Melatonin inhibits the endoplasmic reticulum stress-induced, C/EBP homologous protein-mediated pathway in acute pancreatitis

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Abstract. Melatonin, which is mainly secreted by the pineal gland, appears to have anti-inflammatory activities. Acute pancreatitis (AP) is characterized by inflammation and acinar cell death, and is associated with a high mortality rate. It has been reported that melatonin can alleviate cerulein (Cer) or Cer + lipopolysaccharide (LPS)-induced inflammatory responses in AR42J rat pancreatic acinar cells (AR42J cells). CCAAT/enhancer binding protein homologous protein (CHOP) is a specific transcription factor involved in endoplasmic reticulum (ER) stress-induced apoptosis, and regulates ER stress responses. However, the mechanisms of the anti-inflammatory effects of melatonin' are unknown, particularly the relationship between melatonin and ER stress. Therefore, the present study aimed to investigate the anti-inflammatory activity of melatonin in AR42J cells and analyze its molecular mechanisms during ER stress. The RNA interference method was used to determine the potential role of CHOP in AR42J cells during AP. *In vitro* models of AP were induced by treating AR42J cells with Cer + LPS, and pre-treatment with melatonin was used to identify the potential anti-inflammatory mechanisms. The cells also underwent Cell Counting Kit-8, western blotting and reverse transcription-quantitative PCR analyses. The expression levels of ER stress-related molecules were rapidly activated in the early stage and increased over time in the AR42J AP models, with significant pancreatic inflammation and apoptosis. However, knockdown of CHOP expression significantly reduced apoptosis, the activation of NF-κB and the downstream signal pathway. Moreover, cells treated with melatonin exhibited attenuated inflammation, decreased expression levels of ER stress-associated proteins and inhibition of apoptosis. Thus, the present results suggested that melatonin may attenuate the inflammatory response by inhibiting the activation of the CHOP-mediated pathway in AR42J cells.

Introduction

Acute pancreatitis (SAP) is a prevalent and serious disease caused by activation of digestive proteases in the pancreas, which may result in pancreatic tissue autodigestion, tissue edema, hemorrhage or acinar necrosis, although most are mild patients (mild acute pancreatitis, MAP), ~25% of patients worldwide are severe (severe acute pancreatitis, SAP), which is clinically dangerous and often complicated by systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (1). Although gallstones are known to be the main cause of acute pancreatitis, its diagnosis and management remain challenging, and the pathogenesis of this disease is not fully understood despite the proposal of several explanatory theories (2).

The exocrine pancreas is one of the main organs responsible for digestion; thus, acinar cells have abundant endoplasmic reticulum (ER) to support their central role in the synthesis of digestive enzymes (3). Therefore, acinar cells are extremely sensitive to external stimuli in response to ER perturbations (4). A previous study reported that ER stress serves an important role during the development of AP (5). Furthermore, Hartley *et al* (6) identified morphological changes in the ER caused by ER stress. Pancreatic acinar cell injury, characterized by the formation of vesiculation in the ER a few minutes after a retrograde injection of taurocholate into the biliopancreatic duct in rats, has also been reported by Bhatia *et al* (7). Moreover, the ER is one of the largest cell organelles and is recognized as a vital site in the regulation of protein synthesis, folding and assembly, in addition to regulation of calcium ion levels and cellular stress response (8). Various external stimuli, such as calcium homeostasis imbalance, insufficient energy supply or the production of reactive oxygen species can all lead to excessive accumulation of unfolded or misfolded proteins in the ER, causing ER stress (6). To overcome ER stress, cells activate a series of specific signal transductionpathways, which are collectively

known as the unfolded protein response (UPR) (9). The UPR in the ER is regulated by three ER membrane-associated proteins [PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6)] (10), with the initial intention to alter cellular transcriptional and translational programs, enhance protein folding ability and accelerate protein degradation, in order to restore homeostasis and normal ER function (8,11). However, when ER injury is irreversible, the UPR pathways initiate apoptosis (11). Moreover, induction of the CCAAT/enhancer binding protein (C/EBP)-homologous protein (CHOP), a member of the C/EBP family of transcription factors, is a signaling event involved in ER stress-induced apoptosis (12-14).

CHOP plays a crucial role in ER stress-induced apoptosis, and was first revealed to be one of the main mediating factors in growth arrest and damage response (15). It has been reported that CHOP expression is significantly increased in severe ER stress, and thus promotes cell cycle arrest and/or apoptosis (16,17). However, knockdown of CHOP was revealed to confer resistance to ER stress-induced apoptosis in various experimental models (14). The involvement of CHOP-mediated apoptosis has also been revealed in numerous diseases, such as brain ischemia, diabetes, neurodegenerative abnormalities and some cardiovascular diseases (14,18,19).

Previous studies have suggested that ER stress can initiate an inflammatory response via NF-κB activation in several cells and diseases, including Alzheimer's disease and pancreatic acinar cells (20-24). Allagnat *et al* (25) reported that CHOP plays a pivotal role in the pathogenesis of the inflammatory response by directly contributing to NF-κB pathway activation and subsequent cytokine and chemokine expression, leading to ER stress and CHOP expression, and in turn, CHOP facilitates and amplifies NF-κB pathway activity. Furthermore, NF-κB is a key transcriptional factor that has an important role in the initial stage of inflammation (26), which controls the expression of numerous inflammatory mediators and cell apoptosis genes (27,28). However, continued NF-κB activity and excessive inflammatory responses lead to the development of AP (29,30). Thus, it was speculated that CHOP may have a central role in the pathogenesis of inflammatory responses.

