL-6 mRNA expression, Fig. 4B), or 24 h (for IL-6 levels in the medium, Fig. 4C) in the absence or presence of α-lipoic acid (2 or 5 μM). α-lipoic acid decreased intracellular ROS levels in cerulein/resistin-stimulated cells in a dose-dependent manner (Fig. 4A). In addition, α-lipoic acid dose-dependently inhibited the cerulein/resistin-induced increase in IL-6 mRNA and protein levels (Fig. 4B and C).

GW9662 abolishes the effect of α-lipoic acid on intracellular ROS levels and expression of IL-6, HO-1 and catalase in cerulein/resistin-stimulated AR42J cells. To confirm the role of PPAR-γ in the antioxidant mechanism of α-lipoic acid, cells were simultaneously treated with the PPAR-γ antagonist GW9662 and α-lipoic acid (5 μM) for 2 h prior to stimulation with resistin and cerulein for 45 min (for intracellular ROS levels, western blot analysis and catalase activity; Fig. 5A, C and D, respectively) or 24 h (for IL-6 levels in the medium, Fig. 5B). In the presence of GW9662, the ability of α-lipoic acid to ameliorate the cerulein/resistin-induced increase in intracellular ROS levels (Fig. 5A) and IL-6 protein levels (Fig. 5B) was decreased. In addition, GW9662 hindered the α-lipoic acid-induced increase in HO-1 and catalase expression levels (Fig. 5C) and catalase activity (Fig. 5D). These findings provided corroborating evidence that PPAR-γ is responsible for mediating the α-lipoic acid-induced downregulation of IL-6 expression, decreased ROS levels, and upregulation of HO-1 and catalase expression in cerulein/resistin-stimulated cells.

HO-1 inhibitor ZnPP abolishes the inhibitory effect of α-lipoic acid on IL-6 expression and increases ROS levels in cerulein/resistin-stimulated AR42J cells. It was next sought to determine whether the inhibitory effect of α-lipoic acid on ROS and IL-6 levels is mediated by increased HO-1 expression level in cerulein/resistin-stimulated cells. Cerulein/resistin-stimulated AR42J cells were treated with α-lipoic acid in the presence or absence of the HO-1 inhibitor ZnPP. As revealed in Fig. 6, α-lipoic acid decreased the cerulein/resistin-induced increase in intracellular ROS levels and IL-6 expression level. ZnPP treatment reversed the effect of α-lipoic acid on IL-6 expression and intracellular ROS levels in cerulein/resistin-stimulated cells. Therefore, α-lipoic acid

downregulated IL-6 expression and reduced ROS levels by inducing the expression of the PPAR-γ target gene HO-1 in cerulein/resistin-treated cells.

Discussion

In the present study, it was determined whether α-lipoic acid ameliorates obesity-linked acute pancreatitis in cerulein/resistin-stimulated pancreatic acinar AR42J cells. Acute pancreatitis is a severe inflammatory disease with high mortality and morbidity rates. Obesity is a risk factor for acute pancreatitis. Patients with obesity tend to possess excess adipose tissue, which leads to higher levels of adipokine secretion such as resistin (4,5). Thus, resistin is recognized as a novel marker for predicting the severity of acute pancreatitis (13). It has been previously demonstrated that resistin aggravates IL-6 expression and zymogen activation in cerulein-stimulated pancreatic acinar cells (14). It was revealed that cerulein/resistin could initiate Ca^{2+} overload, leading to NADPH oxidase-mediated ROS production, resulting in NF-κB activation in cerulein/resistin-stimulated AR42J cells. Accordingly, a cocktail of resistin and cerulein was used to establish an *in vitro* obesity-associated acute pancreatitis model in the present study.

α-lipoic acid is an organosulfur compound commonly found in plants and animals, including humans (18). α-lipoic acid functions as a powerful antioxidant, and accumulating evidence has confirmed that α-lipoic acid exhibits potential therapeutic effects against diseases such as diabetes, atherosclerosis, tumors and neurodegenerative diseases (30). In the present study, α-lipoic acid decreased the cerulein/resistin-induced increase in intracellular ROS levels and IL-6 expression level in a dose-dependent manner.

