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Nicotinic acetylcholine receptor signaling regulates inositol-requiring enzyme 1α activation to protect β-cells against terminal unfolded protein response under irremediable endoplasmic reticulum stress

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ABSTRACT
Aims/Introduction: Under irremediable endoplasmic reticulum (ER) stress, hyperactivated inositol-requiring enzyme 1α (IRE1α) triggers the terminal unfolded protein response (T-UPR), causing crucial cell dysfunction and apoptosis. We hypothesized that nicotinic acetylcholine receptor (nAChR) signaling regulates IRE1α activation to protect β-cells from the T-UPR under ER stress.

Materials and Methods: The effects of nicotine on IRE1α activation and key T-UPR markers, thioredoxin-interacting protein and insulin/proinsulin, were analyzed by real-time polymerase chain reaction and western blotting in rat INS-1 and human EndoC-βH1 β-cell lines. Doxycycline-inducible IRE1α overexpression or ER stress agents were used to induce IRE1α activation. An α7 subunit-specific nAChR agonist (PNU-282987) and small interfering ribonucleic acid for α7 subunit-specific nAChR were used to modulate nAChR signaling.

Results: Nicotine inhibits the increase in thioredoxin-interacting protein and the decrease in insulin 1/proinsulin expression levels induced by either forced IRE1α hyperactivation or ER stress agents. Nicotine attenuated X-box-binding protein-1 messenger ribonucleic acid site-specific splicing and IRE1α autophosphorylation induced by ER stress. Furthermore, PNU-282987 attenuated T-UPR induction by either forced IRE1α activation or ER stress agents. The effects of nicotine on attenuating thioredoxin-interacting protein and preserving insulin 1 expression levels were attenuated by pharmacological and genetic inhibition of α7 nAChR. Finally, nicotine suppressed apoptosis induced by either forced IRE1α activation or ER stress agents.

Conclusions: Our findings suggest that nAChR signaling regulates IRE1α activation to protect β-cells from the T-UPR and apoptosis under ER stress partly through α7 nAChR. Targeting nAChR signaling to inhibit the T-UPR cascade may therefore hold therapeutic promise by thwarting β-cell death in diabetes.

INTRODUCTION
Endoplasmic reticulum (ER)-resident enzymatic activities, such as chaperones, play a critical role in maintaining cellular homeostasis during both constitutive secretory protein folding and assembly, and under conditions of excessive protein folding demand (i.e., ER stress). Under ER stress, intracellular signaling pathways termed the unfolded protein response (UPR)
be activated to restore cellular homeostasis. Under remediable levels of ER stress, cytoprotective adaptive-UPR (A-UPR) programs become activated, thereby enhancing protein folding capacity, reducing upstream secretory protein loads and degrading ER unfolded proteins. However, under irremediably high and chronic ER stress, these adaptive systems become attenuated as alternative terminal-UPR (T-UPR) programs trigger apoptosis; this switch from the A-UPR to a T-UPR might underlie many human cell degenerative diseases, including diabetes mellitus.

The UPR is triggered by the activation of three ER transmembrane sensors – protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 and inositol-requiring enzyme 1α (IRE1α) – by changing their oligomerization state. Under ER stress, IRE1α, the most highly conserved UPR sensor, dimerizes and undergoes trans-autophosphorylation and activation of endoribonuclease (RNase) to site-specifically splice X-box-binding protein-1 (XBP1) messenger ribonucleic acid (mRNA) to trigger A-UPR transcriptional programs. However, under high (irremediable) ER stress, oligomerized and autophosphorylated IRE1α activates the RNase catalytic rate further, causing cleavage of myriad ER-localized mRNAs, ribosomal RNAs and microRNAs, which ultimately induces apoptosis. Therefore, modulation of IRE1α activities is central to the A-UPR to T-UPR “switch,” and thereby to control cell fate. This regulation of stability of multiple RNAs by IRE1α is known as regulated IRE1α-dependent decay. Indeed, we have shown in vivo, that IRE1α-specific inhibitors, kinase-inhibitory RNase attenuators (KIRA), can spare pancreatic β-cells from T-UPR and ultimate apoptosis to reverse or ameliorate diabetes in Akita and NOD mice. Notably, the switch from A-UPR to T-UPR “switch,” and thereby to control cell fate. This regulation of stability of multiple RNAs by IRE1α is known as regulated IRE1α-dependent decay. Indeed, we have shown in vivo, that IRE1α-specific inhibitors, kinase-inhibitory RNase attenuators (KIRA), can spare pancreatic β-cells from T-UPR and ultimate apoptosis to reverse or ameliorate diabetes in Akita and NOD mice. These findings suggest thatmodulating the activity of IRE1α in β-cells might have a general therapeutic potential against type 2 and autoimmune diabetes by rescuing β-cells from proapoptotic T-UPR signaling.

