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Original articles

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MELATONIN INFLUENCES THE EXPRESSION AND OLIGOMERIZATION OF AMYLIN IN RAT INS-1E CELLS

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The present study investigated whether melatonin influences the expression/oligomerization of amylin with endoplasmic reticulum (ER) stress in rat insulinoma INS-1E cells. No change in cell survival after exposure to thapsigargin- and tunicamycin-combined melatonin treatment or melatonin-only treatment was observed when compared with the normal control cells. With thapsigargin-only or combined tunicamycin-melatonin treatments, phosphorylation of extracellular signal-regulated kinase (ERK) was significantly increased compared with control and melatonin-only treatments. A significant increase was observed in the levels of ER stress markers, namely, phosphorylated inositol-requiring protein 1α (p-IRE1α), CCAAT enhancer binding proteins (C/EBP)-homologous protein, p-eukaryotic translation initiation factor 2α and cleaved caspase-12, in the thapsigargin-combined melatonin-treated cells as compared with the tunicamycin-combined or only melatonin treatment. The melatonin-only treatment resulted in increased levels of amylin expression/oligomerization in 15-25 kDa and insulin proteins, compared with the thapsigargin- and tunicamycin-combined melatonin treatments. Treatment with ER stress inhibitor 4-phenylbutyric acid (4-PBA) did not suppress amylin expression/oligomerization or insulin production with thapsigargin or tunicamycin treatment. Levels of cleaved caspase-12 were significantly decreased in the thapsigargin- or tunicamycin-4-PBA combination treatments. Therefore, whether melatonin regulates the amylin expression/oligomerization in thapsigargin- or tunicamycin-combined with Bafilomycin A1 (autophagy inhibitor) or MG132 (proteasome inhibitor) treatments were investigated. Amylin expression/oligomerization with melatonin treatment was significantly decreased in the thapsigargin- or tunicamycin-combined Bafilomycin A1 or MG132 treatments. Since these outcomes are involved in cell viability, they indicate that increased cell death leads to decreased amylin expression/oligomerization, however, the effects of melatonin treatment on amylin expression/oligomerization induce proliferation of pancreatic β cells and improve the cellular functions of pancreatic β cells.

Key words: *melatonin, amylin, endoplasmic reticulum stress, thapsigargin, tunicamycin, Bafilomycin A1, MG132, rat insulinoma INS-1E cells, insulin*

INTRODUCTION

Endoplasmic reticulum (ER) stress can perturb physiological and pathological conditions and thereby lead to metabolic disorders and several human diseases, including obesity, atherosclerosis, type 2 diabetes (T2D), neurodegenerative diseases, liver disease and cancer (1, 2). Obesity and diabetes are associated with ER stress-induced dysfunction of pancreatic β cells, and research has been undertaken to ameliorate and protect against β cell dysfunction related to ER stress.

The ER stress inducers thapsigargin and tunicamycin influence pancreatic β-cell dysfunction through the expression of *Wfs1* gene or the activating transcription factor 6 (ATF6) (3, 4). Yusta *et al* (5) demonstrated that exendin-4 directly relieves translational repression and increases insulin biosynthesis under ER stress in the isolated rat β cells or INS-1 cells, indicating glucagon-like peptide-1 receptor (GLP-1R)-dependent restoration of β-cell function in T2D. Moreover, ER stress-mediated induction of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) has been shown to maintain pancreatic β-cell homeostasis (6). Orexin-A improves T2D in rat and rat insulinoma INS-1E cells and Sorafenib-induced ER stress may be serving as the promising mechanism against advanced hepatocellular carcinoma (7, 8).

Hyperglycemia, dyslipidemia, inflammation, autoimmunity, islet amyloid levels and insulin resistance influence the functioning of pancreatic β cells. Pancreatic β-cell dysfunction results in reductions in insulin synthesis/secretion, cell survival and insulin sensitivity, thereby inducing diabetes mellitus (9, 10). In animal models and human patients, decreases in insulin production/secretion related to diabetes and obesity have been associated with a reduction of β cell mass due to apoptosis or autophagy related to ER stress (11, 12).

Amylin or islet amyloid polypeptide (IAPP) is a 37-amino acid peptide, synthesized and secreted with insulin in the pancreatic β cells, and activates the G protein-coupled receptor signaling (13).

Amylin as a circulating glucoregulatory hormone regulates energy homeostasis in rodents (13, 14) and is involved in the cardiovascular system and bone metabolism (13, 15). Amylin mainly acts in the circumventricular organs of the rat brain and functionally interacts with cholecystokinin, leptin, and estradiol. The impaired N-terminal process of proIAPP or amylin of humans results in the formation of amyloid fibrils in islets and induces pancreatic β dysfunction and cell death (16, 17). The toxic potency of human amylin is associated with the intrinsic capacity of the peptide to form amyloid fibrils; however, rat amylin is not toxic to islet cells due to the absence of fibril formation. Nevertheless, fibrillar amylin exerts its toxicity on insulin-producing β cells of the rat and human pancreas, indicating that amylin fibril formation may be important in the pathogenesis of T2D.

The pineal gland hormone melatonin and melatonin metabolite, N-acetyl-N-formyl-5-methoxykynuramine (AFMK) perform antioxidant, antiapoptotic and neuroprotective functions (18-21). In particular, melatonin inhibits the amyloid β-induced neurotoxicity *in vivo* and *in vitro* studies (22, 23) and amyloid-β peptide fibrillogenesis (24, 25). However, human fibrillar amylin can act as its toxic in the rat and human pancreas (16, 17). Aarabi and Mirhashemi (26) demonstrated that melatonin reduces human amylin amyloidogenesis. Therefore, this study was investigated whether melatonin regulates the expression/oligomerization of amylin under ER stress conditions in rat insulinoma INS-1E cells.

MATERIALS AND METHODS

Cell culture

Cell culture was performed following the methods reported by Yoo (27) at 37°C and with 5% CO_2. Briefly, the INS-1E cells were cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 11 mM glucose, 10 mM HEPES (pH 7.3), 10% heat-inactivated fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 50 µM β-mercaptoethanol, 1 mM sodium pyruvate, 50 µg/ml penicillin and 100 µg/ml streptomycin at 37°C and with 5% CO_2. The INS-1E cells were cultured in RPMI 1640 medium plus 2% heat-inactivated FBS with/without melatonin (10 and 50 nM; Sigma-Aldrich; Merck KGaA) with thapsigargin (1 µM; Calbiochem) for 6 hours or with tunicamycin (2 µg/ml; Calbiochem) for 16 hours at 37°C with 5% CO_2. In experiments to determine the effects of 0.1 µM Bafilomycin A1 (Baf A1; Calbiochem) or 1 µM MG132 (Calbiochem), cells were treated with these inhibitors for 24 hours. To investigate the inhibition of ER stress, cells were treated with 5 µM 4-phenylbutyric acid (4-PBA; Sigma-Aldrich; Merck KGaA) and thapsigargin or tunicamycin for 24 hours.

