



# Nicotinic acetylcholine receptor signaling regulates inositol-requiring enzyme 1 $\alpha$ activation to protect $\beta$ -cells against terminal unfolded protein response under irremediable endoplasmic reticulum stress

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## Keywords

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## ABSTRACT

**Aims/Introduction:** Under irremediable endoplasmic reticulum (ER) stress, hyperactivated inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) triggers the terminal unfolded protein response (T-UPR), causing crucial cell dysfunction and apoptosis. We hypothesized that nicotinic acetylcholine receptor (nAChR) signaling regulates IRE1 $\alpha$  activation to protect  $\beta$ -cells from the T-UPR under ER stress.

**Materials and Methods:** The effects of nicotine on IRE1 $\alpha$  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- $\beta$ H1  $\beta$ -cell lines. Doxycycline-inducible IRE1 $\alpha$  overexpression or ER stress agents were used to induce IRE1 $\alpha$  activation. An  $\alpha$ 7 subunit-specific nAChR agonist (PNU-282987) and small interfering ribonucleic acid for  $\alpha$ 7 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 IRE1 $\alpha$  hyperactivation or ER stress agents. Nicotine attenuated X-box-binding protein-1 messenger ribonucleic acid site-specific splicing and IRE1 $\alpha$  autophosphorylation induced by ER stress. Furthermore, PNU-282987 attenuated T-UPR induction by either forced IRE1 $\alpha$  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  $\alpha$ 7 nAChR. Finally, nicotine suppressed apoptosis induced by either forced IRE1 $\alpha$  activation or ER stress agents.

**Conclusions:** Our findings suggest that nAChR signaling regulates IRE1 $\alpha$  activation to protect  $\beta$ -cells from the T-UPR and apoptosis under ER stress partly through  $\alpha$ 7 nAChR. Targeting nAChR signaling to inhibit the T-UPR cascade may therefore hold therapeutic promise by thwarting  $\beta$ -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)<sup>1</sup>. Under ER stress, intracellular signaling pathways termed the unfolded protein response (UPR)

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become 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 irretrievably 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<sup>2–4</sup>.

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 1 $\alpha$  (IRE1 $\alpha$ ) – by changing their oligomerization state<sup>4,5</sup>. Under ER stress, IRE1 $\alpha$ , 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<sup>4,6–8</sup>. However, under high (irremediable) ER stress, oligomerized and autophosphorylated IRE1 $\alpha$  activates the RNase catalytic rate further, causing cleavage of myriad ER-localized mRNAs, ribosomal RNAs and microRNAs, which ultimately induces apoptosis. Therefore, modulation of IRE1 $\alpha$  activities is central to the A-UPR to T-UPR “switch,” and thereby to control cell fate<sup>2</sup>. This regulation of stability of multiple RNAs by IRE1 $\alpha$  is known as regulated IRE1 $\alpha$ -dependent decay<sup>4,9</sup>. Indeed, we have shown *in vivo*, that IRE1 $\alpha$ -specific inhibitors, kinase-inhibitory RNase attenuators (KIRA), can spare pancreatic  $\beta$ -cells from T-UPR and ultimate apoptosis to reverse or ameliorate diabetes in Akita and NOD mice<sup>2,10</sup>. Notably, the switch from A-UPR to T-UPR has recently been shown in NOD mice<sup>10</sup>. These findings suggest that modulating the activity of IRE1 $\alpha$  in  $\beta$ -cells might have a general therapeutic potential against type 2 and autoimmune diabetes by rescuing  $\beta$ -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<sup>11</sup>. 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<sup>12</sup>. Similarly, it was also shown that nAChR is expressed in murine islets and rodent pancreatic  $\beta$ -cell lines<sup>13</sup>. Interestingly, recent reports emphasize the importance of nAChR signaling by showing that although cholinergic innervation of human islets is limited,  $\beta$ -cells can substitute to secrete ACh to stimulate surrounding endocrine cells in a paracrine manner<sup>14</sup>. 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<sup>15–19</sup>. Furthermore, stimulation of nAChR by nicotine increases insulin secretion in rodent  $\beta$ -cells, and decreases cytokine-induced

apoptosis in human and rodent islets at least through the  $\alpha 7$  nAChR subunit<sup>13,20</sup>.

Regarding the link between UPR and nAChR signaling, a limited number of studies show controversial evidence of this link in non- $\beta$  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<sup>21</sup>; however, another study showed the induction of ER stress by nicotine in rat placental trophoblast giant cells<sup>22</sup>. 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 IRE1 $\alpha$  signaling, are linked in  $\beta$ -cells. Here, we aimed to determine whether and if so, how nAChR signaling regulates IRE1 $\alpha$  signaling, and subsequently T-UPR, in rat and human pancreatic  $\beta$ -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).  $\alpha$ -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<sup>3</sup>. 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  $\mu$ g/L streptomycin, 292  $\mu$ g/mL of L-glutamine; Invitrogen) and 1 mmol/L sodium pyruvate. EndoC- $\beta$ H1 cells (Univercell Biosolutions, Toulouse, France) were cultured in accordance with the manufacturer's protocol<sup>23</sup>. Briefly, the cells were plated on a coat with  $\beta$ COAT (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 OPTI $\beta$ 1 complete premixed culture medium (Univercell Biosolutions). Both INS-1 and EndoC- $\beta$ H1 cells were cultured in a humidified atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. 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 IRE1 $\alpha$  overexpressing INS-1 cells*  
Transgenic WT mouse IRE1 $\alpha$  overexpressing INS-1 cells line in this study are tightly inducible by Dox, and maintained as described previously<sup>3</sup>.

#### 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  $\mu$ g of total RNA was reverse transcribed to complementary deoxyribonucleic acid (cDNA) using a reverse transcription kit (Qiagen) after DNase digestion. For quantitative 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  $\beta$ -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'-AAACAGAGTAGCACAGACTGC-3', R:5'-GGATCTCTAAGACTAGAGGCTTGGTG-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<sup>10</sup>. PCR fragments were digested with PstI and resolved on 3% agarose gels.