The nicotinic acetylcholine receptor (nAChR) is a cation channel receptor that responds to acetylcholine and several ligands, including nicotine. nAChR was believed to exist only in the central and peripheral nervous system, and neuromuscular junction of muscles. However, recent studies showed that nAChR also exists in the cells of many kinds of non-neuronal tissues, such as the liver and muscle, and its stimulation shows functional effects. Similarly, it was also shown that nAChR is expressed in murine islets and rodent pancreatic β-cell lines. Interestingly, recent reports emphasize the importance of nAChR signaling by showing that although cholinergic innervation of human islets is limited, -cells can substitute to secrete ACh to stimulate surrounding endocrine cells in a paracrine manner. Supporting an important role of nAChR signaling in glucose metabolism, in vivo, it was reported that nicotine ameliorates the diabetic phenotype in db/db mice, Zucker fatty rats, streptozotocin-induced diabetic mice and even NOD mice. Furthermore, stimulation of nAChR by nicotine increases insulin secretion in rodent β-cells, and decreases cytokine-induced apoptosis in human and rodent islets at least through the α7 nAChR subunit.

Regarding the link between UPR and nAChR signaling, a limited number of studies show controversial evidence of this link in non-β cells. Although, it is reported that pharmacological chaperoning of nAChR signaling could suppress ER stress and UPR, leading to the neuroprotection of Neuro-2a cells; however, another study showed the induction of ER stress by nicotine in rat placental trophoblast giant cells. These findings might be attributed to the tissue-specific effect of nAChR signaling on a specific UPR arm, and further questions are raised as to whether these two factors, nAChR and IRE1α signaling, are linked in β-cells. Here, we aimed to determine whether and if so, how nAChR signaling regulates IRE1α signaling, and subsequently T-UPR, in rat and human pancreatic β-cell lines.

METHODS

Chemicals and reagents

Nicotine, thapsigargin (Tg), doxycycline hyclate (Dox) and PNU-282987 (PNU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tunicamycin (Tm) was from Merk (Darmstadt, Germany). α-Bungarotoxin was from Tocris Bioscience (Bristol, UK).

Cell experiments

Cell and tissue culture

Rat insulinoma cell line, INS-1 cells were maintained as described previously. Briefly, the cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 11.1 mmol/L glucose, 10 mmol/L HEPES (Invitrogen), 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), penicillin-streptomycin-glutamine (which contains 100 units/L penicillin, 100 µg/L streptomycin, 292 µg/mL of l-glutamine; Invitrogen) and 1 mmol/L sodium pyruvate. EndoC-BH1 cells (Univercell Biosolutions, Toulouse, France) were cultured in accordance with the manufacturer’s protocol. Briefly, the cells were plated on a coat with βCOAT (Univercell Biosolutions) or MaxGel ECM coat (Sigma-Aldrich) containing Dulbecco’s modified Eagle’s medium with 4.5 g/L glucose (Invitrogen) and cultured in OPTIB1 complete premixed culture medium (Univercell Biosolutions). Both INS-1 and EndoC-BH1 cells were cultured in a humidified atmosphere of 95% O2 and 5% CO2. As all the experiments are limited to in vitro cultured cell work, the local ethics committee of Wakayama Medical University does not require approval for them.

Doxycyclin-inducible transgenic WT IRE1α overexpressing INS-1 cells

Transgenic WT mouse IRE1α overexpressing INS-1 cells line in this study are tightly inducible by Dox, and maintained as described previously.

Reverse transcription quantitative polymerase chain reaction

Total RNA was extracted from whole cells using a RNeasy Plus kit (Qiagen, Venlo, the Netherlands). For standard mRNA
detection, 1 μg of total RNA was reverse transcribed to complementary deoxyribonucleic acid (cDNA) using a reverse transcription kit (Qiagen) after DNase digestion. For qualitative polymerase chain reaction (PCR), we used SYBR green (Applied Biosystems, Warrington, UK) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Gene expression levels were normalized to β-actin expression level. The primers used for quantitative PCR were as shown in Table 1.

**Measurements of XBP-1 mRNA splicing**

XBP-1 splicing mRNA was isolated from whole cells and reverse transcribed as described above to obtain total cDNA. Then, XBP-1 primers (F: 5'-AAAAACAGATGGACAGACTGC-3', R: 5'-GGATCTCTAAGACTAGGCTTGGTG-3') were used to amplify an XBP-1 amplicon spanning the 26 nt intron from the cDNA samples in reverse transcription PCR as previously described. PCR fragments were digested with PstI and resolved on 3% agarose gels.