Cell viability assay

Cell survival was determined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. The INS-1E cells were cultured in 96-well plates (Corning, Inc.) at a density of 5×10^3/well. The cells were treated with the 10 µl kit solution, incubated for 30 min and their absorbance was measured at 450 nm. The percentage of viable cells per sample was calculated by: Viability (%) = [(total signal-background signal)/control signal] × 100.

Western blot analysis

Western blotting was performed following the methods reported by Yoo (27). Briefly, cells was prepared using a buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris-HCl, (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml pepstatin A, 1 µg/ml chymostatin, 5 mM Na_3VO_4 and 5 mM NaF. Protein concentration was determined using the BCA assay (Sigma-Aldrich; Merck KGaA). Proteins (40 µg) were separated by 12% SDS-PAGE and then transferred to PVDF membranes (Sigma-Aldrich; Merck KGaA). The PVDF membranes were blocked with 5% non-fat dry milk (Santa Cruz Biotechnology, Inc.) in TSB-0.001% Tween 20 (Sigma-Aldrich; Merck KGaA) for 1 hour at room temperature and then incubated with the following primary antibodies overnight at 4°C. Phosphorylated extracellular signal-regulated kinase p-ERK (catalog no. sc-7380; 1:500), ERK (catalog no. sc-93; 1:500), insulin (catalog no. sc-9168; 1:50), C/EBP-homologous protein (CHOP; catalog no. sc-575; 1:500) and GAPDH (catalog no. sc-25778; 1:500) obtained from Santa Cruz Biotechnology, Inc. p-IRE1α (catalog no. PA1-16927; 1:1,000) was obtained from Thermo Fisher Scientific, Inc. Caspase-12 (catalog no. 2202; 1:1000), p-eukaryotic translation initiation factor 2α (eIF2α; catalog no. 3597; 1:1,000) and p-stress activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK; catalog no. 9251; 1:1,000) were provided by Cell Signaling Technology, Inc. The amylin antibody (catalog no. LS-C352341; 1:1000) was provided from LifeSpan BioSciences, Inc. Subsequently, the membranes were incubated with anti-mouse IgG (catalog no. 7076; 1:1000; Cell Signaling Technology, Inc.) or anti-rabbit IgG secondary antibodies conjugated to HRP (catalog no. 7074; 1:1000; Cell Signaling Technology, Inc.) for 1 hour in room temperature. Protein bands were detected with chemiluminescent substrate (Thermo Fisher Scientific, Inc.) and then measured using ImageJ software (version 1.37; National Institute of Health) and were normalized to GAPDH.

Statistical analysis

Significant differences were identified using one-way ANOVA with Tukey's test for multiple comparisons. Analysis was performed using GraphPad Prism v4.0 (GraphPad Software, Inc.). Values are expressed as the mean ± standard deviation of at least three separated experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Cell survival following melatonin and/or endoplasmic reticulum stress treatments

Cell survival was investigated in the presence/absence of melatonin and/or thapsigargin (1 µM) for 6 hours or tunicamycin (2 µg/ml) for 16 hours in INS-1E cells. Cell viability showed no change after exposure to thapsigargin- and tunicamycin-combined melatonin treatment, compared with only melatonin treatment (*Fig. 1*), suggesting that treatment with melatonin and/or thapsigargin or tunicamycin does not influence cell survival of INS-1E cells.

Phosphorylation of extracellular signal-regulated kinase protein following melatonin and/or endoplasmic reticulum stress treatments

Compared with the control treatment, melatonin treatment (10 and 50 µM) significantly increased phosphorylation of ERK in a dose-dependent manner ($P < 0.001$; *Fig. 2*). With thapsigargin-only or the combined tunicamycin-melatonin treatment, phosphorylation of ERK was significantly increased compared with in the control ($P < 0.001$; *Fig. 2*) and melatonin-only treatments ($P < 0.05$ and $P < 0.001$; *Fig. 2*).

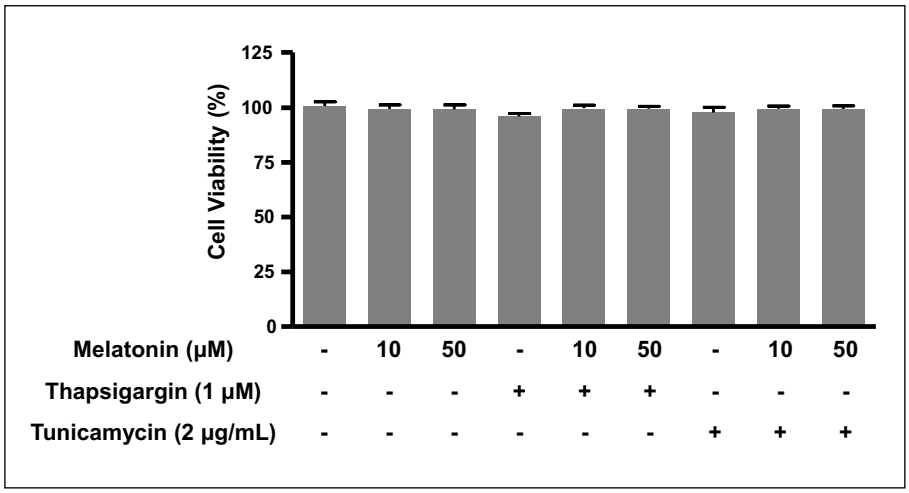

Fig. 1. Cell viability under the condition of endoplasmic reticulum stress and/or melatonin in rat insulinoma INS-1E cells. INS-1E cells were incubated in RPMI 1640 medium supplemented with 2% fetal bovine serum with/without melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours at 37°C with 5% CO_2. Cell viability assay was performed by Cell Counting Kit-8. Values are presented as the mean ± standard deviation from three independent experiments.