#### Western blot analysis

We carried out western blot as previously described<sup>10</sup>. Briefly, for protein analysis, cells were lysed in M-PER buffer (Thermo Scientific, Rockford, IL, USA) mixed with a protease inhibitor, containing 4  $\mu$ g/mL aprotinin (Wako, Osaka, Japan), 4  $\mu$ g/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 g for 5 min at 4°C. The supernatant was collected and quantified by colorimetric protein assay (BioRad, Berkeley, CA, USA). Loading samples were prepared by mixing the protein extract with

NuPAGE sample buffer (Invitrogen) and heated at 98°C for 5 min to denature the proteins.

Blocking, incubation with antibodies and washing were carried out in Tris-buffered saline with 0.05% (v/v) Tween-20 and 5% (w/v) 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).  $\beta$ -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-IRE1 $\alpha$  antibody (1:1,000; Cell Signaling Technology Inc.), mouse anti-phosphorylated eIF2 $\alpha$  (Ser51) antibody (1:1,000; Cell Signaling Technology Inc.), mouse anti-eIF2 $\alpha$  antibody (1:1,000; Cell Signaling Technology Inc.) and mouse anti- $\beta$ -Actin antibody (1:5,000; Sigma-Aldrich).

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

For measuring phosphorylated IRE1 $\alpha$ , 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 $\times$  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 $\alpha$ 7 subunit in EndoC- $\beta$ H1 cells

Total RNA was extracted from EndoC- $\beta$ H1 cells and was reverse transcribed to cDNA as described above. Human CHRNA7 primers (F: 5'-CCGACTCTGGGTAGTGTGT-3', R: 5'-ATGGTGCAGATGATGGTGAA-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<sup>24</sup>.

#### RNA interference

A negative control (Qiagen, #1022563) or two small interfering RNAs (siRNAs) against rat CHRNA7 (Qiagen, #SI00253365, #SI03045028), encoding nAChR  $\alpha$ 7 subunit, were transfected using lipofectamine RNAiMAX lipid reagent (Invitrogen) in Dox-inducible IRE1 $\alpha$ -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

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

Gene	Primer
Rat TXNIP	F: 5'-CTGATGGAGGCACAGTGA-3' R: 5'-CTCGGGTGGAGTGCTTAGAG-3'
Rat Ins1	F: 5'-GTCCTCTGGGAGCC CAAG-3' R: 5'-ACAGAGCCTCCACCAGG-3'
Rat sXBP1	F: 5'-CTGAGTCCGAATCAGGTGCAG-3' R: 5'-ATCCATGGGAAGATGTTCTGG-3'
Human sXBP1	F: 5'-GAGTCCGCAGCAGGTG-3' R: 5'-TCCTTCTGGGTA GACCTCTGGGAG-3'
Human TXNIP	F: 5'-CCTCTGGGAACATCCTTCAA-3' R: 5'-GGGGTATTGACATCCACCAG-3'
Rat CHRNA7	F: 5'-TGGCCAACGACTCGCAGCCG-3' R: 5'-ACTCCGGGTACTCAGACAT-3'
Rat CHOP	F: 5'-CCAGCAGAGGTCACAAGCAC-3' R: 5'-CGCACTGACCACTCTGTTTC-3'
Rat $\beta$ -actin:	F: 5'-GCAATGCTTCTAGGCGGAC-3' R: 5'-AAGAAAGGGTGTAAACGCAGC-3'
Human $\beta$ -actin:	F: 5'-AGAGCTACGAGCTGCCTGAC-3' R: 5'-AGCACTGTGTTGGCGTACAG-3'

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 IRE1 $\alpha$  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 FACSVerser (BD Biosciences), and the results were analyzed using FACSsuite (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  $\pm$  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<sup>®</sup> 14 (SAS Institute Inc., Cary, NC, USA).

## RESULTS

### Nicotine reverses the T-UPR induced by IRE1 $\alpha$ hyperactivation

To elucidate whether nAChR signaling has the cell protective effect against T-UPR induced by IRE1 $\alpha$  signaling in pancreatic  $\beta$ -cells, we first examined T-UPR markers in Dox-inducible FLAG-tagged IRE1 $\alpha$ -overexpressing INS-1 cells. We previously validated this cell system as a tool to determine the sufficiency of specific and focal hyperactivation of IRE1 $\alpha$  (without the need to resort to upstream ER stress inducing agents)<sup>3</sup>. Overexpression of IRE1 $\alpha$  increased a key T-UPR marker, TXNIP, which activates the NLRP3 inflammasome to promote  $\beta$ -cell death (Figure 1a, Figure S1)<sup>25,26</sup>. Furthermore, it decreased insulin 1 (Ins1) mRNAs, which was identified as IRE1 $\alpha$  RNase substrate, and consecutive proinsulin protein levels over time (Figure 1b, Figure S1)<sup>3</sup>. The effect of IRE1 $\alpha$  overexpression on PERK arm is limited in this system, based on its substrate eIF2 $\alpha$  phosphorylation levels and CHOP mRNA expression being unchanged (Figure S2a,b). Using this IRE1 $\alpha$ -overexpressing INS-1 cell line, nicotine reversed the increase of TXNIP mRNA expression levels in a dose-dependent manner, starting at 10  $\mu$ mol/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,