**Western blot analysis**

We carried out western blot as previously described. Briefly, for protein analysis, cells were lysed in M-PER buffer (Thermo Scientific, Rockford, IL, USA) mixed with a protease inhibitor, containing 4 μg/mL aprotinin (Wako, Osaka, Japan), 4 μg/mL leupeptin (Nacalai Tesque, Kyoto, Japan), 1 mmol/L PMSF (Wako), 1 mg/mL pepstatin (Wako) and a phosphatase inhibitor cocktail (Sigma-Aldrich). The lysis solution was mixed and centrifuged at 15,000 rpm for 20 min with gentle mixing, and the buffer was exchanged three times before loading samples. Western blot was carried out as described above.

**Table 1 | Forward and reverse sequences for the primers used for reverse transcription polymerase chain reaction gene**

| Gene          | Primer                                      |
|---------------|---------------------------------------------|
| Rat TXNIP     | F: 5'-CTGATGGAGCAGACTGAG-3'                 |
|               | R: 5'-CTCCCTGGAGGAGCAGA-3'                 |
| Rat Ins1      | F: 5'-GTCTCTGGGAGCCCGAAG-3'                |
|               | R: 5'-ACAGACCTCTCCACCAGG-3'                |
| Rat sXBP1     | F: 5'-CTGATCGGACATCGGATCGAC-3'             |
|               | R: 5'-ATCCATGGAAGATGTTGAG-3'               |
| Human sXBP1   | F: 5'-GAGTCCCGGACAGGGT-3'                  |
|               | R: 5'-TCCTCTGGGATGACCCCTGGGAG-3'           |
| Human TXNIP   | F: 5'-CCTGAGGAGCAGACTCCA-3'                |
|               | R: 5'-GGGCTTGGAGGAGCAGAC-3'                |
| Rat CHRNA7    | F: 5'-TGGCAGAAGGTCCAGCGCG-3'               |
|               | R: 5'-ACTCCGCGGATCAGACAT-3'                |
| Rat CHOP      | F: 5'-CCACAGAAGGTCCAGCGCG-3'               |
|               | R: 5'-CCACAGAAGGTCCAGCGCG-3'               |
| Rat β-actin:  | F: 5'-GAAAGCTCTTCTGAGGAGCAG-3'             |
|               | R: 5'-AGAAGCTCCTTCTGAGGAGCAG-3'            |
| Human β-actin:| F: 5'-AGAAGCTCTGAGGAGCAG-3'                |
|               | R: 5'-AGAAGCTCCTGAGGAGCAG-3'               |

**Detection of nAChR α7 subunit in EndoC-βH1 cells**

Total RNA was extracted from EndoC-βH1 cells and was reverse transcribed to cDNA as described above. Human CHRNA7 primers (F: 5'-CCGACTCTGGTGATGGTGTG-3', R: 5'-ATGGTGCGATGGTGTG-3') were used for a regular three-step PCR, as previously described. The pcDNA3.1-human CHRNA7 plasmid (Addgene, Watertown, MA, USA) was a gift from Sherry Leonard and Henry Lester (Addgene, #62276), and as a positive control.

**RNA interference**

A negative control (Qiagen, #1022563) or two small interfering RNAs (siRNAs) against rat CHRNA7 (Qiagen, #SI00253365, #SI03405208), encoding nAChR α7 subunit, were transfected using lipofectamine RNAiMAX lipid reagent (Invitrogen) in Dox-inducible IRE1α-overexpressing INS-1 cells, in accordance with the manufacturer’s protocol. A total of 24 h after the transfection, cells were treated with Dox with or without blocking, incubation with antibodies and washing were carried out in Tris-buffered saline with 0.05% Tween-20 and 5% non-fat dry milk. Primary antibodies against target proteins were diluted in the blocking solution. Secondary antibodies were diluted in a blocking solution to detect the species-specific portion of the primary antibodies. Antibody-binding was detected using LAS-3000 mini (FUJI Film, Tokyo, Japan), and quantified by densitometry using ImageJ (NIH, Bethesda, MD, USA). β-Actin was used as the loading control.

**Antibody**

As primary antibodies, we used mouse anti-insulin antibody (1:1,000; Cell Signaling Technology Inc., Danvers, MA, USA), mouse anti-TXNIP antibody (1:1,000, JY2; MBL International, Woburn, MA, USA), rabbit anti-IRE1α antibody (1:1,000; Cell Signaling Technology Inc.), mouse anti-phosphorylated eIF2α (Ser51) antibody (1:1,000; Cell Signaling Technology Inc.), mouse anti-eIF2α antibody (1:1,000; Cell Signaling Technology Inc.) and mouse anti-β-Actin antibody (1:5,000; Sigma-Aldrich).