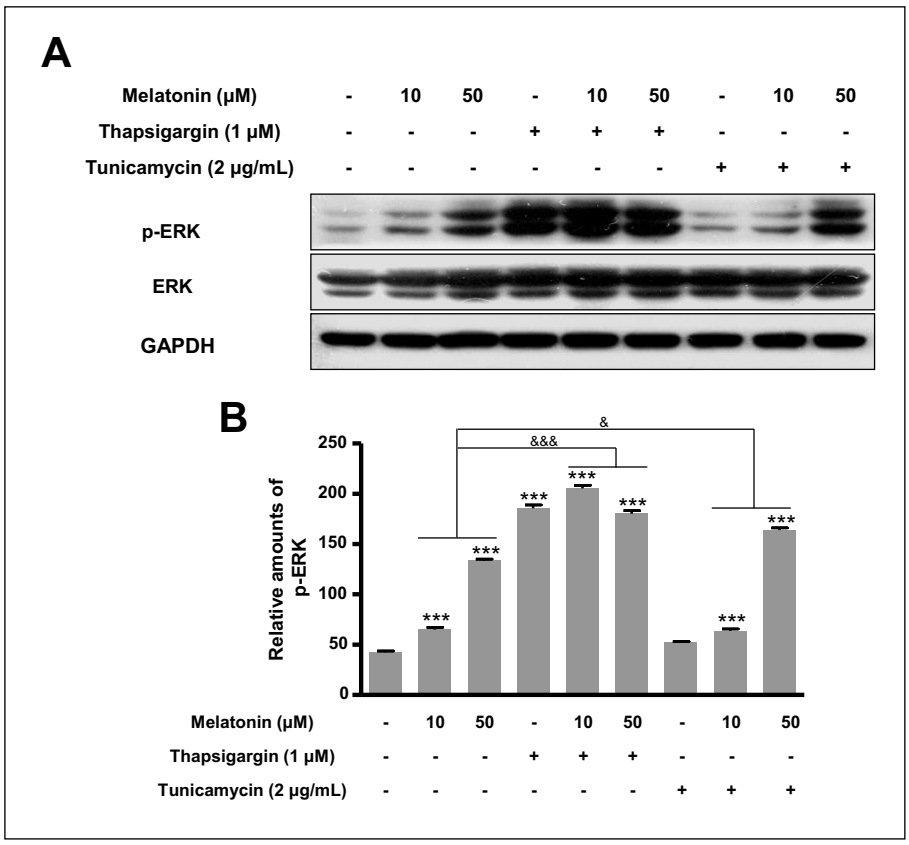

Fig. 2. Phosphorylation of phosphorylated-extracellular signal regulated kinase (p-ERK) protein under the condition of endoplasmic reticulum (ER) stress and/or melatonin in rat insulinoma INS-1E cells. INS-1E cells were incubated in RPMI 1640 medium supplemented with 2% fetal bovine serum with/without melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours at 37°C with 5% CO_2. (A) p-ERK expression was then detected by Western blotting. The relative amount of p-ERK (B) was quantified as described in the materials and methods section. Data presents the mean ± standard deviation of three experiments. $^{***}P < 0.001$ versus the control; $^{\&}P < 0.05$ and $^{\&\&\&}P < 0.001$ versus melatonin.

Expression of endoplasmic reticulum stress proteins following melatonin and/or endoplasmic reticulum stress treatments

The expression of ER stress markers p-IRE1α, CHOP, p-eIF2α, p-SAPK/JNK and cleaved caspase-12 in the combined thapsigargin-melatonin treatment were significantly increased compared with in the combined thapsigargin-melatonin treatment ($P < 0.01$ and $P < 0.001$; *Fig. 3A-3E*) or melatonin-only treatment ($P < 0.001$; *Fig. 3A-3E*). Furthermore, compared with the melatonin-only treatment, exposure to a tunicamycin-melatonin combination resulted in significantly decreased levels of p-IRE1α ($P < 0.001$; *Fig. 3B*), whereas p-eIF2α was significantly increased ($P < 0.01$; *Fig. 3D*).

Expression of amylin and insulin proteins following melatonin and/or endoplasmic reticulum stress treatments

Expression levels of amylin and insulin proteins were investigated in rat INS-1E cells in the presence/absence of melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours. Amylin expression/oligomerization in 15-25 kDa was significantly increased under the combined thapsigargin- and tunicamycin-melatonin treatments compared with the control ($P < 0.001$; *Fig. 4A* and *4B*) and significantly decreased under the combined tunicamycin-melatonin treatment compared with following the melatonin-only treatment ($P < 0.01$; *Fig. 4A* and *4B*). Insulin synthesis under melatonin (10

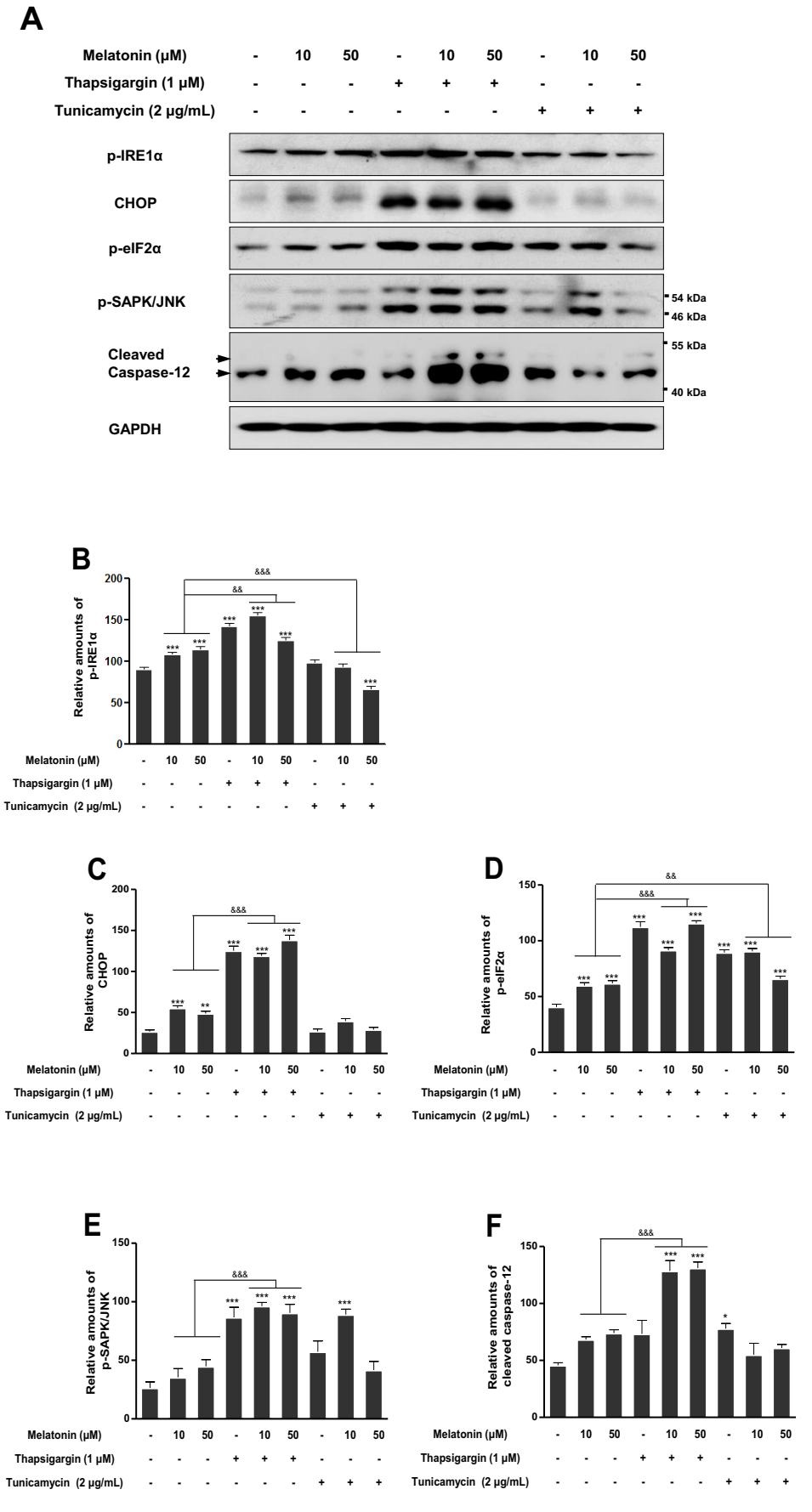

Fig. 3. Expression of p-IRE1α, CHOP, p-eIF2α, p-SAPK/JNK and cleaved caspase-12 proteins under the condition of ER stress and/or melatonin in rat insulinoma INS-1E cells. INS-1E cells were incubated in RPMI 1640 medium supplemented with 2% fetal bovine serum with/without melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours at 37°C with 5% CO_2. (A) p-IRE1α, CHOP, p-eIF2α, p-SAPK/JNK and cleaved caspase-12 proteins were then detected by Western blotting. The relative amounts of p-IRE1α (B), CHOP (C), p-eIF2α (D), p-SAPK/JNK (E) and cleaved caspase-12 (F) proteins were quantified as described in the materials and methods section. Data are presented as the mean ± standard deviation of three experiments. $^{***}P < 0.001$ versus the control; $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ versus melatonin. *Abbreviations*: ER, endoplasmic reticulum; CHOP, C/EBP-homologous protein; eIF2α, eukaryotic translation initiation factor 2α; JNK, c-Jun N-terminal kinase; p, phosphorylated; SAPK, stress activated protein kinase.