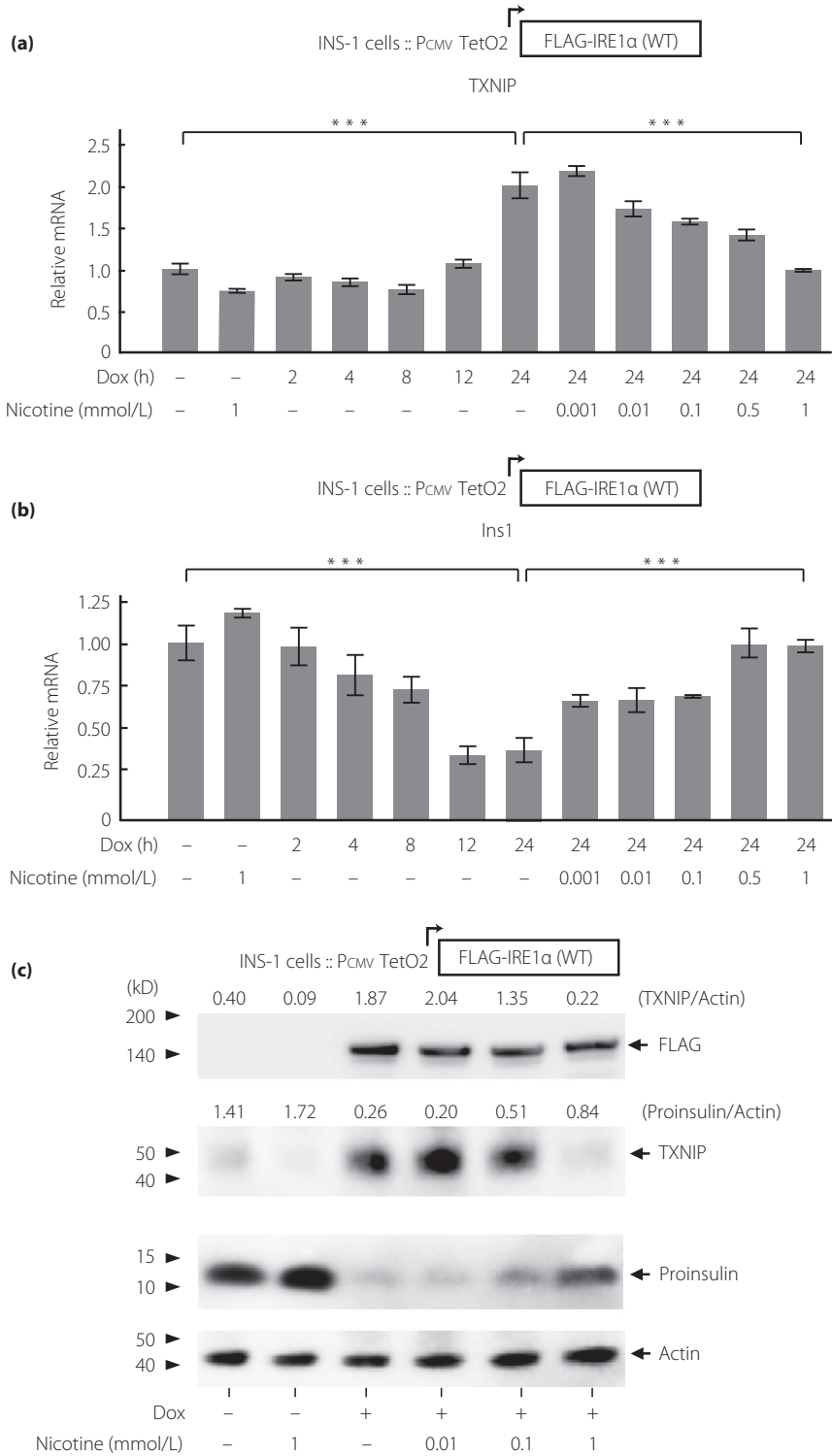
nAChR signaling protects T-UPR signaling driven by IRE1 $\alpha$ -specific activation in INS-1 cells.

### Nicotine modulates IRE1 $\alpha$ signaling under ER stress

Encouraged by the protective effect of nicotine against the T-UPR by IRE1 $\alpha$ -specific hyperactivation, we next examined the effect of nicotine on IRE1 $\alpha$  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 Ca<sup>2+</sup> ATPase, which activates calcium-dependent ER chaperones, leading to the accumulation of unfolded proteins<sup>27</sup>. Tm blocks glycoprotein biosynthesis in the ER causing accumulation of unfolded glycoproteins, which in turn leads to ER stress<sup>28</sup>. 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 IRE1 $\alpha$  drives RNase activity into the hyperactivation state, we next investigated the phosphorylation levels of IRE1 $\alpha$  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 level of phosphorylated IRE1 $\alpha$  to that of non-phosphorylated IRE1 $\alpha$ , and nicotine decreased it (Figure 2d). Then, as shown in the rodent  $\beta$ -cell line, we confirmed for the first time the expression of the  $\alpha 7$  nAChR subunit in the well-validated human  $\beta$ -cell line, EndoC- $\beta$ H1 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 eIF2 $\alpha$  phosphorylation levels being unchanged (Figure S3). Therefore, nicotine modulates kinase and RNase activation of IRE1 $\alpha$  under ER stress in pancreatic  $\beta$ -cells.

### Nicotine attenuates T-UPR induced by ER stress

As nicotine showed the effect of reducing the direct IRE1 $\alpha$  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- $\beta$ H1 cells (Figure 3f,g). To evaluate the effect of nicotine under physiological ER stress, we investigated it at high glucose levels<sup>29</sup>. A high ambient glucose concentration (28 mmol/L) increased the protein expression levels of TXNIP and reduced those of proinsulin protein levels as previously reported<sup>10</sup>, and nicotine attenuated these effects (Figure 3h). These results suggest that nicotine attenuates T-UPR induced by ER stress.