Sechovcová *et al* (49) have reported that endogenous plasma levels of lipoic acid range between <4.9 and 197.0 nmol/l, determined using a conventional method. The authors developed a new method using high-performance liquid chromatography to determine endogenous lipoic acid levels. The endogenous lipoic acid concentration in the plasma of non-supplemented voluntary blood donors was <1.85 nmol/l. Borowczyk *et al* (50) have reported that the human plasma concentration of α-lipoic acid is 0.12-5.0 nmol/ml. If the human plasma concentration of
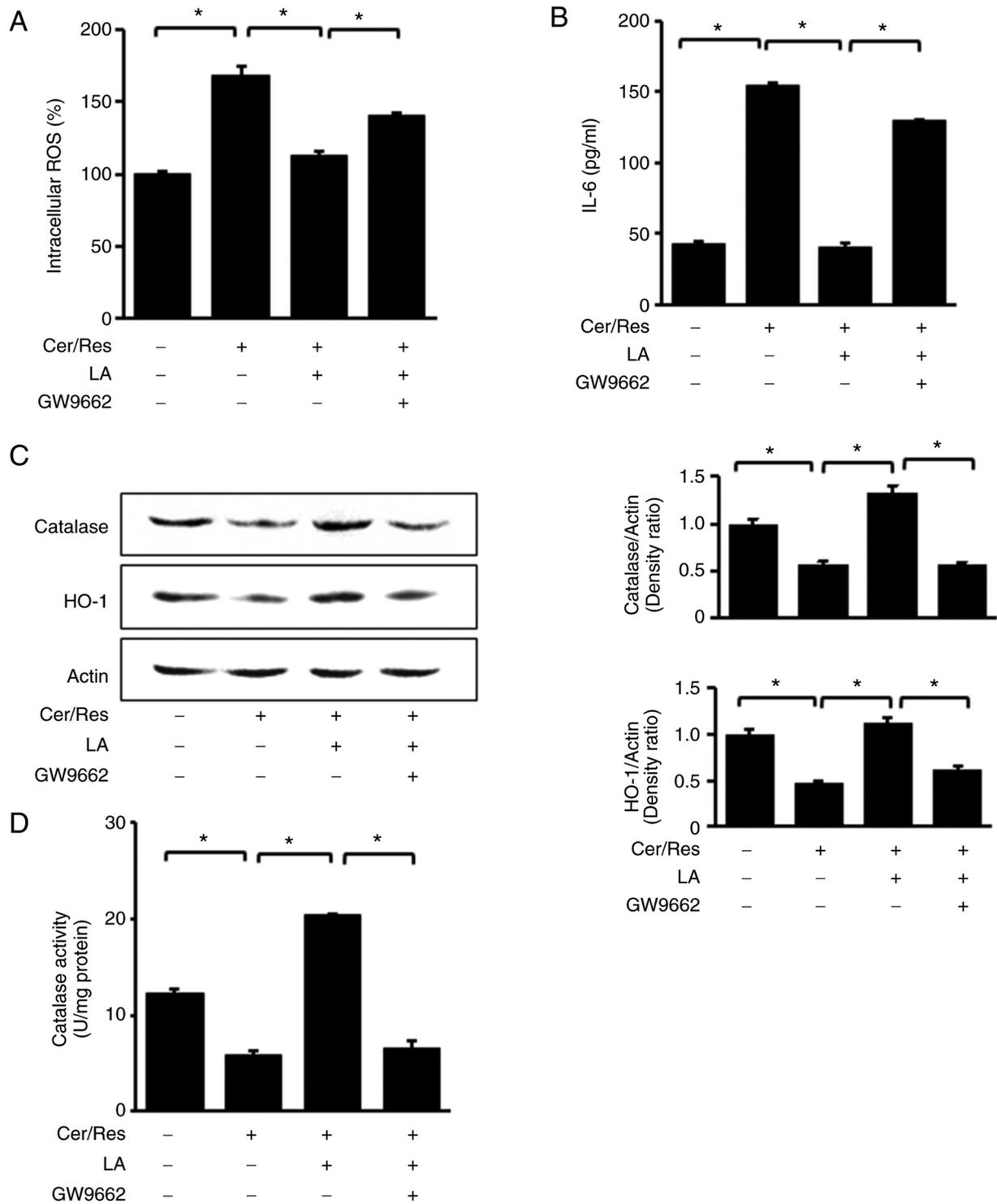
Figure 5. Impact of PPAR-γ antagonist GW9662 on the levels of intracellular ROS, IL-6, HO-1, and catalase in cerulein/resistin-stimulated AR42J cells treated with α-lipoic acid. Cells were co-treated with 10 μM GW9662 and 5 μM LA for 2 h, followed by stimulation with Cer and Res for 45 min (A,C and D), or 24 h (B). (A) Intracellular ROS levels were measured using dichlorofluorescein diacetate. Data are expressed as the mean ± SE (n=12 for each group). (B) The protein level of IL-6 in the media was determined by ELISA. (C) Protein levels of HO-1 and catalase in whole-cell extracts were determined by western blot analysis. Actin was used as the loading control (left panel). Densitometry data represent mean ± SE from three immunoblots and are shown as the relative density of protein band normalized to actin level (right panel). (D) Catalase activity was determined using a catalase assay kit. Data are expressed as the mean ± SE (n=12 for each group). *P<0.05. PPAR-γ, peroxisome proliferator-activated receptor-γ; ROS, reactive oxygen species; IL-6, intereukin-6; HO-1, heme oxygenase-1; LA, α-lipoic acid; Cer, cerulein; Res, resistin; SE, standard error.