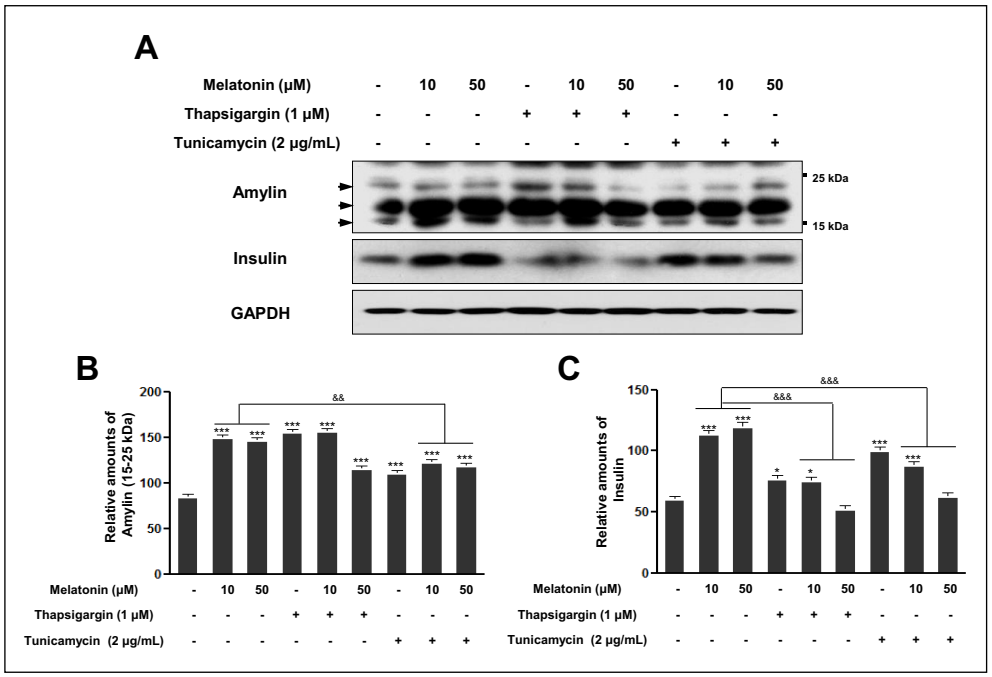

Fig. 4. Expression of amylin and insulin proteins under the condition of endoplasmic reticulum (ER) stress and/or melatonin in rat insulinoma INS-1E cells. (A) Amylin and insulin proteins were then detected by Western blotting. The relative amounts of (B) amylin and (C) insulin proteins were quantified as described in the materials and methods section. Data are presented as the mean ± standard deviation of three experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the control; $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ versus melatonin.