Melatonin, which is mainly secreted by the pineal gland, plays a key role in inflammation and immune defense (31). In addition, melatonin is able to attenuate oxidative stress damage and inhibit inflammatory activities (32), and has exhibited anti-inflammatory effects via the NF-κB signaling pathway (33). As early as 1999, studies have reported the beneficial effects of melatonin, which attenuates tissue edema and lipid peroxidation in cerulein (Cer)-induced AP (34). Moreover, previous studies revealed that melatonin can reduce liver fibrosis and cirrhosis by inhibiting ER stress (35,36). However, it remains unknown whether melatonin exhibits its anti-inflammatory effect via the ER stress-induced CHOP-mediated pathway.

The present study investigated the potential role of melatonin in the CHOP-mediated signaling pathway to decrease inflammation and apoptosis in AR42J cell models of AP.

Materials and methods

Lentivirus (LV)-mediated stable RNA interference (RNAi) of CHOP in AR42J cells. AR42J cells (American Type Culture Collection) were cultured in DMEM with 20% FBS (Sigma-Aldrich; Merck KGaA) and antibiotics (100 U/ml penicillin and 100 μl/ml streptomycin) at 37°C in a humidified incubator with 5% CO_2. The LV (vehicle information: hU6-MCS-Ubiquitin-EGFP-IRES-puromycin; Shanghai GeneChem Co., Ltd.), which carried short hairpin RNA (shRNA) that targeted the CHOP gene (LV-shCHOP; GenBank NM_024134) or that did not have an RNAi effect (LV-control; product no. GCNL89264) were both purchased from Shanghai GeneChem Co., Ltd., and designated as LV-shCHOP and LV-control cells, respectively.

AR42J wild-type cells (3-5x10⁴ cells/well) were seeded in 12-well plates and incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. The medium in each well was subsequently replaced with 100 μl viral suspension (1x10⁸ TU/ml) and 400 μl DMEM without FBS and antibiotics in the presence of 10 μg/ml polybrene (Shanghai GeneChem Co., Ltd.). The multiplicity of infection was ~100. This was followed by 8 h of culture under standard conditions and then replacement with 1 ml fresh medium. During this period, cell passaging and medium refreshment were routinely performed. After 72-96 h of virus infection, green fluorescent protein (GFP)-positive cells were observed under a Nikon inverted fluorescence microscope (Nikon Corporation) at x40 magnification. The effects of knockdown of CHOP expression in AR42J cells were analyzed by western blotting and reverse transcription-quantitative PCR (RT-qPCR).

Cell treatment and groups. In total, three types of AR42J cell lines (wild-type, LV-control and LV-shCHOP cells) were seeded in 6-well plates (5x10⁵ cells/well). Following incubation for 24 h at 37°C in a humidified incubator with 5% CO_2, the groups were defined as follows: Group I, three types of AR42J cells were added to PBS alone and used as the negative control (control); Group II, three types of AR42J cells were treated with Cer (10 mM, Sigma-Aldrich; Merck KGaA) + LPS (10 mg/l, Sigma-Aldrich; Merck KGaA) to induce AP (Cer + LPS) for 8 h at 37°C in a humidified incubator with 5% CO_2; Group III, LV-control cells were treated with a low dose of melatonin (0.5 mmol/l, Sigma-Aldrich; Merck KGaA) 30 min before AP was induced (0.5 mM) at 37°C in a humidified incubator with 5% CO_2; and Group IV, LV-control cells were treated with a high dose of melatonin (2 mmol/l) 30 min before AP was induced (2 mM) at 37°C in a humidified incubator with 5% CO_2. Then 9 h later, cell extracts were collected for western blotting and RT-qPCR.

Furthermore, to assess the effects of Cer + LSP treatment on the protein expression levels of ER stress markers, apoptosis and inflammatory-related molecules, wild-type cells were treated with Cer + LPS for 0 (control), 3, 6, 9 and 12 h at 37°C in a humidified incubator with 5% CO_2, and then analyzed by western blotting. Each experiment was performed in triplicate.

Cell viability assay. The effects of melatonin on the viability of AR42J wild-type cells after Cer + LPS treatment was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Wild-type cells (4x10³ cells/well) were seeded into 96-well (100 μl/well) plates. After 24 h, the cells were treated with various concentrations of melatonin (0.5, 1, 2 and

4 mM) 30 min before AP was induced (melatonin treatment groups; 0.5-4 mM), 0.01 M PBS (negative control group) and Cer + LPS (AP group) at 37°C in a humidified incubator with 5% CO_2. Each group consisted of five parallel wells. Following incubation for 0, 3, 6, 9 and 12 h at 37°C in a humidified incubator with 5% CO_2, 100 μl of culture medium containing 10 μl CCK-8 was then added to the culture media. An enzyme standard instrument (Infinite® 200 PRO NanoQuant; Tecan Austria GmbH) was used to measure the supernatant of each well at a wavelength of 450 nm. Cell viability was analyzed in each group by the optical density value. Each experiment was performed in triplicate.

Western blot analysis. Western blotting was performed to assess the protein expression levels of β-actin, 78 kDa glucose-regulated protein (GRP78), CHOP, tumor necrosis factor-α (TNF-α), Bcl-2, Bax, caspase-3, phospho-NF-κB inhibitor α (P-IκBα) and phospho-NF-κB p65 (p-p65).