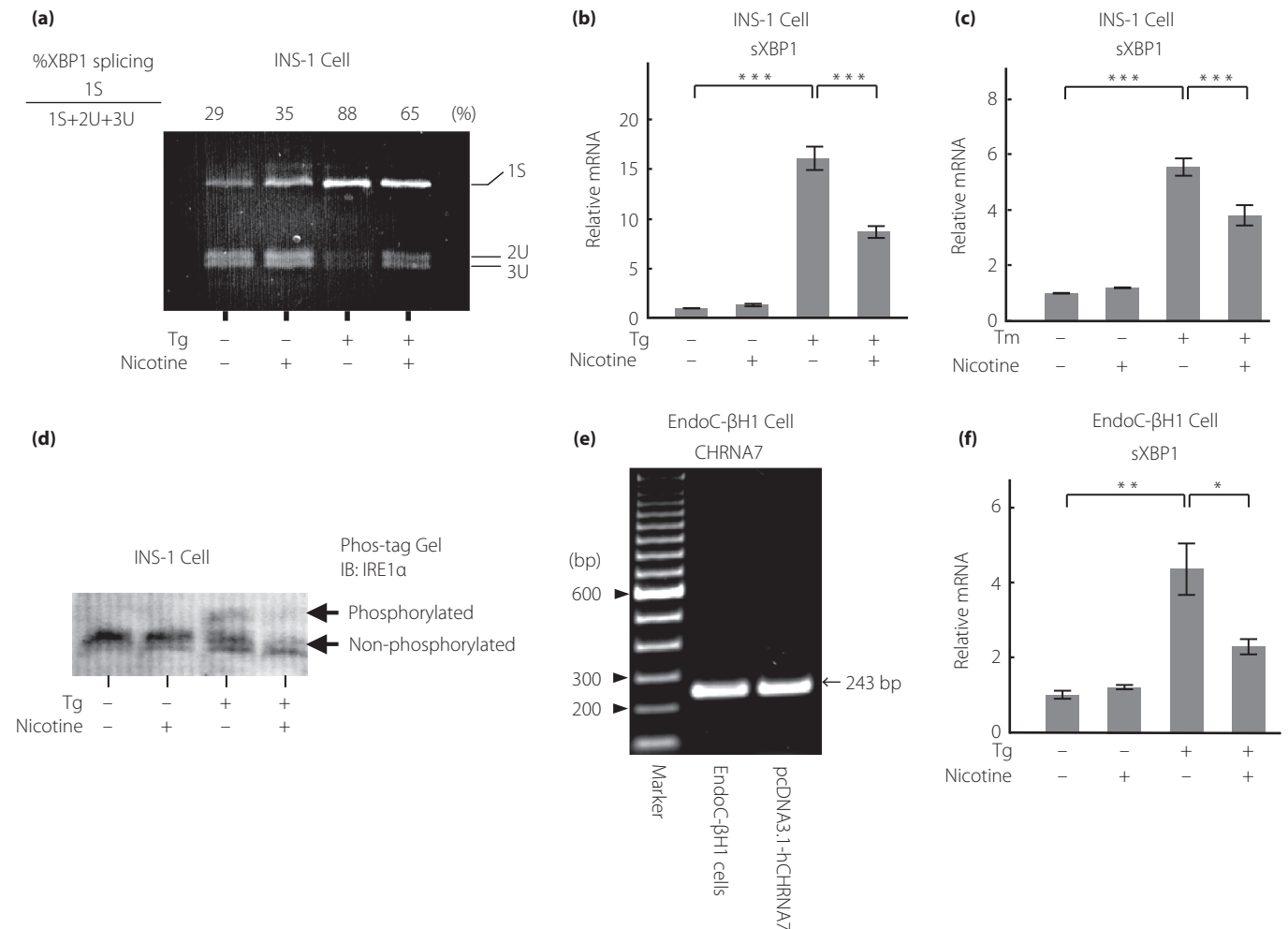


**Figure 1** | Nicotine reverses terminal unfolded protein response induced by overexpression of Flag-tagged full-length inositol-requiring enzyme 1 $\alpha$  in INS-1 cells. (a,b) Relative messenger ribonucleic acid (mRNA) expression levels of (a) TXNIP and (b) insulin 1 (Ins1) in IRE1 $\alpha$  overexpressing INS-1 cells ( $n = 3-8$ ). The cells were treated with doxycycline hydrochloride (Dox; 1  $\mu$ g/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 IRE1 $\alpha$  overexpressing INS-1 cells treated with Dox with or without 1 mmol/L nicotine for 24 h. All data are expressed as the mean  $\pm$  standard error of the mean. \*\*\* $P < 0.001$  versus control.

### nAChR signaling regulates IRE1 $\alpha$ signaling through the $\alpha$ 7 subunit

To investigate nAChR signaling in detail, we focused on signaling through the  $\alpha$ 7 subunit for the following reasons. First, it is the most commonly expressed subunit among the nAChR subunits in rodent  $\beta$ -cells<sup>11,30</sup>. Second, the mechanisms underlying the recently revealed anti-apoptotic effects of a complete and highly specific nAChR  $\alpha$ 7 agonist, PNU, in  $\beta$ -cells are still unknown. PNU dose-dependently attenuated IRE1 $\alpha$ -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- $\beta$ H1 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  $\alpha$ 7-specific antagonist,  $\alpha$ -bungarotoxin (Figure 5a). Finally, the protective effects of nicotine and PNU on T-UPR signaling were canceled by two specific small interfering RNAs targeting  $\alpha$ 7 nAChR with approximately 60% reduction of CHRNA7