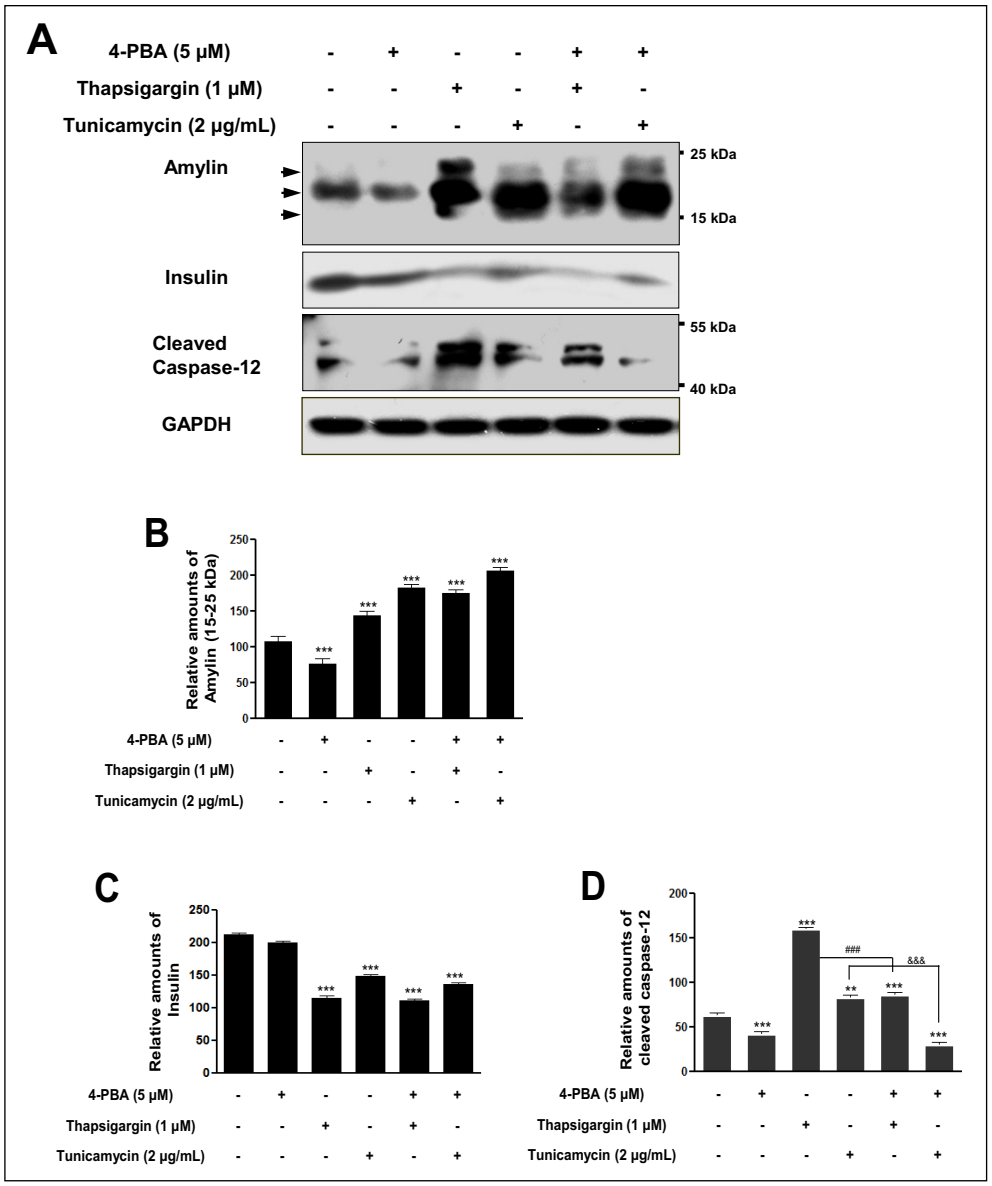

Fig. 5. Expression of amylin, insulin and cleaved caspase-12 proteins under the condition of endoplasmic reticulum (ER) stress and/or 4-phenylbutyric acid (4-PBA) in rat insulinoma INS-1E cells. (A) Amylin, insulin and cleaved caspase-12 proteins were then detected by Western blotting. INS-1E cells were incubated in RPMI 1640 medium supplemented with 2% fetal bovine serum with/without melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours in the presence/absence of the ER stress inhibitor, 5 μM 4-PBA at 37°C with 5% CO_2. The relative amounts of (B) amylin, (C) insulin and (D) cleaved caspase-12 proteins were quantified as described in the materials and methods section. Data are presented as the mean ± standard deviation of three experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the control; $^{\#\#\#}P < 0.001$, thapsigargin versus thapsigargin/4-PBA; $^{\&\&\&}P < 0.001$, tunicamycin versus tunicamycin/4-PBA.

μM), thapsigargin and tunicamycin-only treatments and thapsigargin- and tunicamycin-melatonin combination treatments was significantly increased compared with the control ($P < 0.05$ and $P < 0.001$; *Fig. 4A* and *4C*). Insulin synthesis was significantly decreased following the combined thapsigargin- and tunicamycin-melatonin treatments compared with the melatonin-only treatment (10 and 50 μM) ($P < 0.001$; *Fig. 4A* and *4C*).

Expression of amylin and insulin proteins following 4-PBA and endoplasmic reticulum stress treatments

To investigate whether 4-PBA treatment inhibits the expression of amylin and insulin proteins in rat INS-1E cells, 5 μM 4-PBA was added in combination with thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours. The combined 4-PBA-thapsigargin or -tunicamycin treatments did not suppress

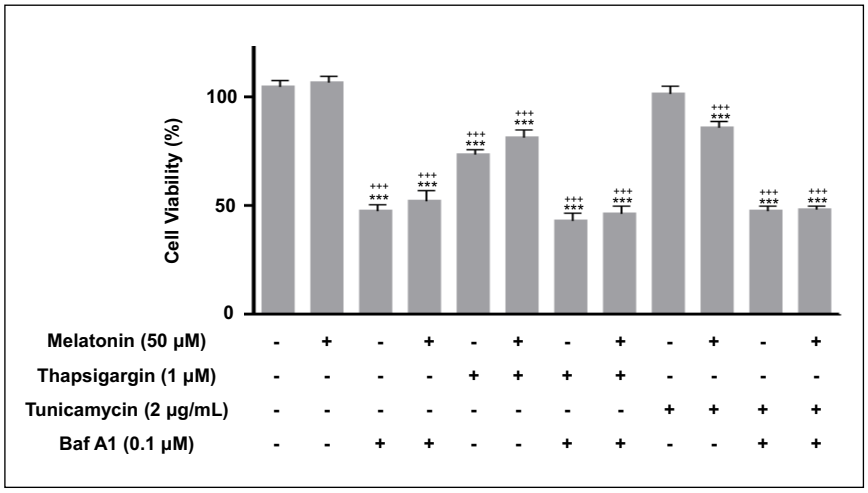

Fig. 6. Cell viability under the condition of endoplasmic reticulum (ER) stress and/or Bafilomycin A1 (Baf A1) in rat insulinoma INS-1E cells. INS-1E cells were incubated in RPMI 1640 medium supplemented with 2% fetal bovine serum with/without melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours at 37°C with 5% CO_2. In experiments to determine the effects of 0.1 μM Baf-A1, cells were treated with thapsigargin or tunicamycin. Cell viability assay was performed by Cell Counting Kit-8. Values are presented as the mean ± standard deviation from three independent experiments. $^{***}P < 0.001$ versus the control; $^{+++}P < 0.001$ versus melatonin.