α-lipoic acid is 5 nmol/ml, a plasma level of 5 μM α-lipoic acid can be obtained from human participants consuming α-lipoic acid-rich foods. A single oral supplementation of α-lipoic acid (600 mg) is rapidly absorbed (peak at 58 min with plasma concentration of 6.86±1.29 μg/ml), exhibiting high bioavailability and low toxicity (51). Concentrations at 2 and 5 μM were used for the cell culture system in the present study. Given that the human plasma concentration of α-lipoic acid is
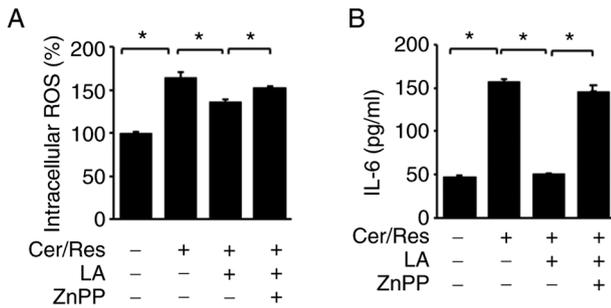
Figure 6. Effect of the HO-1 inhibitor ZnPP on the levels of intracellular ROS and IL-6 in cerulein/resistin-stimulated AR42J cells treated with LA. Cells were co-treated with 1 μM ZnPP and 5 μM LA for 2 h, followed by stimulation with Cer and Res for (A) 45 min or (B) 24 h. (A) Intracellular ROS levels were measured using dichlorofluorescein diacetate. (B) The protein level of IL-6 in the media was determined by ELISA. Data are expressed as the mean ± standard error (n=12 for each group). *P<0.05. HO-1, heme oxygenase-1; ZnPP, zinc protoporphyrin; ROS, reactive oxygen species; IL-6, intereukin-6; LA, α-lipoic acid; Cer, cerulein; Res, resistin.

variable, oral supplementation with α-lipoic acid or consumption of α-lipoic acid-rich foods may prevent obesity-associated acute pancreatitis.

Considering the antioxidant mechanism of α-lipoic acid, it was revealed that the treatment with α-lipoic acid increased the expression levels of PPAR-γ and its target genes HO-1 and catalase in unstimulated AR42J cells. Furthermore, in cerulein/resistin-stimulated AR42J cells, α-lipoic acid treatment restored the cerulein/resistin-induced decrease in PPAR-γ expression level and nuclear localization. A similar tendency was observed in western blot analysis, where α-lipoic acid treatment restored protein levels of PPAR-γ, HO-1, and catalase in a dose-dependent manner in cerulein/resistin-stimulated AR42J cells.

To examine the effect of PPAR-γ on cerulein/resistin-stimulated AR42J cells, the PPAR-γ antagonist, GW9662, which covalently modifies the ligand-binding site of PPAR-γ and inhibits PPAR-γ-mediated transcription, was used (52). Combined treatment with α-lipoic acid and GW9662 reversed the suppressive effect of α-lipoic acid on the cerulein/resistin-induced increase in ROS and IL-6 levels and decreased expression levels of HO-1 and catalase in AR42J cells. These results confirmed that PPAR-g upregulates its target genes HO-1 and catalase, thereby establishing their role in the protective mechanism of α-lipoic acid in cerulein/resistin-treated AR42J cells. To further investigate the effect of the PPAR-γ target gene HO-1, the HO-1 inhibitor ZnPP, a metabolite generated during heme biosynthesis, was used. High levels of ZnPP competitively inhibit HO-1 (53). Therefore, ZnPP has been widely used as a potent HO-1 inhibitor in experimental studies. Treatment with ZnPP reversed the inhibitory effect of α-lipoic acid on the cerulein/resistin-induced increase in IL-6 expression and ROS levels in cerulein/resistin-stimulated AR42J cells.

The present results revealed that α-lipoic acid inhibited cerulein/resistin-induced increment of ROS and IL-6 levels in AR42J cells. α-lipoic acid could activate PPAR-γ and upregulate the expression levels of its target genes HO-1 and catalase in AR42J cells. Inhibition of PPAR-γ or HO-1 by GW9662 or ZnPP, respectively, suppressed the inhibitory

effect of α-lipoic acid on cerulein/resistin-induced increase in ROS levels and IL-6 expression level. These results indicated that α-lipoic acid may reduce ROS levels and IL-6 expression level by upregulating the PPAR-γ signaling pathway in cerulein/resistin-stimulated AR42J cells. Since other factors than ROS may mediate the development of cerulein/resistin-induced pancreatitis, further study should be performed to investigate whether α-lipoic acid alleviates the overall symptoms of cerulein/resistin-induced pancreatitis.