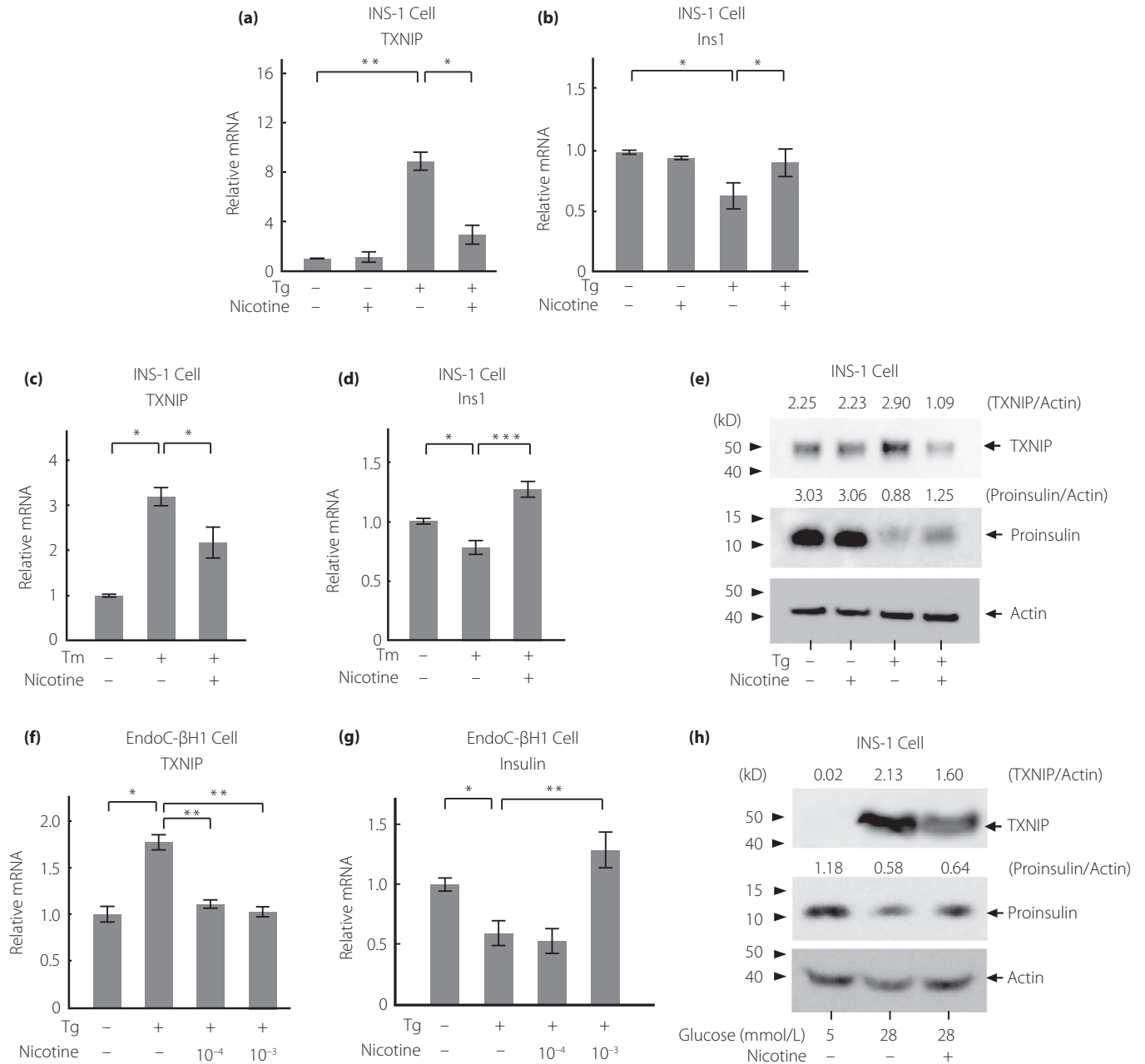


**Figure 2** | Nicotine regulates inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) activation induced by endoplasmic reticulum stress in INS-1 and EndoC- $\beta$ H1 cells. (a) EtBr-stained agarose gel electrophoresis of Pst1-digested X-box-binding protein-1 (XBP1) amplicons obtained by polymerase 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  $\mu$ g/mL, 3 h;  $n = 3$ ) with or without nicotine (1 mmol/L) for 6 h in INS-1 cells. (d) Immunoblots with anti-IRE1 $\alpha$  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- $\beta$ H1 cells. The expression of the in pcDNA3.1-human CHRNA7 vector is shown as the positive control. (f) Relative sXBP1 mRNA expression in EndoC- $\beta$ H1 cells treated with Tg (10  $\mu$ mol/L) with or without nicotine (1 mmol/L) for 6 h ( $n = 3$ ). All data are expressed as the mean  $\pm$  standard error of the mean. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control.

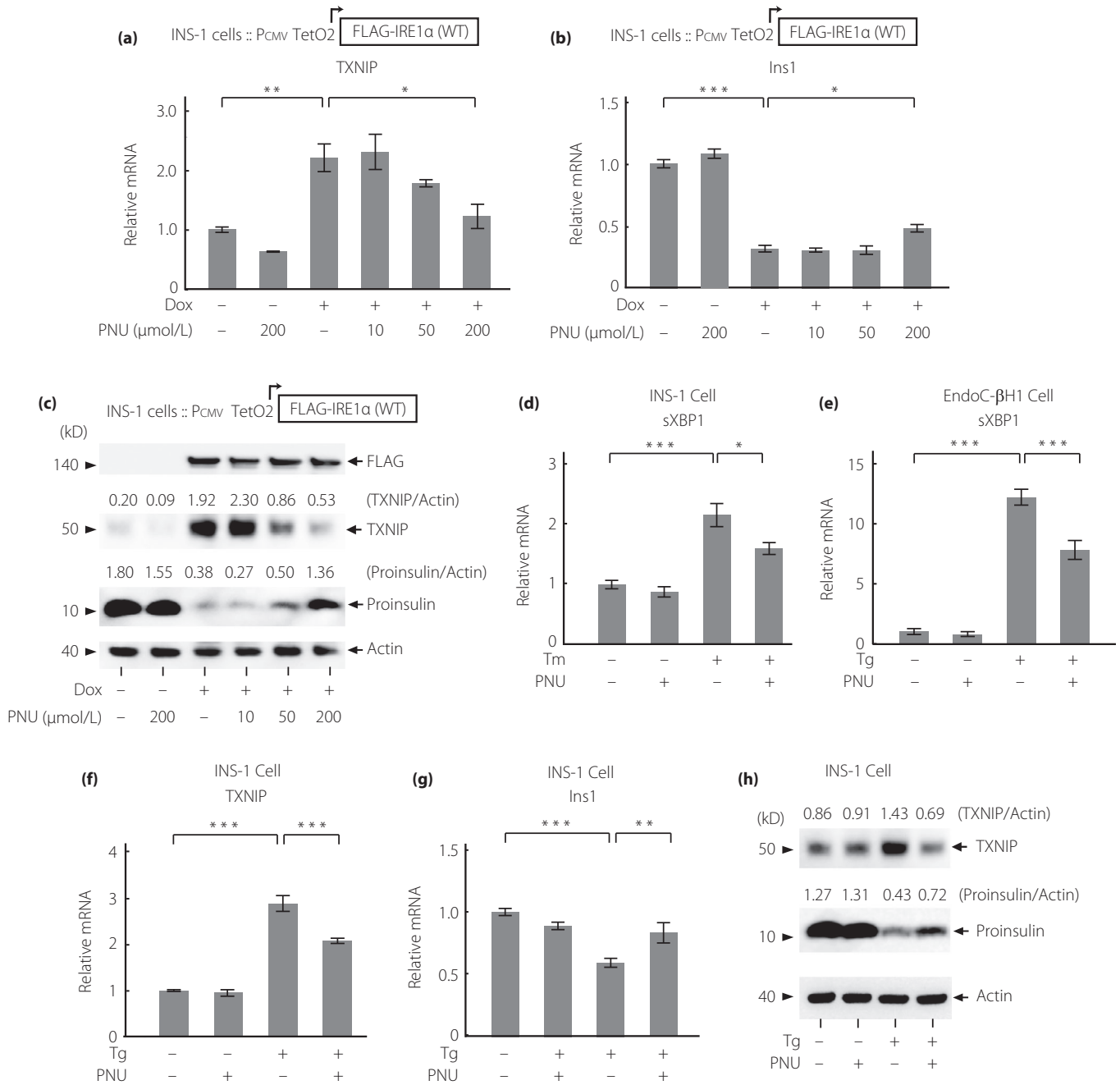
mRNA expression levels under overexpression of IRE1 $\alpha$  in INS-1 cells (Figure 5b,c, Figure S5). Taken together, the effects of nAChR signaling on T-UPR were, at least in part, through the  $\alpha 7$  nAChR in pancreatic  $\beta$ -cells.