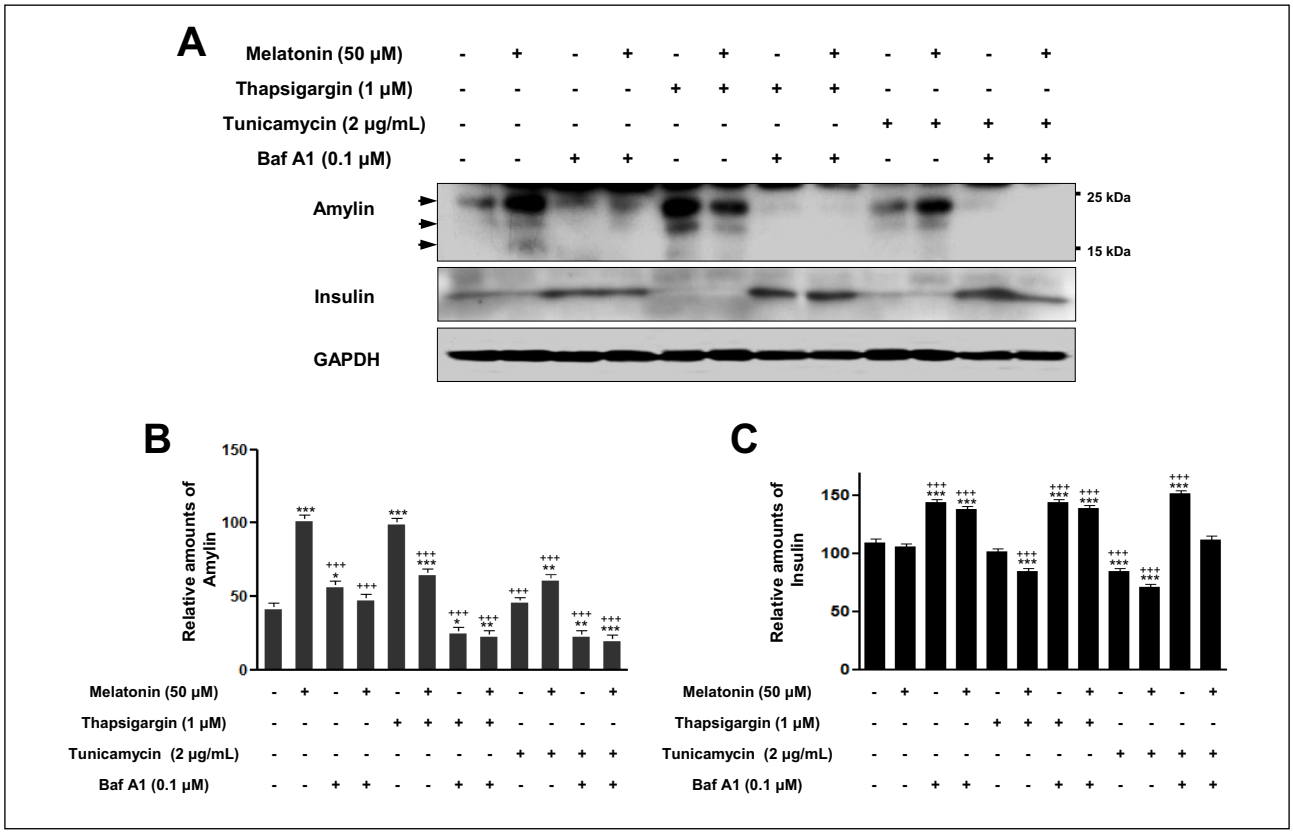

Fig. 7. Expression of amylin and insulin proteins under the condition of endoplasmic stress and/or Bafilomycin A1 (Baf A1) in rat insulinoma INS-1E cells. INS-1E cells were incubated in RPMI 1640 medium supplemented with 2% fetal bovine serum with/without melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours at 37°C with 5% CO_2. In experiments to determine the effects of 0.1 μM Baf A1, cells were treated with thapsigargin or tunicamycin. (A) Amylin and insulin proteins in cells were then detected by Western blotting. The relative amounts of (B) amylin and (C) insulin proteins in cells were quantified as described in the materials and methods section. Data are presented as the mean ± standard deviation of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the control; $^{+++}P < 0.001$ versus melatonin.

amylin expression/oligomerization and insulin production compared with the control (*Fig. 5A-5C*). However, compared with the thapsigargin-only or tunicamycin-only treatments, the expression of ER stress marker cleaved caspase-12 was significantly decreased in the 4-PBA-thapsigargin ($P < 0.001$; *Fig. 5A* and *5D*) and 4-PBA-tunicamycin ($P < 0.001$; *Fig. 5A* and *5D*) treatments.

Expression of amylin and insulin proteins in melatonin with thapsigargin- or tunicamycin-combined Baf A1

The amylin expression/oligomerization and expression levels of insulin protein were further evaluated in rat INS-1E cells after exposure to melatonin or thapsigargin- or tunicamycin-melatonin treatments with/without Baf A1, an autophagy inhibitor or autophagosome fusion blocker. Compared with the control treatments with/without melatonin, treatment with Baf A1, as well as the combined thapsigargin- and tunicamycin-Baf A1 treatments with/without melatonin significantly decreased the cell viability ($P < 0.001$; *Fig. 6*). In addition, the amylin expression/oligomerization of INS-1E cells was significantly decreased in the Baf A1-only and thapsigargin- or tunicamycin-Baf A1 treatments with/without melatonin compared with the melatonin-only treatment ($P < 0.001$; *Fig. 7A* and *7B*). However, protein levels of insulin were significantly increased in the Baf A1-only and thapsigargin- and tunicamycin-Baf A1 treatments compared with the melatonin treatment ($P < 0.001$; *Fig. 7A* and *7C*).

Expression of amylin and insulin proteins in melatonin with thapsigargin- or tunicamycin-combined MG132

Lastly, the amylin expression/oligomerization and expression of insulin protein in rat INS-1E cells in thapsigargin- or tunicamycin-melatonin treatments with/without added MG132, a proteasome inhibitor was evaluated. Cell viability was significantly decreased with MG132 treatment alone and in the thapsigargin- or tunicamycin-melatonin treatments with MG132, compared with the viability levels in the with/without melatonin treatment ($P < 0.001$; *Fig. 8*). Compared with the with/without melatonin treatments, amylin expression/oligomerization and insulin protein levels of INS-1E cells were significantly decreased in the MG132-only treatment as well as in the thapsigargin- or tunicamycin-melatonin treatments with MG132 ($P < 0.05$, $P < 0.01$ and $P < 0.001$; *Fig. 9*).

DISCUSSION

The results of the present study showed that an increase in cell death results in a decrease in amylin expression/oligomerization and an increase in insulin production following treatment with the autophagy inhibitor Baf A1, indicating that amylin expression/oligomerization with melatonin treatment induces the proliferation of pancreatic β cells and improves the cellular functions of pancreatic β cells. ER dysfunction has been implicated in insulin resistance and is an important cause of T2D (27-30). Autophagy in ER stress-induced pancreatic β cells is an important regulator of insulin production and secretion (27-31). In addition, autophagy in pancreatic β cells is essential in the maintenance of normal morphology, mass and functioning of β-cells, and is regarded as a crucial stress response in the protection of β cells under an insulin-resistant state (30, 31). A previous report showed that ER stress caused by melatonin, especially in the presence of thapsigargin, decreased intracellular insulin biosynthesis and that extracellular secretion of insulin may be regulated by melatonin in rat insulinoma INS-1E cells (27). In the present study, the ER stress inhibitor 4-PBA was observed to not affect insulin production when combined with thapsigargin or tunicamycin treatments and that melatonin influenced insulin production in the presence of the autophagy inhibitor Baf A1 and the proteasome inhibitor MG132 *via* ER stress in rat insulinoma INS-1E cells. Rapamycin, an mTOR inhibitor, has been reported to induce autophagy and, subsequently, decrease insulin production (32). Autophagy can regulate insulin production at the cellular level through physiological and biochemical changes in ER homeostasis *via* the unfolded protein response (33, 34).

Kim *et al*. (35) identified the accumulation of human amylin or IAPP oligomers in autophagy-deficient mouse pancreatic β cells and detected 6-20 kDa-sized amylin oligomers by performing Western blot analysis. They also reported that exposure of INS-1 cells to the autophagy inhibitor 3-methyladenine markedly increased the accumulation of human amylin or IAPP protein but there was no such effect on murine amylin or IAPP. In the present study, Baf A1 (autophagy inhibitor) and MG132 (proteasome inhibitor) were used to detect the 15-40 kDa size of murine IAPP expression/oligomerization in pancreatic INS-1E cells. Amylin oligomerization/expression was decreased in the present study in the thapsigargin- or tunicamycin-BafA1 or -MG132 treatment groups. In particular, cell survival in the Baf A1 and MG132 treatments was decreased, indicating that amylin oligomerization/expression may act as a survival factor, thereby improving the viability of pancreatic β cells.

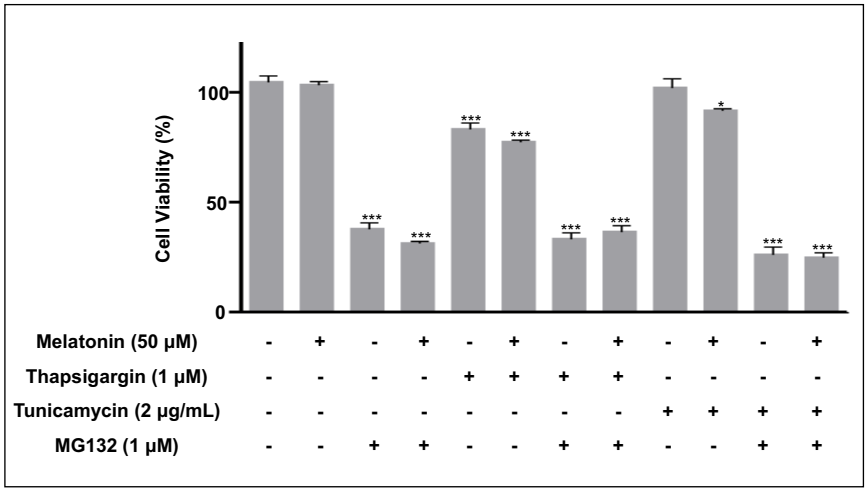

Fig. 8. Cell viability under the condition of endoplasmic reticulum stress and/or MG132 in rat insulinoma INS-1E cells. INS-1E cells were incubated in RPMI 1640 medium supplemented with 2% fetal bovine serum with/without melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours at 37°C with 5% CO_2. In experiments to determine the effects of 1 μM MG132, cells were treated with thapsigargin or tunicamycin. Cell viability assay was performed by Cell Counting Kit-8. Values are presented as the mean ± standard deviation from three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the control; $^{+++}P < 0.001$ versus melatonin.