Total proteins from pancreatic acinar cells were extracted and homogenized in ice-cold RIPA buffer (Shanghai Biyuntian Bio-Technology Co., Ltd.) supplemented with protease and phosphatase inhibitors for 30 min on ice. The extracts were then transferred to a microcentrifuge tube and centrifuged at 1.2×10^4 x g for 20 min at 4°C, and the protein concentrations were determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). Subsequently, 45 μg total protein per lane was separated by 10% SDS-PAGE and then transferred to PVDF membranes (EMD Millipore) at 300 mA for 0.5-1 h. The membranes were blocked at 20-25°C for 2 h with 5% non-fat milk and then immunoblotted with specific primary antibodies overnight at 4°C. The following primary antibodies were used: GRP78 (product code ab108615), CHOP (also known as DDIT3; product code ab179823), TNF-α (product code ab6671), Bcl-2 (product code ab32124), Bax (product code Ab182733) and caspase-3 (product code Ab32351; all from Abcam), p-IκBα (Ser 32; product no. 2859), IκBα (44D4; product code 4812), p-p65 (Ser 536; product code 3033), p65 (D14E12; product code 8242) and β-actin (D6A8; product code 8457) all from Cell Signaling Technology, Inc.) at 1:1,000 dilution. β-actin was used as an internal control. The following day, the membranes were washed three times with TBST containing 1% Tween-20 for 10 min each time and incubated for 1 h at 20-25°C with a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:5,000; product code BS10003; Bioworld Technology, Inc.), and then washed with TBST as before. Protein bands were visualized using a Western Bright ECL detection kit (Advansta, Inc.). Images of the protein bands were captured using the ChemiDoc MP imaging densitometer (Bio-Rad Laboratories, Inc.) and the density of the bands was quantified using Image Lab software 4.1 (Bio-Rad Laboratories, Inc.). Each experiment was performed in triplicate.

RT-qPCR. Total RNA from pancreatic acinar cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then synthesized to cDNA using a RT kit (product code K1691; Invitrogen; Thermo Fisher Scientific, Inc.) at 25°C for 5 min, 42°C for 60 min and 70°C for 5 min. PCR was subsequently performed in the presence of specific primers to the cDNA of rat genes. The PCR primers were synthesized by Sangon Biotech Co., Ltd., and the sequences were as follows: GRP78 forward, 5'-CAAGAACCAACT CACGTCCA-3' and reverse, 5'-ACCACCTTGAATGGCAAG AA-3'; CHOP forward, 5'-CCAGGAAACGAAGAGGAAG A-3' and reverse, 5'-CTTTGGGAGGTGCTTGTGA-3'; TNF-α forward, 5'-TGATCCGAGATGTGGAACTG-3' and reverse, 5'-CGAGCAGGAATGAGAAGAGG-3'; interleukin (IL)-6, forward 5'-TACCCCAACTTCCAATGCTC-3' and reverse, 5'-GGTTTGCCGAGTAGACCTCA-3'; Bcl-2 forward, 5'-AGG ATTGTGGCCTTCTTTGA-3' and reverse, 5'-CAGATGCCG GTTCAGGTACT-3'; Bax forward, 5'-CAGGATCGAGCA GAGAGGAT-3' and reverse, 5'-GTCCAGTTCATCGCCAAT TC-3'; caspase-3 forward, 5'-ACTGGACTGTGGCATTGA GA-3' and reverse, 5'-AATTTCGCCAGGAATAGTAACC-3'; and β-actin, forward 5'-CGTGAAAAGATGACCCAGAT-3' and reverse, 5'-ACCCTCATAGATGGGCACA-3'. The qPCR procedure was conducted using a RT qPCR system (Bio-Rad Laboratories, Inc.) with the following: Initial denaturation at 50°C for 2 min; 40 cycles of 95°C for 30 sec, 95°C for 5 sec and 60°C for 34 sec) and the Takara Power SYBR-Green PCR Master mix (cat. no. DRR820A; Takara Bio, Inc.). β-actin was used as the internal standard, and quantified relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (37). Each experiment was performed in triplicate.

Statistical analysis. The results were analyzed using SPSS software 20.0 (SPSS, Inc.), and data are presented as the mean ± SEM. One-way ANOVA followed by a Tukey's post-hoc test was performed for comparisons of ≥3 groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of melatonin on the viability of wild-type cells after Cer + LPS treatment. Cell viability of the Cer + LPS group was significantly reduced compared with the control group after 12 h of treatment (70% viability; Fig. 1). However, when the concentration of melatonin was increased, cell viability was gradually enhanced (Fig. 1), with a maximum effect observed in the 4-mM group compared with the Cer + LPS group at the same time period (Fig. 1). Thus, it was speculated that melatonin may have a dose-dependent effect on cell viability.

Effects of Cer + LPS on the CHOP-mediated pathway at different treatment times. The expression levels of factors related to the CHOP-mediated pathway were examined by western blotting in cells cultured with Cer + LPS for 0, 3, 6, 9 and 12 h. It was demonstrated that expression levels of the inflammatory markers, p-p65, P-IκBα, and TNF-α, and ER stress markers, GRP78 and CHOP, were increased over time (Fig. 2). Moreover, the expression levels of pro-apoptosis-related molecules caspase-3 and Bax increased, while the anti-apoptotic protein Bcl-2 decreased in the early stage of inflammation. However, at the 12-h time-point, Bax expression was decreased (Fig. 2).