**Nicotine inhibits apoptosis induced by IRE1 $\alpha$  activation and ER stress**

Finally, if indeed nicotine attenuates T-UPR, we hypothesized that nicotine could regulate ultimate apoptosis induced by

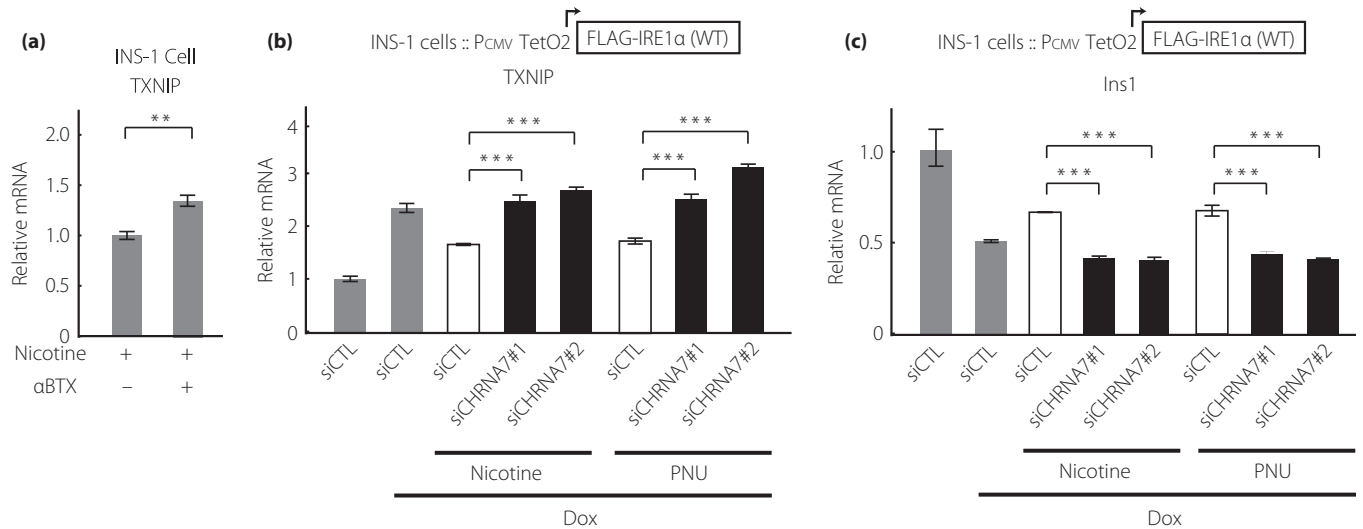


**Figure 3** | Nicotine attenuates terminal unfolded protein response induced by endoplasmic reticulum stress in INS-1 and EndoC- $\beta$ H1 cells. (a–d) Relative messenger ribonucleic acid (mRNA) levels of (a,c) TXNIP and (b,d) Ins1 by quantitative polymerase chain reaction in INS-1 cells treated with 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- $\beta$ H1 cells treated with Tg (10  $\mu$ mol/L) with or without nicotine (100  $\mu$ mol/L) for 6 h. All data are expressed as the mean  $\pm$  standard error of the mean. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control.



**Figure 4** | The nicotinic acetylcholine receptor (nAChR)  $\alpha 7$  subunit-specific agonist, PNU-282987, regulates terminal unfolded protein response induced by inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) overexpression and endoplasmic reticulum stress in INS-1 and EndoC- $\beta$ H1 cells. (a,b) Relative messenger ribonucleic acid (mRNA) expression levels of (a) TXNIP and (b) Ins1 in IRE1 $\alpha$ -overexpressing INS-1 cells treated with doxycycline hydrochloride (Dox; 1  $\mu$ g/mL) with or without PNU-282987 (PNU), the agonist of nAChR  $\alpha 7$  at indicated doses for 24 h ( $n = 4$ ). (c) Western blots of TXNIP and proinsulin in IRE1 $\alpha$ -overexpressing INS-1 cells treated with Dox (1  $\mu$ g/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- $\beta$ H1 cells with thapsigargin (Tg; 10  $\mu$ mol/L, 6 h) with or without PNU (10  $\mu$ mol/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  $\mu$ mol/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  $\mu$ mol/L) for 6 h. All data are expressed as the mean  $\pm$  standard error of the mean. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control.