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis, phosphatase gel separation**

For measuring phosphorylated IRE1α, we used SuperSep Phos-tag gel (Wako), which separates phosphorylated proteins from their unphosphorylated counterparts in sodium dodecyl sulfate polyacrylamide gel electrophoresis. In accordance with the manufacturer’s instructions, the gel was soaked in 1× transfer buffer with 10 mmol/L ethylenediaminetetraacetic acid for 20 min with gentle mixing, and the buffer was exchanged three times before loading samples. Western blot was carried out as described above.

**Detection of nAChR α7 subunit in EndoC-βH1 cells**

Total RNA was extracted from EndoC-βH1 cells and was reverse transcribed to cDNA as described above. Human CHRNA7 primers (F: 5'-CCGACTCTGGTGATGGTGTG-3', R: 5'-ATGGTGCGATGGTGTG-3') were used for a regular three-step PCR, as previously described. The pcDNA3.1-human CHRNA7 plasmid (Addgene, Watertown, MA, USA) was a gift from Sherry Leonard and Henry Lester (Addgene, #62276), and as a positive control.

**RNA interference**

A negative control (Qiagen, #1022563) or two small interfering RNAs (siRNAs) against rat CHRNA7 (Qiagen, #SI00253365, #SI03405208), encoding nAChR α7 subunit, were transfected using lipofectamine RNAiMAX lipid reagent (Invitrogen) in Dox-inducible IRE1α-overexpressing INS-1 cells, in accordance with the manufacturer’s protocol. A total of 24 h after the transfection, cells were treated with Dox with or without blocking, incubation with antibodies and washing were carried out in Tris-buffered saline with 0.05% Tween-20 and 5% non-fat dry milk. Primary antibodies against target proteins were diluted in the blocking solution. Secondary antibodies were diluted in a blocking solution to detect the species-specific portion of the primary antibodies. Antibody-binding was detected using LAS-3000 mini (FUJI Film, Tokyo, Japan), and quantified by densitometry using ImageJ (NIH, Bethesda, MD, USA). β-Actin was used as the loading control.

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**Sodium dodecyl sulfate polyacrylamide gel electrophoresis, phosphatase gel separation**

For measuring phosphorylated IRE1α, we used SuperSep Phos-tag gel (Wako), which separates phosphorylated proteins from their unphosphorylated counterparts in sodium dodecyl sulfate polyacrylamide gel electrophoresis. In accordance with the manufacturer’s instructions, the gel was soaked in 1× transfer buffer with 10 mmol/L ethylenediaminetetraacetic acid for 20 min with gentle mixing, and the buffer was exchanged three times before loading samples. Western blot was carried out as described above.

**Detection of nAChR α7 subunit in EndoC-βH1 cells**

Total RNA was extracted from EndoC-βH1 cells and was reverse transcribed to cDNA as described above. Human CHRNA7 primers (F: 5'-CCGACTCTGGTGATGGTGTG-3', R: 5'-ATGGTGCGATGGTGTG-3') were used for a regular three-step PCR, as previously described. The pcDNA3.1-human CHRNA7 plasmid (Addgene, Watertown, MA, USA) was a gift from Sherry Leonard and Henry Lester (Addgene, #62276), and as a positive control.

**RNA interference**

A negative control (Qiagen, #1022563) or two small interfering RNAs (siRNAs) against rat CHRNA7 (Qiagen, #SI00253365, #SI03405208), encoding nAChR α7 subunit, were transfected using lipofectamine RNAiMAX lipid reagent (Invitrogen) in Dox-inducible IRE1α-overexpressing INS-1 cells, in accordance with the manufacturer’s protocol. A total of 24 h after the transfection, cells were treated with Dox with or without blocking, incubation with antibodies and washing were carried out in Tris-buffered saline with 0.05% Tween-20 and 5% non-fat dry milk. Primary antibodies against target proteins were diluted in the blocking solution. Secondary antibodies were diluted in a blocking solution to detect the species-specific portion of the primary antibodies. Antibody-binding was detected using LAS-3000 mini (FUJI Film, Tokyo, Japan), and quantified by densitometry using ImageJ (NIH, Bethesda, MD, USA). β-Actin was used as the loading control.
nicotine or PNU for 24 h. Then, relative TXNIP or Ins1 mRNA expression levels were determined by quantitative PCR as described above.

Apoptosis
For apoptosis assay, INS-1 cells were treated with Tm, or Dox-inducible transgenic WT IRE1α overexpressing INS-1 cells were incubated with Dox. Translocation of phosphatidylserine to the external cell surface was detected by flow cytometry with annexin V-fluorescein isothiocyanate staining fluorescein isothiocyanate-annexin V apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Flow cytometry was carried out using BD FACSuite (BD Biosciences), and the results were analyzed using FACSuite (BD Biosciences, v.1.0.5.3841). Activation of caspase-3 and -7, which are markers of the early stages of apoptosis, was detected using a Caspase-Glo 3/7 assay kit (Promega Corporation, Madison, WI, USA) through luminescence assay in accordance with the manufacturer’s instructions.