Transduction efficacy of LV-shCHOP-mediated RNAi in AR42J cells. At 72-96 h after infection, highly efficient transduction (>95%) of LV-shCHOP was identified in AR42J
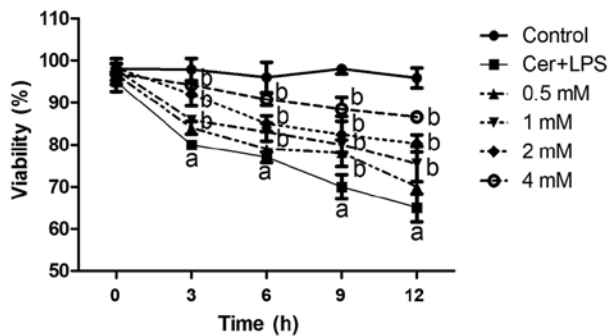
Figure 1. Effects of melatonin on the viability of AR42J cells after Cer + LPS treatment. Wild-type cells were treated with various concentrations of melatonin (0.5, 1, 2 and 4 mM) for 30 min before Cer + LPS treatment (melatonin treatment groups, 0.5-4 mM), 0.01 M PBS (negative control) and Cer + LPS (AP group). Cell viability was examined at 0, 3, 6, 9 and 12 h with a Cell Counting Kit-8 assay. Data are presented as the mean ± SEM of ≥3 independent experiments. [a]$P<0.05$ vs. the control group. [b]$P<0.05$ vs. the Cer + LPS group at the same time period. Cer + LPS, cerulein plus LPS; AP, acute pancreatitis; LPS, lipopolysaccharide.
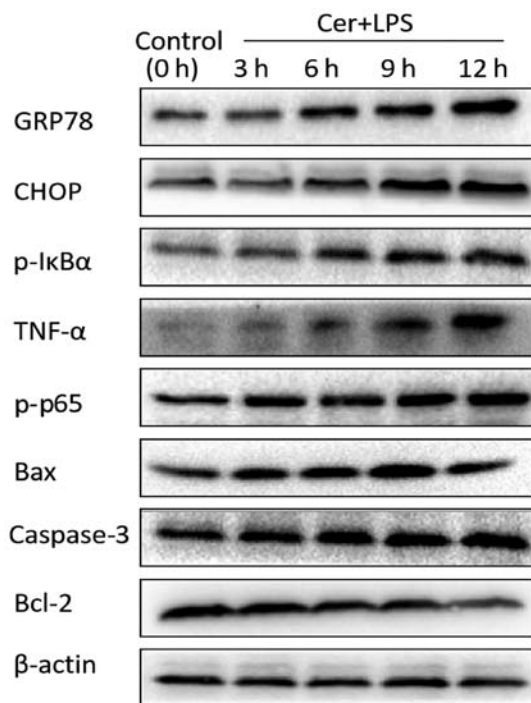

Figure 2. Effects of Cer + LPS treatment on the protein expression levels of ER stress markers, apoptosis and inflammation-related molecules in AR42J cells. Wild-type cells were treated with Cer + LPS for 0, 3, 6, 9 and 12 h. The protein expression levels of GRP78, CHOP, p-p65, TNF-α, p-IκBα, Bcl-2, Bax and caspase-3 were assessed by western blotting, and β-actin was used as an internal control. Cer + LPS, cerulein + LPS; LPS, lipopolysaccharide; GRP78, 78 kDa glucose-regulated protein; CHOP, CCAAT/enhancer binding protein (C/EBP)-homologous protein; p-IκBα, phospho-NF-κB inhibitor α; TNF-α, tumor necrosis factor-α; p-p65, phospho-NF-κB p65.

cells, as GFP expression was observed under a Nikon inverted fluorescence microscope (Fig. 3), and the LV-control and LV-shCHOP cells had similar transduction efficiencies (Fig. 3).

Effects of Cer + LPS and treatment with melatonin on the expression of ER stress-related factors. Western blotting results identified increased GRP78 and CHOP protein expression

levels in LV-control cells treated with Cer + LPS compared with the respective controls, and there were no differences between wild-type cells and LV-control cells. However, LV-shCHOP cells after Cer + LPS treatment and LV-control cells in the melatonin treatment groups both had reduced GRP78 and CHOP expression levels, compared with LV-control cells treated with Cer + LPS (Fig. 4A). These findings were also demonstrated by RT-qPCR (Fig. 4B). LV-shCHOP cells had a greater effect of silencing on CHOP expression in AR42J cells and significantly attenuated ER stress after Cer + LPS treatment. Moreover, pre-treatment of LV-control cells with melatonin also downregulated CHOP expression and inhibited ER stress to protect the cells. Therefore, the extent of inhibition by melatonin was dose dependent.

Effects of Cer + LPS and treatment with melatonin on inflammation and apoptosis-associated molecules. The expression levels of NF-κB pathway and pro-inflammatory molecules were increased following Cer + LPS treatment, compared with the respective controls. However, the expression levels of these molecules in LV-shCHOP cells after Cer + LPS treatment and LV-control cells in the melatonin treatment groups were lower compared with the LV-control cells, after Cer + LPS treatment (Fig. 5A and B).

Furthermore, there were significantly enhanced Bax and caspase-3 expression levels and reduced Bcl-2 expression in LV-control cells after Cer + LPS treatment, compared with the respective controls (Fig. 6A and B). Moreover, LV-shCHOP cells treated with Cer + LPS and LV-control cells in the melatonin treatment groups had significantly reduced expression levels of Bax and caspase-3 and enhanced expression of Bcl-2 compared with the LV-control cells treated with Cer + LPS. Collectively, the results indicated that knockdown of CHOP expression reduced activation of the NF-κB pathway, inflammation and the apoptotic response after Cer + LPS treatment, while pre-treatment with melatonin also attenuated inflammation and apoptosis. Furthermore, the extent of inhibition by melatonin exhibited a dose-dependent effect.

Discussion

The present results indicated that melatonin had an anti-inflammatory effect via the ER stress-induced CHOP-mediated pathway in AR42J cells. Moreover, ER stress was significantly activated in the early stage of AP induced by Cer + LPS. The CHOP-mediated pathway also aggravated acinar cell damage by inducing apoptosis and NF-κB activation. Furthermore, it was indicated that treatment with melatonin significantly attenuated the expression of pro-inflammatory cytokines and ER stress-related molecules, and exerted a protective effect by inhibiting apoptosis of acinar cells.