**Figure 5** | Nicotinic acetylcholine receptor (nAChR) signaling regulates inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) signaling through the  $\alpha$ 7 subunits in INS-1 cells. (a) Relative messenger ribonucleic acid (mRNA) levels of TXNIP in INS-1 cells co-treated with tunicamycin (Tm; 200 nmol/L) and nicotine (1 mmol/L) for 24 h, pretreated with or without  $\alpha$ -bungarotoxin ( $\alpha$ BTX, 1  $\mu$ mol/L), an  $\alpha$ 7-specific nAChR inhibitor, for 3 h ( $n = 6$ ). (b,c) IRE1 $\alpha$ -overexpressing INS-1 cells were transfected with a non-specific control small interfering ribonucleic acid (siCTL) or two independent small interfering ribonucleic acids targeting CHRNA7 (siCHRNA7 #1 and #2). After 24 h, the cells were treated with doxycycline hydrochloride (Dox; 1  $\mu$ g/mL) with or without nicotine (1 mmol/L) or PNU (20  $\mu$ mol/L) for 24 h. Relative (b) TXNIP and (c) Ins1 mRNA levels were determined by quantitative polymerase chain reaction ( $n = 3$ -5). All data are expressed as the mean  $\pm$  standard error of the mean. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control.

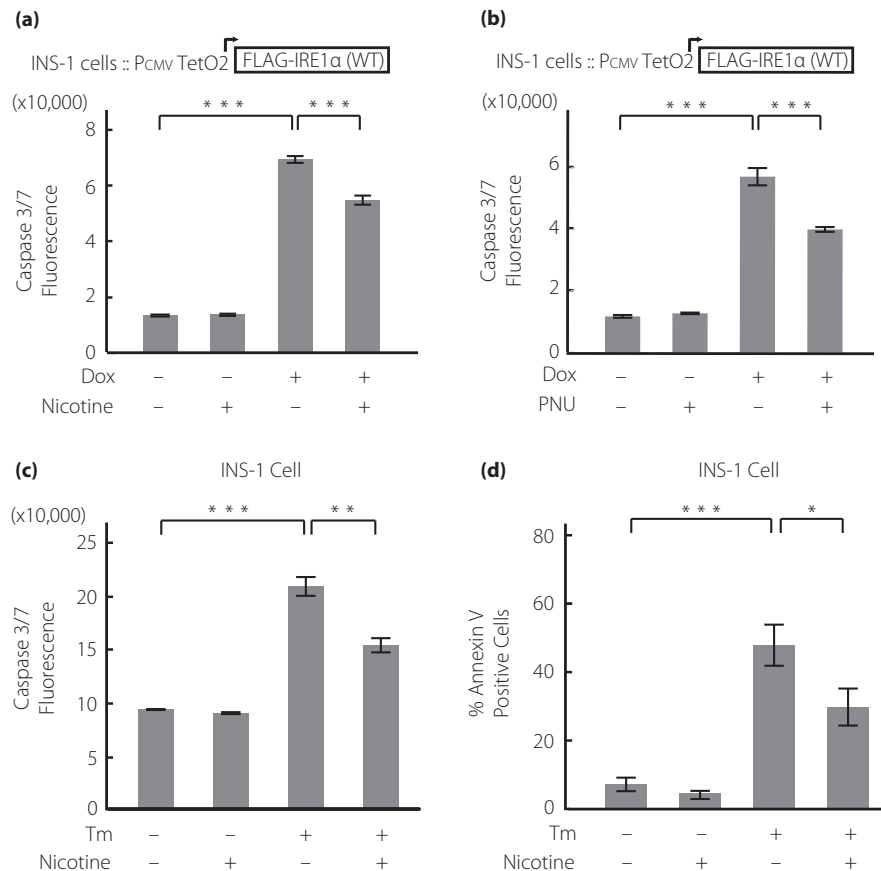
IRE1 $\alpha$  activation or ER stress. To evaluate apoptosis, we measured two markers, caspase-3 and -7 activity and annexin V. Caspase-3 and -7 is an apoptotic marker, and its activation is specific to cell apoptosis. Caspase-3 and -7 are normally present in cells as inactive precursors. When apoptosis occurs, these caspases are proteolytically activated, initiating a caspase cascade that leads to the degradation of proteins essential for cell survival<sup>31</sup>. Annexin V is a marker of apoptotic cells based on its ability to bind to phosphatidylserine<sup>32</sup>. The translocation of phosphatidylserine to the surface of the cell membrane occurs downstream in the apoptotic process<sup>33</sup>. Not only nicotine, but also PNU suppressed caspase-3/7 activity caused by IRE1 $\alpha$  overexpression (Figure 6a,b). Notably, the concentration of PNU was lower than that of nicotine, suggesting that the effect of the  $\alpha$ 7 subunit specifically attenuates IRE1 $\alpha$ -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 IRE1 $\alpha$  activation.

## DISCUSSION

We previously showed the critical role of IRE1 $\alpha$  signaling in diabetes by demonstrating that its endoribonuclease activities trigger cell death and crucial dysfunction in  $\beta$ -cells<sup>3</sup>. Furthermore, we showed that the monoselective IRE1 $\alpha$  inhibitor KIRA8 rescues  $\beta$ -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 IRE1 $\alpha$  output for glucose metabolism *in vivo*<sup>2,10</sup>. In contrast, recent reports have emphasized the protective role of global and  $\alpha$ 7 subunit-specific nAChR signaling against apoptosis in rodent  $\beta$ -cells, but the underlying mechanisms are still unknown<sup>34</sup>. These backgrounds encouraged us to investigate the link between nAChR signaling and IRE1 $\alpha$  signaling. In the present study, we showed that the modulation of global and  $\alpha$ 7 subunit-specific nAChR signaling attenuated IRE1 $\alpha$  activation, which protected rat and human  $\beta$ -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<sup>35</sup>. 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<sup>18,36</sup>. In addition, the specific stimulation of nAChR  $\alpha$ 7 has recently been shown to activate anti-inflammatory and prosurvival pathways in cytokine-treated INS-1 cells and murine islets<sup>34</sup>. In contrast, we and other groups have recently shown that UPR markers are induced in  $\beta$ -cells from type 1 diabetes patients and NOD mice, and that established diabetes in NOD mice could be reversed by KIRA<sup>10,37</sup>. In addition, we previously showed that IRE1 $\alpha$  activation induces TXNIP to activate the NLRP3