In the present study, AR42J cells were used to examine the pathological mechanisms of cerulein/resistin-induced inflammatory events. AR42J cells are derived from chemically induced rat pancreatic acinar carcinoma (54) and maintain the characteristics of normal pancreatic acinar cells, including calcium signaling, synthesis and secretion of digestive enzymes, receptor expression and signal transduction mechanisms (54,55). Thus, AR42J cells have been widely used to study the function of the exocrine pancreas and as an *in vitro* model of cerulein-induced acute pancreatitis (56-61). In addition, several studies have used AR42J cells as an *in vitro* model of resistin- or cerulein/resistin-induced acute pancreatitis (12-14).

Lugea *et al* (62) used human pancreatic acinar cells isolated from cadaveric donor pancreata for transplantation. However, freshly isolated human pancreatic acinar cells rapidly change their phenotype when placed in culture. This includes losses of polarity, secretory responsiveness, calcium mobilization in response to stimulation, and other aspects of differentiation. Until now, there has been no currently available human pancreas-derived cell lines which fully represent the acinar cell phenotype and function.

Regarding the effect of cerulein on cell proliferation, Chao *et al* (63) demonstrated that blockade of cerulein-induced IL-6 accelerates acinar cell apoptosis and attenuates experimental acute pancreatitis *in vivo*. A neutralizing antibody against IL-6 effectively suppressed increase in serum amylase, IL-6 levels, and pancreatitis-associated lung injury and caused induction of apoptosis in the pancreatic acinar cells of mice with acute pancreatitis. Our *in vitro* studies using pancreatic acinar cells treating cerulein showed that 24-h treatment of cerulein (10^{-7} M) increased protein level of apoptosis-inducing factor (64). However, 4-h treatment of cerulein (10^{-8} M) induced the expression of genes related to proliferation and differentiation such as lithostatin, progestin-induced protein and stathin 1 in pancreatic acinar cells (65). In the present study, AR42J cells were treated with cerulein (10^{-8} M). Therefore, further study is necessary to determine whether cerulein (10^{-8} M) affects cell death to determine the relation of IL-6 expression and apoptosis in pancreatic acinar cells.

Although the evidence obtained from the *in vitro* cell culture model fails to precisely represent the events occurring in human obesity-related acute pancreatitis, they could provide a possible pathologic mechanism clarifying how resistin aggravates acute pancreatitis. As previously described, blood levels of resistin are higher in patients with acute pancreatitis than in healthy individuals (15-17) and represent the severity of acute pancreatitis; resistin treatment may increase inflammatory events in cerulein-stimulated pancreatic acinar cells. Further studies are warranted to determine the effect of α-lipoic acid

on levels of IL-6, ROS, PPAR-γ, HO-1 and catalase in pancreatic tissues and nuclear levels of PPAR-γ in the pancreas of animals treated with cerulein and resistin.

For the studies on agonist of PPAR-γ in cerulein-stimulated AR42J cells, it was previously identified that pre-treating cerulein (10^{-8} M)-stimulated AR42J cells with PPAR-γ ligands, 15d-PGJ2 and troglitazone, inhibited ROS-mediated JAK2/STAT3 activation and IL-6 expression (38). In addition, it was revealed that troglitazone inhibited the cerulein (10^{-8} M)-induced increase in ROS and IL-6 expression, but induced catalase expression in AR42J cells (66).

From the previous study, the possible molecular mechanism by which α-lipoic acid increases PPARγ protein expression can be postulated. It was previously demonstrated that docosahexaenoic acid acts as an agonist of PPARγ, which mediates the expression of PPARγ-target catalase expression and reduce ROS levels, leading to the inhibition of JAK2/STAT3 activation and IL-6 expression in cerulein-stimulated acinar cells (66). In the present study, it is evident that α-lipoic acid activates PPARγ and induces catalase and HO-1 expression in AR42J cells stimulated with cerulein/resistin. Further study should be performed to explore whether α-lipoic acid binds to PPARγ to induce its target genes in AR42J cells.

In conclusion, α-lipoic acid activates PPAR-γ and upregulates its downstream target antioxidant genes HO-1 and catalase, thereby reducing ROS levels. Based on this molecular mechanism, α-lipoic acid significantly suppresses cerulein/resistin-induced IL-6 expression in pancreatic acinar cells.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HK conceived and designed the experiments. JWL assisted in the experimental design. YL performed the experiments. YL and JWL analyzed the data. YL and JWL confirm the authenticity of all the raw data. YL wrote the paper. HK reviewed and edited the paper. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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