In cells, heat shock proteins (HSPs) promote the modification of newly synthesized proteins in the ER and their translocation to the cellular membrane (38), and this is essential in the process of recovery to reduce stimulation and injury (39). In addition, under inflammatory conditions, the regulation of HSPs may attenuate the inflammatory response (40). GRP78, which belongs to the HSP family, is important in the response to ER stress (41). When excess unfolded or misfolded proteins
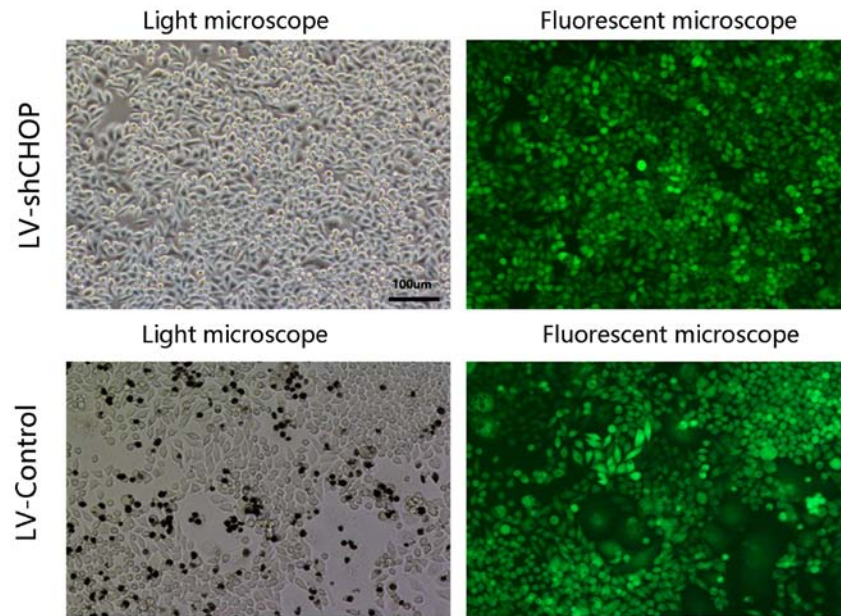
Figure 3. Infection efficacy of LV-shCHOP and LV-control mediated RNA interference in AR42J cells. The transduction efficiencies of these two types of cells were detected using light microscopy and fluorescent microscopy. Magnification, x40. Scale bar, 100 μm. LV, lentivirus; sh, short hairpin RNA; CHOP, CCAAT/enhancer binding protein (C/EBP)-homologous protein.
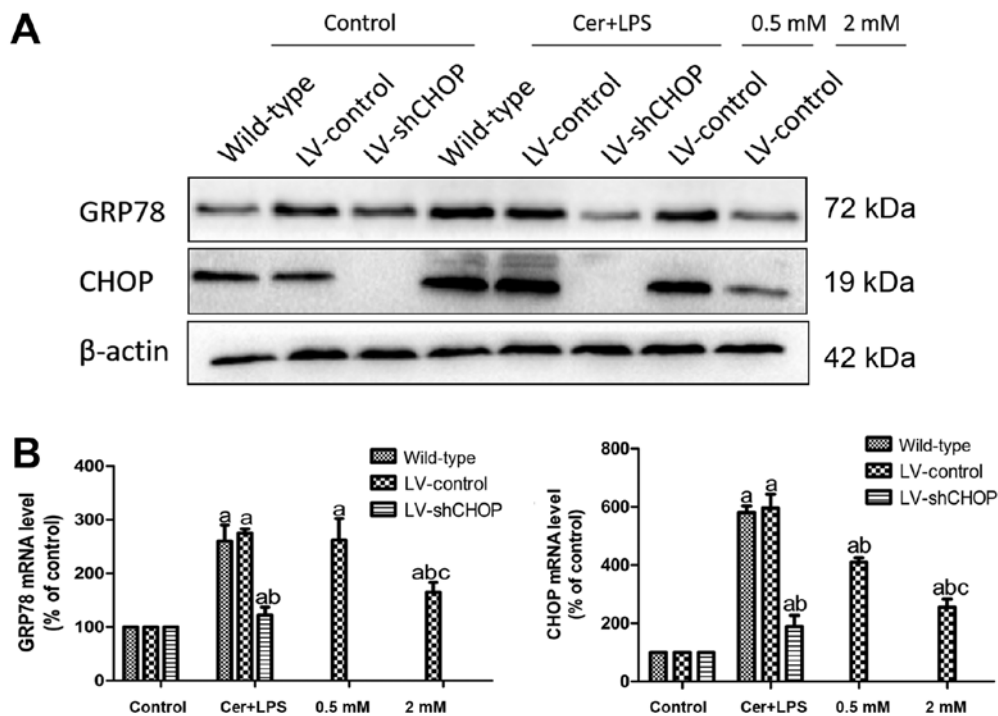