Statistical analysis
The results of the present study are expressed as the mean ± standard error of the mean. All experiments were replicated more than three times. The significance of differences (P < 0.05) between normalized mean values was evaluated by one-way ANOVA followed by Tukey’s post-hoc test or the Student’s t-test, as appropriate. All statistical analyses were carried out using JMP® 14 (SAS Institute Inc., Cary, NC, USA).

RESULTS
Nicotine reverses the T-UPR induced by IRE1α hyperactivation
To elucidate whether nAChR signaling has the cell protective effect against T-UPR induced by IRE1α signaling in pancreatic β-cells, we first examined T-UPR markers in Dox-inducible FLAG-tagged IRE1α-overexpressing INS-1 cells. We previously validated this cell system as a tool to determine the sufficiency of specific and focal hyperactivation of IRE1α (without the need to resort to upstream ER stress inducing agents)3. Overexpression of IRE1α increased a key T-UPR marker, TXNIP, which activates the NLRP3 inflammasome to promote β-cell death (Figure 1a, Figure S1)25,26. Furthermore, it decreased insulin 1 (Ins1) mRNAs, which was identified as IRE1α RNAse substrate, and consecutive proinsulin protein levels over time (Figure 1b, Figure S1)3. The effect of IRE1α overexpression on PERK arm is limited in this system, based on its substrate eIF2α phosphorylation levels and CHOP mRNA expression being unchanged (Figure S2a,b). Using this IRE1α-overexpressing INS-1 cell line, nicotine reversed the increase of TXNIP mRNA expression levels in a dose-dependent manner, starting at 10 μmol/L, with complete reversal at 1 mmol/L (Figure 1a). Similarly, nicotine reversed the decrease in Ins1 mRNA expression levels dose-dependently (Figure 1b).

These effects of nicotine were confirmed at the protein expression levels of TXNIP and proinsulin (Figure 1c). Thus, nAChR signaling protects T-UPR signaling driven by IRE1α-specific activation in INS-1 cells.

Nicotine modulates IRE1α signaling under ER stress
Encouraged by the protective effect of nicotine against the T-UPR by IRE1α-specific hyperactivation, we next examined the effect of nicotine on IRE1α signaling under global ER stress. To induce ER stress, we used two ER stress inducers: Tg and Tm. Tg is an inhibitor of the ER Ca2+-ATPase, which activates calcium-dependent ER chaperones, leading to the accumulation of unfolded proteins27. Tm blocks glycoprotein biosynthesis in the ER causing accumulation of unfolded glycoproteins, which in turn leads to ER stress28. We first showed that nicotine minimized site-specific XBP1 mRNA splicing induced by Tg in INS-1 cells (Figure 2a). Furthermore, this effect of nicotine was confirmed by the quantitative measurement of the spliced form of XBP1 mRNAs in Tg- and Tm-exposed INS-1 cells (Figure 2b,c). As passing the threshold of autophosphorylation of IRE1α drives RNAse activity into the hyperactivation state, we next investigated the phosphorylation levels of IRE1α using Phos-tag gel, which can separate phosphorylated forms from non-phosphorylated forms of protein on the basis of phosphorylation levels. Tm increased the ratio of the phosphorylated IRE1α to that of non-phosphorylated IRE1α, and nicotine decreased it (Figure 2d). Then, as shown in the rodent β-cell line, we confirmed for the first time the expression of the α7 nAChR subunit in the well-validated human β-cell line, EndoC-βH1 cells (Figure 2e). In this cell line, nicotine also reduced Tg-induced spliced XBP1 mRNA expression levels (Figure 2f). The effect of nicotine on the PERK arm was limited under ER stress, based on its substrate eIF2α phosphorylation levels being unchanged (Figure S3). Therefore, nicotine modulates kinase and RNAse activation of IRE1α under ER stress in pancreatic β-cells.