**Figure 6** | Nicotinic acetylcholine receptor (nAChR) signaling inhibits apoptosis induced by inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) activation and endoplasmic reticulum stress in INS-1 cells. (a,b) Caspase-3/7 fluorescence in IRE1 $\alpha$  overexpressing INS-1 cells treated with (a) doxycycline hyclate (Dox; 1  $\mu$ g/mL) with or without nicotine (1 mmol/L) or (b) PNU (10 nmol/L) for 72 h ( $n = 4$ ). (c) Caspase-3/7 fluorescence ( $n = 4$ ) or (d) percentage of annexin V-positive cells ( $n = 6$ ) in INS-1 cells treated with tunicamycin (Tm; 100 ng/mL) with or without nicotine (1 mmol/L) for 48 h. All data are expressed as the mean  $\pm$  standard error of the mean. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control.

inflammasome and promote programmed cell death under irremediable ER stress by reducing the expression levels of a TXNIP mRNA destabilizing microRNA, miR-17<sup>10,25</sup>. The findings of the present study might support the anti-inflammatory role of nAChR signaling in the immune/UPR-mediated pathogenic state of pancreatic  $\beta$ -cells by showing its novel pathway of regulation of proapoptotic TXNIP expression induced by IRE1 $\alpha$  activation and ER stress.

The effect of nAChR signaling on the IRE1 $\alpha$  arm has not been previously investigated. Regarding the effect of nAChR signaling on UPR in non- $\beta$ -cells, one study showed that nicotine inhibits the translocation of endogenously expressed activating transcription factor 6 and the phosphorylation of eIF2 $\alpha$  in Neuro-2a cells<sup>21</sup>; however, another study controversially showed that nicotine dose-dependently induces the phosphorylation of PERK and eIF2 $\alpha$ , and its antagonist blocks the phosphorylation of PERK in rat placental trophoblast cells<sup>22</sup>. One of the reasons for the conflicting observations of the effect of nicotine on UPR might be related to the tissue specificity of the effect of nicotine. In  $\beta$ -cells, previous studies showed the

expressions of the  $\alpha 7$  subunit in rat islet  $\beta$ -cells<sup>13</sup>, and the  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 7$  and  $\beta 2$  subunits in INS-1 cells<sup>34</sup>. We also preliminarily confirmed the expression of the  $\alpha 2$ ,  $\alpha 5$  and  $\beta 2$  subunits, as well as the  $\alpha 7$  subunit (Figure S7; Table S1). The  $\alpha 7$  subunit is the most common subunit in the previous islet studies, and its specific agonist has recently been shown to have a protective effect against cytokine-induced  $\beta$ -cell apoptosis and eventual diabetes *in vivo*<sup>19,34</sup>. On the basis of these findings, we investigated the  $\alpha 7$  subunit-specific effect on T-UPR and apoptosis under ER stress. In addition to previously reported protective mechanisms of modulation of nAChR  $\alpha 7$  signaling against  $\beta$ -cell apoptosis, which were attributed to attenuated JAK2–STAT3 signaling<sup>34</sup>, the results of our present study provide a novel protective role of nAChR  $\alpha 7$  signaling by linking to the regulation of IRE1 $\alpha$  signaling under ER stress.

One of the limitations of the present study is that, although we showed that the modulation of global and  $\alpha 7$ -specific nAChR signaling could regulate IRE1 $\alpha$  signaling, its detailed pathway(s) is still widely unknown. Initially, UPR signaling was viewed as a direct and linear transduction of ER stress levels<sup>4</sup>.

However, recent findings have shown that UPR sensors are tightly regulated through their binding to cofactors and post-translational modifications<sup>4,38</sup>. 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 “UPRosome”<sup>38</sup>. As for IRE1 $\alpha$ , we and other groups showed that ABL kinase, as well as protein-tyrosine phosphatase 1B, BAX inhibitor-1, ASK1 and RACK1, could tune IRE1 $\alpha$  outputs to affect cell fate<sup>10,39–43</sup>. 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 IRE1 $\alpha$  to attenuate the amplification of the T-UPR cascade. Further studies are required to elucidate the detailed mechanism(s) of cross-talk of nAChR-IRE1 $\alpha$  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  $\beta$ -cells or the paracrine effect of other islet component cells on this signaling in *in vivo*  $\beta$ -cells, the present findings regarding the roles of nAChR-IRE1 $\alpha$  signaling should be carefully interpreted. Further studies using animal models are required<sup>35,44</sup>.

In conclusion, we showed that nAChR signaling regulates IRE1 $\alpha$  signaling to protect against T-UPR and eventual apoptosis induced by ER stress in both rat and human pancreatic  $\beta$ -cells. Furthermore, it regulates IRE1 $\alpha$  activation, at least in part, through the  $\alpha 7$  nAChR subunit. We elucidated the novel role of nAChR signaling as a protective factor for  $\beta$ -cells under ER stress. nAChR signaling could be one of the candidates of a therapeutic target to prevent or reverse  $\beta$ -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 1 $\alpha$  (IRE1 $\alpha$ )-overexpressing INS-1 cells.

**Fig. S2** | Overexpression of inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) does not affect phosphorylation of eIF2 $\alpha$  nor CHOP messenger ribonucleic acid expression in INS-1 cells.

**Fig. S3** | Effects of nicotine on phosphorylation of eIF2 $\alpha$  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  $\alpha$ 7 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.