Figure 4. Effects of Cer + LPS and treatment with melatonin on the expression of ER stress-related molecules in AR42J cells. (A) Protein expression levels of GRP78 and CHOP at 9 h after Cer + LPS and melatonin treatment were assessed by western blotting, and β-actin was used as the internal control. (B) mRNA expression levels of GRP78 and CHOP were quantified by reverse transcription-quantitative PCR at 9 h. Data are presented as the mean ± SEM of ≥3 independent experiments. Control, treated with PBS; 0.5 mM, treated with 0.5 mM melatonin 30 min before CER + LPS treatment; 2 mM, treated with 2 mM melatonin 30 min before CER + LPS treatment. [a]P<0.05 vs. the respective controls. [b]P<0.05 vs. the LV-control cells treated with Cer + LPS. [c]P<0.05 vs. the 0.5 mM group. Cer + LPS, cerulein + LPS; LV, lentivirus; sh, short hairpin RNA; CHOP, CCAAT/enhancer binding protein (C/EBP)-homologous protein; LPS, lipopolysaccharide; GRP78, 78 kDa glucose-regulated protein.

accumulate in the ER causing ER stress, GRP78 separates from the three major triggering molecules (ATF6, PERK and IRE1) of the UPR, thus initiating the downstream UPR signaling pathways (42). The present results revealed that

GRP78 was upregulated early after Cer + LPS treatment and exhibited a time-dependent increase, which suggested that ER stress was activated early in AP and became increasingly severe over time.
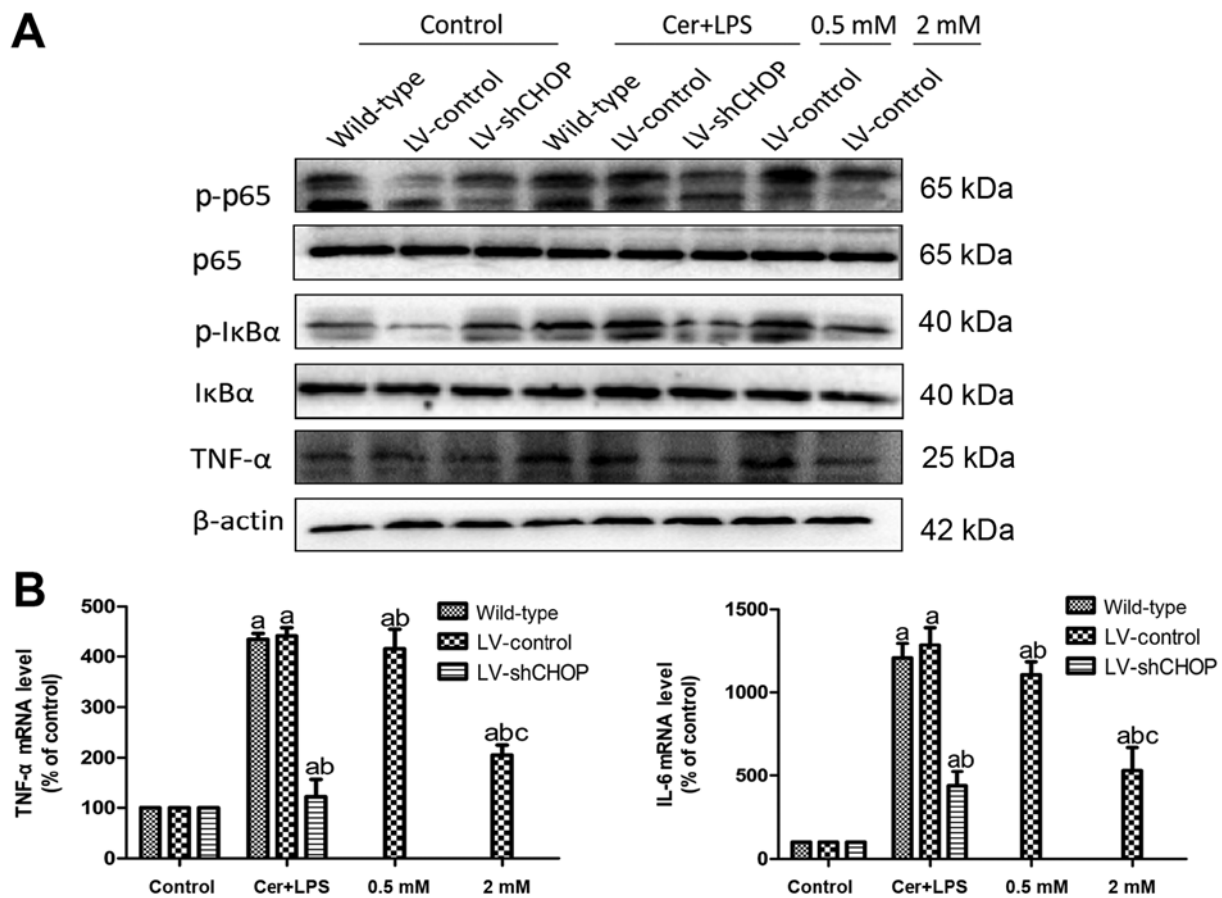
Figure 5. Effects of Cer + LPS and treatment with melatonin on the expression of inflammation-related molecules in AR42J cells. (A) Protein expression levels of p-p65, p65, p-IκBα, IκBα and TNF-α at 9 h after Cer + LPS and melatonin treatment were assessed by western blotting, and β-actin was used as the internal control. (B) mRNA expression levels of IL-6 and TNF-α were quantified by reverse transcription-quantitative PCR at 9 h. Data are presented as the mean ± SEM of ≥3 independent experiments. Control, treated with PBS; 0.5 mM, treated with 0.5 mM melatonin 30 min before Cer + LPS treatment; 2 mM, treated with 2 mM melatonin 30 min before Cer + LPS treatment. [a]$P<0.05$ vs. the respective controls. [b]$P<0.05$ vs. the LV-control cells treated with Cer + LPS. [c]$P<0.05$ vs. the 0.5 mM group. Cer + LPS, cerulein + LPS; LV, lentivirus; sh, short hairpin RNA; CHOP, CCAAT/enhancer binding protein (C/EBP)-homologous protein; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; p-IκBα, phospho-NF-κB inhibitor α; p-p65, phospho-NF-κB p65.