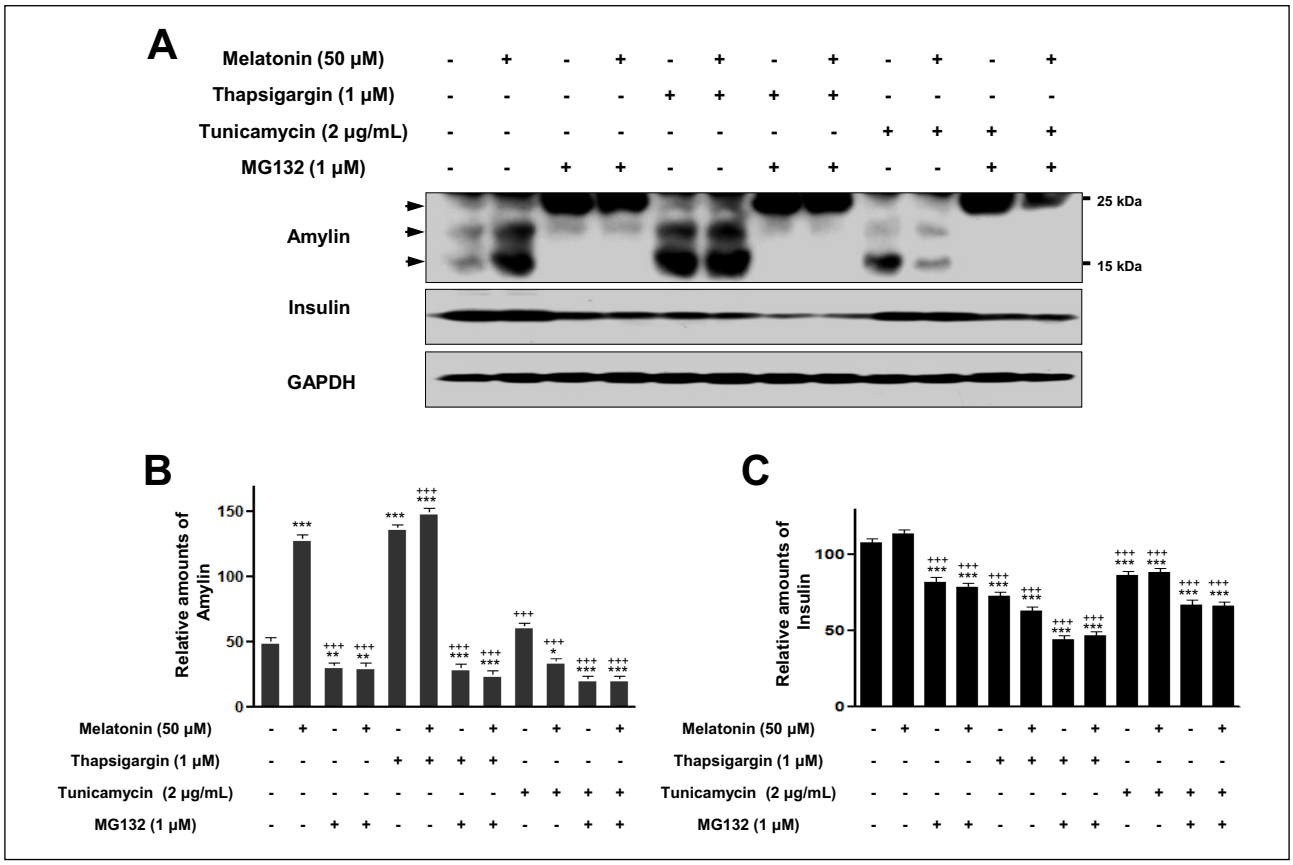

Fig. 9. Expression of amylin and insulin proteins under the condition of endoplasmic reticulum stress and/or MG132 or melatonin in rat insulinoma INS-1E cells. INS-1E cells were incubated in RPMI 1640 medium supplemented with 2% fetal bovine serum with/without melatonin and/or thapsigargin (1 μM) for 6 hours or tunicamycin (2 μg/ml) for 16 hours at 37°C with 5% CO_2. In experiments to determine the effects of 1 μM MG132, cells were treated with thapsigargin or tunicamycin. (A) Amylin and insulin proteins in cells were then detected by Western blotting. The relative amounts of (B) amylin and (C) insulin proteins in cells were quantified as described in the materials and methods section. Data represent the mean ± standard deviation of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the control; $^{+++}P < 0.001$ versus melatonin.

The increase of the amyloid fibrils formed by amyloidogenic human amylin or IAPP induces the toxic potency of cell death in islets and results in pancreatic β dysfunction, and in human, results in progression to T2D (16, 17). Furthermore, the autophagy mechanism in pancreatic β cells promotes the clearance of amyloidogenic human amylin or IAPP and autophagy deficiency exacerbates amyloidogenic amylin or IAPP accumulation-associated human T2D (35-38). However, murine amylin or IAPP do not exhibit amyloidogenic behavior, suggesting that murine amylin is unable to form amyloid fibrils and is therefore not toxic to islet cells (16). Another possibility is the absence of β cell-specific autophagy in rat INS-1 cells, although the accumulation of murine amylin or IAPP oligomers may be non-toxic (36).

Melatonin suppresses the amyloid-β peptide fibrillogenesis (24, 25) and human amylin amyloidogenesis (26). This study demonstrated that melatonin increases the expression/oligomerization of murine amylin under ER stress in rat INS-1E cells. The autophagy inhibitor Baf A1 treatment decreased amylin oligomerization/expression. In contrast, Baf A1 treatment increased insulin production. Nevertheless, human fibrillar amylin can be toxic to insulin-producing β cells of both rat and human pancreases (16), indicating that human amylin fibril formation may be important in the pathogenesis of T2D. In this study, exposure to Baf A1 and MG132 decreased cell survival and results in decreased amylin expression/oligomerization. Therefore, considering the various cell deaths, further research into amylin expression/oligomerization in rats and mice is required to establish the survival factor of pancreatic β cells.