Nicotine attenuates T-UPR induced by ER stress
As nicotine showed the effect of reducing the direct IRE1α signaling under ER stress, we next examined whether nicotine also attenuates T-UPR induced by high and chronic ER stress. ER stress inducers increased mRNA expression levels of TXNIP and decreased those of Ins1, whereas nicotine inhibited these effects of ER stress inducers (Figure 3a-d, Figure S4). Similar results were found for TXNIP and proinsulin at the protein level (Figure 3e). The effects of attenuating the ER stress-induced increase in TXNIP and decrease in insulin mRNA expression levels by nicotine were also confirmed in EndoC-βH1 cells (Figure 3f,g). To evaluate the effect of nicotine under physiological ER stress, we investigated it at high glucose levels29. A high ambient glucose concentration (28 mmol/L) induced increase in TXNIP and decrease in insulin mRNA expression levels by nicotine were also confirmed in EndoC-βH1 cells (Figure 3f,g). To evaluate the effect of nicotine under physiological ER stress, we investigated it at high glucose levels29. A high ambient glucose concentration (28 mmol/L) increased the protein expression levels of TXNIP and reduced those of proinsulin protein levels as previously reported10, and nicotine attenuated these effects (Figure 3h). These results suggest that nicotine attenuates T-UPR induced by ER stress.
Figure 1 | Nicotine reverses terminal unfolded protein response induced by overexpression of Flag-tagged full-length inositol-requiring enzyme 1α in INS-1 cells. (a,b) Relative messenger ribonucleic acid (mRNA) expression levels of (a) TXNIP and (b) insulin 1 (Ins1) in IRE1α overexpressing INS-1 cells (n = 3–8). The cells were treated with doxycycline hyclate (Dox; 1 µg/mL) with or without nicotine at indicated concentrations for indicated times. Relative mRNA levels of those in the cells treated with only nicotine for 24 h are shown in the second lane as a control. (c) Western blots of TXNIP and proinsulin in IRE1α overexpressing INS-1 cells treated with Dox with or without 1 mmol/L nicotine for 24 h. All data are expressed as the mean ± standard error of the mean. ***P < 0.001 versus control.
nAChR signaling regulates IRE1α signaling through the α7 subunit
To investigate nAChR signaling in detail, we focused on signaling through the α7 subunit for the following reasons. First, it is the most commonly expressed subunit among the nAChR subunits in rodent β-cells. Second, the mechanisms underlying the recently revealed anti-apoptotic effects of a complete and highly specific nAChR α7 agonist, PNU, in β-cells are still unknown. PNU dose-dependently attenuated IRE1α-induced T-UPR markers similarly to nicotine (Figure 4a-c). Next, PNU attenuated the increased mRNA expression levels of XBP1 spliced form induced by ER stress in INS-1 and EndoC-βH1 cells (Figure 4d,e). Furthermore, PNU inhibited the increase of TXNIP and decrease of Ins1/ proinsulin expression at mRNA and protein levels (Figure 4f-h). The effect of nicotine was canceled by the α7-specific antagonist, α-bungarotoxin (Figure 5a). Finally, the protective effects of nicotine and PNU on T-UPR signaling were canceled by two specific small interfering RNAs targeting α7 nAChR with approximately 60% reduction of CHRNA7 expression (Figure 5b).

Figure 2 | Nicotine regulates inositol-requiring enzyme 1α (IRE1α) activation induced by endoplasmic reticulum stress in INS-1 and EndoC-βH1 cells. (a) EtBr-stained agarose gel electrophoresis of PstI-digested X-box-binding protein-1 (XBP1) amplicons obtained by polymerase chain reaction in INS-1 cells treated with Tg (500 nmol/L, 6 h) with or without nicotine (1 mmol/L) for 3 h. (b,c) The numbers above are calculated from the ratio metric quantitation of the level of spliced XBP1 complementary deoxyribonucleic acid to that of total XBP1 complementary deoxyribonucleic acids. sXBP1 expression measured by quantitative reverse transcription polymerase chain reaction treated with (b) thapsigargin (Tg; 500 nmol/L, 6 h; n = 10) or (c) tunicamycin (Tm; 100 μg/mL, 3 h; n = 3) with or without nicotine (1 mmol/L) for 6 h in INS-1 cells. (d) Immunoblots with anti-IRE1α antibody for proteins separated using Phos-tag gel for INS-1 cells treated with Tg (500 nmol/L) with or without nicotine (1 mmol/L) for 6 h. (e) Expression of CHRNA7 messenger ribonucleic acid (mRNA) by polymerase chain reaction in human EndoC-βH1 cells. The expression of the in pcDNA3.1-human CHRNA7 vector is shown as the positive control. (f) Relative sXBP1 mRNA expression in EndoC-βH1 cells treated with Tg (10 μmol/L) with or without nicotine (1 mmol/L) for 6 h (n = 3). All data are expressed as the mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.
mRNA expression levels under overexpression of IRE1α in INS-1 cells (Figure 5b,c, Figure S5). Taken together, the effects of nAChR signaling on T-UPR were, at least in part, through the α7 nAChR in pancreatic β-cells.