Apoptosis is the process of programmed cell death. Numerous studies have reported that apoptosis and necrosis occur in clinical and experimental AP (43-45), and some studies have indicated that these are involved in ER stress-induced apoptosis (46,47). Furthermore, the apoptotic signal can be induced when cells are subjected to excessive and prolonged ER stress (11).

Previous studies have revealed that ER stress can exacerbate inflammatory damage in pancreatic tissue by regulating cell death, and thus promote the development of pancreatitis (48-50). ER stress-induced apoptosis includes the following three signaling pathways: i) CHOP-mediated apoptosis via the downregulation of Bcl-2; ii) caspase-12-mediated pathway, which plays a role in apoptosis by activating caspase-9 and caspase-3; and iii) kinase 1-c-Jun N-terminal kinase pathway activation (13,14). Therefore, it is important to identify the extent to which signaling pathways contribute to acinar cell apoptosis after Cer + LPS treatment. CHOP is a pro-apoptotic molecule and can be activated by all three branches of the ER stress pathway, and in turn has been targeted for the modulation of ER stress (51). In the present study, the *in vitro* models of AP demonstrated that CHOP expression was significantly increased after Cer + LPS treatment, indicating that ER

stress was activated early in AR42J cells under the condition of Cer + LPS-induced inflammation. Thus, the LV-mediated RNAi method was used to determine the potential role of CHOP in the regulation of cell apoptosis during AP. The present results indicated that the mRNA and protein expression levels of CHOP could be specifically silenced, while this did not occur using a non-target LV that did not have an RNAi effect on CHOP expression in AR42J cells. Furthermore, knockdown of CHOP led to an almost complete suppression of apoptosis after Cer + LPS treatment, which significantly decreased the expression levels of Bax and caspase-3, and increased the expression of Bcl-2 in the experimental pancreatitis model. This pro-apoptotic potential may be due to CHOP, as it is one of the highest inducible genes underlying ER stress. Moreover, excessively activated IRE1α may result from the recruitment of TNF receptor-associated factor 2 (TRAF2) and its combining with apoptosis signal-regulated kinase 1 (ASK1) to form the IRE-1-TRAF2-ASK1 complex on the outer membrane of ER, which activates the c-Jun amino-terminal kinase and p38 mitogen-activated protein kinase (52). In addition, phosphorylation of p38 mitogen-activated protein kinase can induce the expression of CHOP after transduction of the activation domain serine 78/81 (14,52,53). ER stress-induced
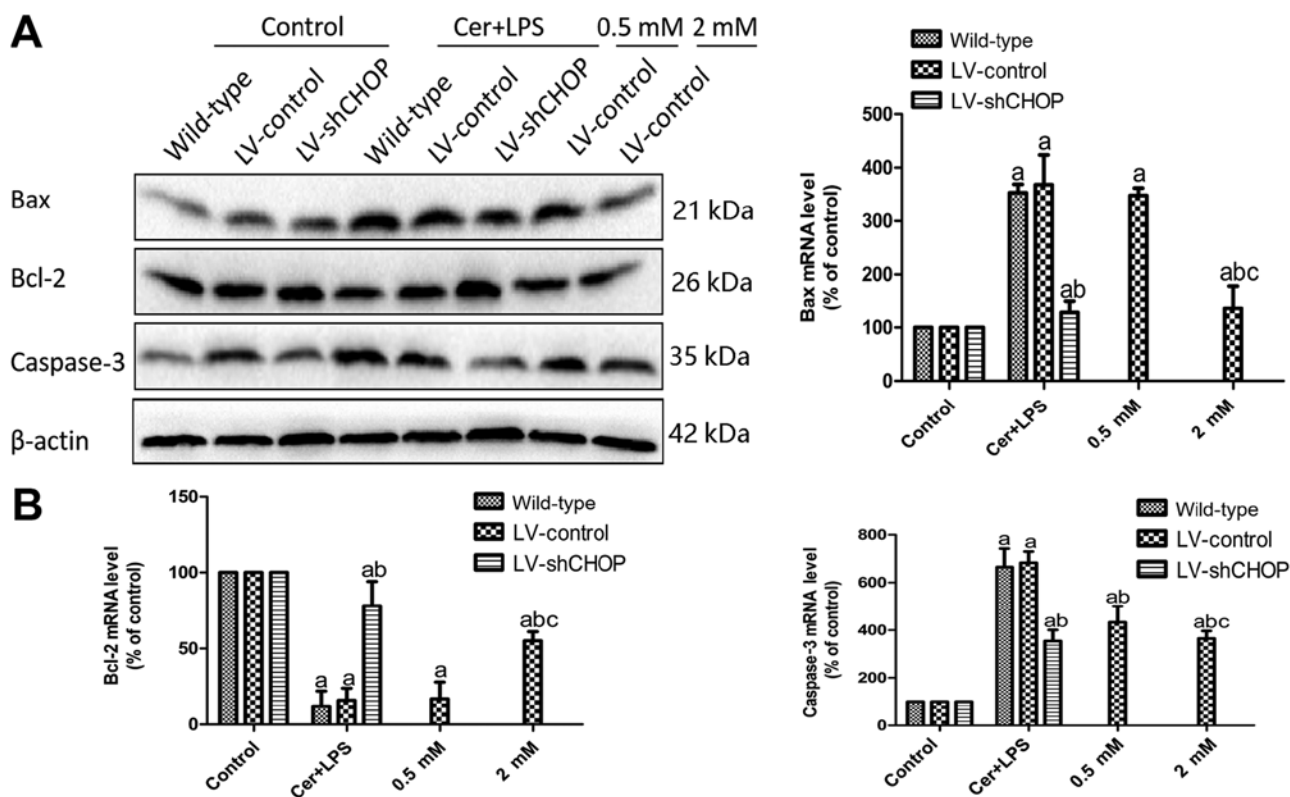
Figure 6. Effects of Cer + LPS and treatment with melatonin on the expression of apoptosis-related molecules in AR42J cells. (A) Protein expression levels of Bax, Bcl-2 and caspase-3 at 9 h after Cer + LPS and melatonin treatment were assessed by western blotting, and β-actin was used as the internal control. (B) mRNA expression levels of Bcl-2, Bax and caspase-3 were quantified by reverse transcription-quantitative PCR at 9 h. Data are presented as the mean ± SEM of ≥3 independent experiments. Control, treated with PBS; 0.5 mM, treated with 0.5 mM melatonin 30 min before cerulein + LPS treatment; 2 mM, treated with 2 mM melatonin 30 min before cerulein + LPS treatment. [a]P<0.05 vs. the respective controls. [b]P<0.05 vs. the LV-control cells treated with Cer + LPS. [c]P<0.05 vs. the 0.5 mM group. Cer + LPS, cerulein + LPS; LV, lentivirus; sh, short hairpin RNA; CHOP, CCAAT/enhancer binding protein (C/EBP)-homologous protein; LPS, lipopolysaccharide.