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REFERENCES

1. Ozcan L, Tabas I. Role of endoplasmic reticulum stress in metabolic disease and other disorders. *Annu Rev Med* 2012; 63: 317-328.
2. Schonthal AH. Endoplasmic reticulum stress: its role in disease and novel prospects for therapy. *Scientifica* (*Cairo*) 2012; 2012: 857516. doi: 10.6064/2012/857516
3. Ueda K, Kawano J, Takeda K, *et al*. Endoplasmic reticulum stress induces Wfs1 gene expression in pancreatic beta-cells

via transcriptional activation. *Eur J Endocrinol* 2005; 153: 167-176.
4. Seo HY, Kim YD, Lee KM, *et al.* Endoplasmic reticulum stress-induced activation of activating transcription factor 6 decreases insulin gene expression via up-regulation of orphan nuclear receptor small heterodimer partner. *Endocrinology* 2008; 149: 3832-3841.
5. Yusta B, Baggio LL, Estall JL, *et al.* GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. *Cell Metab* 2006; 4: 391-406.
6. Yamaguchi S, Ishihara H, Yamada T, *et al.* ATF4-mediated induction of 4E-BP1 contributes to pancreatic beta cell survival under endoplasmic reticulum stress. *Cell Metab* 2008; 7: 269-276.
7. Kaczmarek P, Skrzypski M, Pruszynska-Oszmalek E, *et al*. Chronic orexin-A (hypocretin-1) treatment of type 2 diabetic rats improves glucose control and beta-cell functions. *J Physiol Pharmacol* 2017; 68: 669-681.
8. Liu M, Zhou R, Wu X, *et al*. Clinicopathologic charcterization of sorafenib-induced endoplasmic reticulum stress in human liver cancer cells. *J Physiol Pharmacol* 2018; 69: 573-577.
9. Cernea S, Dobreanu M. Diabetes and beta cell function: from mechanisms to evaluation and clinical implication. *Biochem Med* (*Zagreb*) 2013; 23: 266-280.
10. Donath MY, Dalmas E, Sauter NS, Boni-Schnetzler M. Inflammation in obesity and diabetes: islet dysfunction and therapeutic opportunity. *Cell Metab* 2013; 17: 860-872.
11. Prentki M, Nolan CJ. Islet β cell failure in type 2 diabetes. *J Clin Invest* 2006; 116: 1802-1812.
12. Marrif HI, Al-Sunousi SI. Pancreatic β cell mass death. *Front Pharmacol* 2016; 7: 83. doi: 10.3389/fphar.2016.00083
13. Hay DL, Chen S, Lutz TA, Parkes DG, Roth JD. Amylin: pharmacology, physiology, and clinical potential. *Pharmacol Rev* 2015; 67: 564-600.
14. Lutz TA. Control of energy homeostasis by amylin. *Cell Mol Life Sci* 2012; 69: 1947-1965.
15. Wookey PJ, Lutz TA, Andrikopoulos S. Amylin in the periphery II: an updated mini-review. *ScientificWorldJournal* 2006; 6: 1642-1655.
16. Lorenzo A, Razzaboni B, Weir GC, Yankner BA. Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature* 1994; 368: 756-760.
17. Marzban L, Rhodes CJ, Steiner DF, Haataja L, Halban PA, Verchere CB. Impaired NH2-terminal processing of human proislet amyloid polypeptide by the prohormone convertase PC2 leads to amyloid formation and cell death. *Diabetes* 2006; 55: 2192-2201.
18. Jaworek J, Szklarczyk J, Bonior J, *et al*. Melatonin metabolite, N(1)-acetyl-N(1)-formyl-5-methoxykynuramine (AFMK), attenuates acute pancreatitis in the rat: in vivo and in vitro studies. *J Physiol Pharmacol* 2016; 67: 411-421.
19. Murawska-Cialowicz E, Januszewska L, Zuwala-Jagiello J, et al. Melatonin decreases homocysteine level in blood of rats. *J Physiol Pharmacol* 2008; 59: 717-729.
20. Luchetti F, Canonico B, Betti M, *et al*. Melatonin signaling and cell protection function. *FASEB J* 2010; 24: 3603-3624.
21. Joshi N, Biswas J, Nath C, Singh S. Promising role of melatonin as neuroprotectant in neurodegenerative pathology. *Mol Neurobiol* 2015; 52: 330-340.
22. Ansari Dezfouli M, Zahmatkesh M, Farahmandfar M, Khodagholi F. Melatonin protective effect against amyloid β-induced neurotoxicity mediated by mitochondrial biogenesis; involvement of hippocampal Sirtuin-1 signaling pathway. *Physiol Behav* 2019; 204: 65-75.
23. Corpas R, Grinan-Ferre C, Palomera-Avalos V, *et al.* Melatonin induces mechanisms of brain resilience against neurodegeneration. *J Pineal Res* 2018; 65: e12515. doi: 10.1111/jpi.12515
24. Pappolla M, Bozner P, Soto C, *et al.* Inhibition of Alzheimer β-fibrillogenesis by melatonin. *J Biol Chem* 1998; 273: 7185-7188.
25. Masilamoni JG, Jesudason EP, Dhandayuthapani S, *et al.* The neuroprotective role of melatonin against amyloid β peptide injected mice. *Free Radic Res* 2008; 42: 661-673.
26. Aarabi MH, Mirhashemi SM. To estimate effective antiamyloidogenic property of melatonin and fisetin and their actions to destabilize amyloid fibrils. *Pak J Pharm Sci* 2017; 30: 1589-1593.
27. Yoo YM. Melatonin-mediated insulin synthesis during endoplasmic reticulum stress involves HuD expression in rat insulinoma INS-1E cells. *J Pineal Res* 2013; 55: 207-220.
28. Eizirik DL, Cardozo AK, Cnop M. The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr Rev* 2008; 29: 42-61.
29. Back SH, Kaufman RJ. Endoplasmic reticulum stress and type 2 diabetes. *Annu Rev Biochem* 2012; 81: 767-793.
30. Yin JJ, Li YB, Wang Y, Liu GD, *et al.* The role of autophagy in endoplasmic reticulum stress-induced pancreatic β cell death. *Autophagy* 2012; 8: 158-164.
31. Bartolome A, Guillen C, Benito M. Autophagy plays a protective role in endoplasmic reticulum stress-mediated pancreatic β cell death. *Autophagy* 2012; 8: 1757-1768.
32. Kim HS, Yoo YM. Data of intracellular insulin protein reduced by autophagy in INS-1E cells. *Data Brief* 2016; 8: 1151-1156.
33. Rutkowski DT, Hegde RS. Regulation of basal cellular physiology by the homeostatic unfolded protein response. *J Cell Biol* 2010; 189: 783-794.
34. Cao SS, Kaufman RJ. Unfolded protein response. *Curr Biol* 2012; 22: R622-R626.
35. Kim J, Cheon H, Jeong YT, *et al*. Amyloidogenic peptide oligomer accumulation in autophagy-deficient β cells induces diabetes. *J Clin Invest* 2014; 124: 3311-3324.
36. Rivera JF, Costes S, Gurlo T, Glabe CG, Butler PC. Autophagy defends pancreatic β cells from human islet amyloid polypeptide-induced toxicity. *J Clin Invest* 2014; 124: 3489-3500.
37. Shigihara N, Fukunaka A, Hara A, *et al*. Human IAPP-induced pancreatic β cell toxicity and its regulation by autophagy. *J Clin Invest* 2014; 124: 3634-3644.
38. Lee MS. Role of islet β cell autophagy in the pathogenesis of diabetes. *Trends Endocrinol Metab* 2014; 25: 620-627.

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