Nicotine inhibits apoptosis induced by IRE1α activation and ER stress
Finally, if indeed nicotine attenuates T-UPR, we hypothesized that nicotine could regulate ultimate apoptosis induced by

![Graphs showing mRNA expression levels](image)

**Figure 3** | Nicotine attenuates terminal unfolded protein response induced by endoplasmic reticulum stress in INS-1 and EndoC-βH1 cells. (a–d) Relative messenger ribonucleic acid (mRNA) levels of (a,c) TXNIP and (b,d) Ins1 by quantitative polymerase chain reaction in INS-1 cells treated with thapsigargin (Tg; 500 nmol/L, 6 h) or tunicamycin (Tm; 200 nmol/L, 24 h) with or without nicotine (1 mmol/L; n = 6–9). (e,h) Western blots of TXNIP and proinsulin in INS-1 cells incubated with (e) Tg (500 nmol/L, 6 h; n = 3) or with (h) high medium glucose (28 mmol/L, 24 h) with or without nicotine (1 mmol/L). (f,g) Relative mRNA levels of (f) TXNIP and (g) insulin in EndoC-βH1 cells treated with Tg (10 μmol/L) with or without nicotine (100 μmol/L) for 6 h. All data are expressed as the mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.
Figure 4 | The nicotinic acetylcholine receptor (nAChR) α7 subunit-specific agonist, PNU-282987, regulates terminal unfolded protein response induced by inositol-requiring enzyme 1α (IRE1α) overexpression and endoplasmic reticulum stress in INS-1 and EndoC-βH1 cells. (a, b) Relative messenger ribonucleic acid (mRNA) expression levels of (a) TXNIP and (b) Ins1 in IRE1α-overexpressing INS-1 cells treated with doxycycline hyclate (Dox; 1 μg/mL) with or without PNU-282987 (PNU), the agonist of nAChR α7 at indicated doses for 24 h (n = 4). (c) Western blots of TXNIP and proinsulin in IRE1α-overexpressing INS-1 cells treated with Dox (1 μg/mL) with or without PNU at indicated doses for 24 h. (d, e) Relative mRNA expression levels of splice X-box-binding protein-1 (sXBP1) in (d) INS-1 cells treated with Tm (200 nmol/L, 24 h) or in (e) EndoC-βH1 cells with thapsigargin (Tg; 10 μmol/L, 6 h) with or without PNU (10 μmol/L) (n = 3–4). (f, g) Relative mRNA levels of (f) TXNIP (n = 3) and (g) Ins1 (n = 6) in INS-1 cells treated with Tg (500 nmol/L) with or without PNU (10 μmol/L) for 6 h. (h) Western blots of TXNIP and proinsulin in INS-1 cells treated with Tg (500 nmol/L) with or without PNU (10 μmol/L) for 6 h. All data are expressed as the mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.
IRE1α activation or ER stress. To evaluate apoptosis, we measured two markers, caspase-3 and -7 activity and annexin V. Caspase-3 and -7 is an apoptotic marker, and its activation is specific to cell apoptosis. Caspase-3 and -7 are normally present in cells as inactive precursors. When apoptosis occurs, these caspases are proteolysed and activated, initiating a caspase cascade that leads to the degradation of proteins essential for cell survival. Annexin V is a marker of apoptotic cells based on its ability to bind to phosphatidylserine. The translocation of phosphatidylserine to the surface of the cell membrane occurs downstream in the apoptotic process. Not only nicotine, but also PNU suppressed caspase-3/7 activity caused by IRE1α overexpression (Figure 6a,b). Notably, the concentration of PNU was lower than that of nicotine, suggesting that the effect of the α7 subunit specifically attenuates IRE1α-induced apoptosis. Furthermore, nicotine attenuated the increased caspase-3/7 activation induced by ER stress (Figure 6c). Finally, nicotine suppressed the number of Annexin V-positive cells induced by ER stress (Figure 6d, Figure S6). Taken together, nAChR signaling suppresses ultimate apoptosis induced by ER stress and IRE1α activation.

**DISCUSSION**

We previously showed the critical role of IRE1α signaling in diabetes by demonstrating that its endoribonuclease activities trigger cell death and crucial dysfunction in β-cells. Furthermore, we showed that the monoselective IRE1α inhibitor KIRA8 rescues β-cells from T-UPR and ultimate apoptosis to ameliorate or reverse diabetes in Akita or NOD mice, as shown by the observation that KIRA spares cell survival A-UPR, but shuts down T-UPR, showing the essential role of tuning IRE1α output for glucose metabolism in vivo. In contrast, recent reports have emphasized the protective role of global and α7 subunit-specific nAChR signaling against apoptosis in rodent β-cells, but the underlying mechanisms are still unknown. These backgrounds encouraged us to investigate the link between nAChR signaling and IRE1α signaling. In the present study, we showed that the modulation of global and α7 subunit-specific nAChR signaling attenuated IRE1α activation, which protected rat and human β-cells from T-UPR and ultimate apoptosis under ER stress.