CHOP-mediated apoptosis is speculated to upregulate the pro-apoptotic molecule Bax and translocation of Bax to the mitochondria, downregulate anti-apoptotic molecules such as Bcl-2 and Bcl-xl (14,54,55), and activate growth arrest and DNA damage inducible 34 and ER oxidoreductin-1a (51). Collectively, these findings indicated that CHOP plays a central role in pancreatitis acinar cell apoptosis and leads to acinar cell injury in AP. Therefore, in the future, CHOP inhibitors may be developed as novel therapeutic drugs for AP.

In recent years, the ER stress-induced, CHOP-mediated pathway has been reported in various inflammatory diseases (8,56-58), but to the best of our knowledge, it has not been demonstrated in a cell model of pancreatic injury. The present results revealed that the CHOP-mediated pathway enhances pancreatitis inflammation, possibly via the transcriptional regulation of NF-κB, and this was consistent with the findings from Allagnat et al (25). NF-κB plays an important role in the inflammatory response (29). When cells are subjected to pathological stimulation such as inflammation or viral infection, IκB kinase is activated and phosphorylates IκB, which is subsequently degraded and exposes a nuclear-localization signal for NF-κB, allowing NF-κB to rapidly migrate into the nucleus, where it initiates the transcription and activation of numerous inflammation-associated genes (12). In addition, the association between ER stress and the inflammatory response are interconnected, and inflammation can

regulate and activate ER stress in cells (12). Zhang et al (59) revealed that ER stress can activate the cAMP-responsive element-binding protein H (CREBH) to induce an acute inflammatory response, and pro-inflammatory cytokines such as IL-6, IL-1β or TNF-α can also exacerbate ER stress and promote the production of CREBH in hepatoma cells in vivo. Previous studies have also reported that inflammatory factors can lead to insulin resistance, promote the occurrence and development of metabolic syndrome, and aggravate tissue and cell stress and damage (60,61). In line with these previous findings, the present results demonstrated that the NF-κB pathway and pro-inflammatory cytokines were significantly reduced by CHOP knockdown after Cer + LPS treatment. Therefore, it was speculated that CHOP knockdown inhibited NF-κB signaling pathway activation and attenuated pancreatitis inflammation and acinar cell injury. Thus, the present results suggested that ER stress-induced, CHOP-mediated pathway had a detrimental role in the pathogenesis of AP.

Other pro-inflammatory mechanisms of CHOP have also been investigated in previous studies. For example, one study using a CHOP-deficient mouse model of experimental pancreatitis identified a reduction in pancreatic tissue inflammation and IL-1β activity by inhibiting the induction of inflammation-associated caspases, caspase-11 and caspase-1 (56). It has also been revealed that in a mouse model of myocardial ischemia/reperfusion injury, knockdown of

CHOP led to reduced myocardial inflammation, possibly via the regulation of the transcription of specific pro-inflammatory cytokine genes, such as IL-6 (8). However, the underlying molecular mechanisms of the CHOP-mediated pathway and pro-inflammatory activity are not fully understood and require further investigation.

The anti-inflammatory effects of melatonin on pancreatitis have been reported in numerous cellular and animal studies, which also functions as a protectant and antioxidant; however, the potential mechanisms are largely unknown (32). It has also been revealed that melatonin could reduce cell injury in different models via the inhibition of ER stress (58). Moreover, the present results revealed that treatment with melatonin significantly decreased the protein expression of CHOP, inhibited the activation of the NF-κB signaling pathway and the release of pro-inflammatory factors, as well as attenuated the ER stress and suppressed apoptosis of AR42J cells. Thus, it was speculated that melatonin protected acinar cells against AP, and alleviated acinar cell injury and inflammatory response by inhibiting the ER stress-induced, CHOP-mediated pathway.

However, the present study had some limitations. The clinical application of melatonin is still not universal, and further clinical trials are required. Moreover, the underlying anti-inflammatory molecular mechanism and the optimal concentration of melatonin need to be identified, in order to further reduce pancreatitis mortality and improve survival prognosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZ and HZ performed the majority of the research. JH and XL designed the study. XJ and JW analyzed the data. XL, JW and HZ collected the information. QZ wrote the manuscript. All authors read and approved the final 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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