The vagus nerve system was previously shown to have a cytoprotective effect by balancing and attenuating the pro-inflammatory activation in islets. It has also been shown that nicotine or the systemic increase in ACh levels ameliorates the diabetic phenotypes by stimulating the Th1 to Th2 cytokine switch or pancreatic T-cell differentiation in NOD mice and multiple low-dose streptozotocin mice. In addition, the specific stimulation of nAChR α7 has recently been shown to activate anti-inflammatory and prosurvival pathways in cytokine-treated INS-1 cells and murine islets. In contrast, we and other groups have recently shown that UPR markers are induced in β-cells from type 1 diabetes patients and NOD mice, and that established diabetes in NOD mice could be reversed by KIRA. In addition, we previously showed that IRE1α activation induces TXNIP to activate the NLRP3 inflammasome.
inflammasome and promote programmed cell death under irre-
mediable ER stress by reducing the expression levels of a
TXNIP mRNA destabilizing microRNA, miR-1710,25. The
findings of the present study might support the anti-in-
flammatory role of nAChR signaling in the immune/UPR-mediated patho-
genic state of pancreatic β-cells by showing its novel pathway
of regulation of proapoptotic TXNIP expression induced by
IRE1α activation and ER stress.

The effect of nAChR signaling on the IRE1α arm has not
been previously investigated. Regarding the effect of nAChR
signaling on UPR in non-β-cells, one study showed that nicotine
does not influence the expression of c-jun NH2-terminal
kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK)
in cultured rat mesangial cells14; however, another study
controversially showed that nicotine dose-dependently
induces the phosphorylation of JNK and p38 MAPK, and its
antagonist blocks the phosphorylation of JNK in HepG2 cells21.

One of the limitations of the present study is that, although
we showed that the modulation of global and α7-specific
nAChR signaling could regulate IRE1α signaling, its detailed
pathway(s) is still widely unknown. Initially, UPR signaling
was viewed as a direct and linear transduction of ER stress levels4.
However, recent findings have shown that UPR sensors are tightly regulated through their binding to cofactors and post-translational modifications. For example, it has recently been reported that many cytosolic proteins characterized as components of other signaling pathways interact with UPR sensors to regulate their outputs, and their complexes are conceptually termed "UPRosomes." As for IRE1α, we and other groups showed that ABL kinase, as well as protein-tyrosine phosphatase 1B, BAX inhibitor-1, ASK1 and RACK1, could tune IRE1α outputs to affect cell fate. As nAChR is a type of ligand-gated ion channel, it could be speculated that a change in intracellular calcium flux through nAChR might widely affect myriad molecules, including UPRosome-related proteins, or the post-translational modulation of IRE1α to attenuate the amplification of the T-UPR cascade. Further studies are required to elucidate the detailed mechanism(s) of cross-talk of nAChR-IRE1α signaling. Another limitation is that all the experiments were in vitro studies. Thus, as we could speculate the effect of vagus systems on nAChR signaling in β-cells or the paracrine effect of other islet component cells on this signaling in vivo β-cells, the present findings regarding the roles of nAChR-IRE1α signaling should be carefully interpreted. Further studies using animal models are required.

In conclusion, we showed that nAChR signaling regulates IRE1α signaling to protect against T-UPR and eventual apoptosis induced by ER stress in both rat and human pancreatic β-cells. Furthermore, it regulates IRE1α activation, at least in part, through the α7 nAChR subunit. We elucidated the novel role of nAChR signaling as a protective factor for β-cells under ER stress. nAChR signaling could be one of the candidates of a therapeutic target to prevent or reverse β-cell loss and dysfunction in diabetes.

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DISCLOSURE
The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** | Time courses of FLAG, TXNIP and proinsulin expressions in doxycycline hyclate (Dox)-inducible inositol-requiring enzyme 1α (IRE1α)-overexpressing INS-1 cells.

**Fig. S2** | Overexpression of inositol-requiring enzyme 1α (IRE1α) does not affect phosphorylation of eIF2α nor CHOP messenger ribonucleic acid expression in INS-1 cells.

**Fig. S3** | Effects of nicotine on phosphorylation of eIF2α is limited under endoplasmic reticulum (ER) stress in INS-1 cells.

**Fig. S4** | Nicotine does not affect basal TXNIP expression in INS-1 cells.

**Fig. S5** | Knock down of rat α7 nicotinic acetylcholine receptor (CHRNA7) by specific small interfering ribonucleic acids in INS-1 cells.

**Fig. S6** | Representative histograms of fluorescein isothiocyanate annexin V-stained INS-1 cells.

**Fig. S7** | Messenger ribonucleic acid expression levels of nicotinic acetylcholine receptor subunits in INS-1 cells.

**Table S1** | Primers used for Figure S7.