4μ8C Inhibits Insulin Secretion Independent of IRE1α RNase Activity

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ABSTRACT. IRE1α plays an important role in the unfolded protein response (UPR), which is activated by the accumulation of unfolded proteins in the endoplasmic reticulum. 4μ8C, a well-known inhibitor of IRE1α RNase activity, is commonly used to analyze IRE1α function during ER stress in cultured mammalian cells. However, the off-target effects of 4μ8C remain elusive. Pancreatic β-cells synthesize a large amount of insulin in response to high glucose stimulation, and IRE1α plays an important role in insulin secretion from pancreatic β-cells. Here, to analyze the role of IRE1α in pancreatic β-cells, we examined insulin secretion after 4μ8C treatment. Although 4μ8C inhibited insulin secretion within 2 hr, neither insulin synthesis nor maturation was inhibited by 4μ8C under the same conditions. This result prompted us to examine the precise effects of 4μ8C on insulin secretion in pancreatic β-cells. Unexpectedly, with just 5 min of treatment, 4μ8C blocked insulin secretion in cultured pancreatic β-cells as well as in pancreatic islets. Furthermore, insulin secretion was prevented by 4μ8C, even in pancreatic β-cells lacking the IRE1α RNase domain, suggesting that 4μ8C blocked the late stage of the insulin secretory process, independent of the IRE1α-XBP1 pathway. Our results indicate that 4μ8C has an off-target effect on insulin secretion in pancreatic β-cells. These findings inform the researchers in the field that the use of 4μ8C requires the special consideration for the future studies.

Key words: 4μ8C, XBP1, insulin, IRE1α, pancreatic β-cells

Introduction

Pancreatic β-cells are specialized cells that secrete insulin to regulate blood glucose levels. Preproinsulin, a precursor of insulin, is synthesized in the cytosol and co-translationally inserted into the endoplasmic reticulum (ER) (Liu et al., 2014; Rhodes et al., 2005; Sun et al., 2015). Its signal peptide is cleaved and proinsulin is released into the lumen of the ER. Proinsulin forms three disulfide bonds in the ER, and properly folded proinsulin is then transported to the Golgi apparatus. Proinsulin is packaged into the secretory granules and converted into mature insulin. The secretory granules fuse with the plasma membrane upon high glucose stimulation (exocytosis) and insulin is released from β-cells. High glucose stimulates insulin synthesis as well as exocytosis. Insulin biosynthesis is triggered by 2–4 mM glucose, whereas exocytosis is stimulated by more than 5 mM glucose (Sun et al., 2015).

Insulin biosynthesis accounts for 10–50% of total protein synthesis in β-cells (Schuit et al., 1988; Van Lommel et al., 2006). High demand for insulin biosynthesis causes ER stress in β-cells, characterized by the accumulation of unfolded proteins in the ER. During ER stress, the unfolded protein response (UPR) mediates a reduction in the total protein load in the ER and increases the expression of ER chaperones and ER-associated protein degradation (ERAD) machinery to restore ER homeostasis (Korennykh and Walter, 2012; Ron and Walter, 2007; Kimata and Kohno, 2011). IRE1α is a type-I transmembrane protein localized in the ER; it plays an important role as an ER stress sensor for the UPR in mammalian cells (Tirasophon et al., 1998). IRE1α has a sensor domain in the luminal region and a kinase and RNase domain in the cytoplasmic region (Korennykh and Walter, 2012). The luminal region detects
the accumulation of unfolded proteins and facilitates the
oligomerization of IRE1α, leading to self-phosphorylation
in its kinase domain to activate RNase activity (Korenykh
and Walter, 2012). IRE1α RNase activity cleaves a part of
XBP1u mRNA (u; unspliced) to produce XBP1s (s; spliced)
mRNA. After translation, the XBP1s protein enters the
nucleus and acts as a transcription factor to induce the transcrip-
tion of ER chaperones and the components of the
ERAD machinery (Kimata and Kohno, 2011; Ron and
Walter, 2007).

IRE1α is ubiquitously expressed in various mammalian
tissues. It is particularly highly expressed and activated in
the pancreas (Iwawaki et al., 2004; Tirasophon et al., 1998;
Tsuchiya Y., Saito M., Kadokura H., Miyazaki J., Tashiro F.,
Imagawa Y., Iwawaki T., Kohno K. submitted). However,
the precise roles of IRE1α and XBP1s/u in pancreatic β-
cells remain undefined. Lipson et al. reported that the
depletion of IRE1α by siRNA and the inhibition of IRE1α
phosphorylation by the overexpression of its
phosphorylation-deficient mutant (K599A) impaired proin-
sulin protein synthesis (Lipson et al., 2006). They proposed
that proinsulin biosynthesis is dependent on the phosphory-
lization of IRE1α, rather than XBP1s production, as XBP1s
mRNA expression was not increased by “physiological”
levels of ER stress induced by high glucose (Lipson et al.,
2006). On the other hand, Hassler et al. reported that the
overexpression of the K907A IRE1α mutant that lacks
RNase activity, decreases proinsulin protein synthesis (Hassler et al.,
2015), indicating that the RNase domain of
IRE1α also plays an important role in proinsulin biosynthe-
sis. However, the precise role of the RNase domain of
IRE1α in insulin secretion remains unknown.

4μ8C (4-methyl umbelliferone 8-carbaldehyde) inhibits
IRE1α RNase activity (Cross et al., 2012) by covalently
modifying the K907 lysine residue in the RNase domain. It
inhibits XBP1u mRNA splicing, but does not inhibit RNase
L, which supports the idea that 4μ8C is a specific inhibitor
of IRE1α RNase activity (Cross et al., 2012). Here, to
examine whether IRE1α RNase activity has a direct role in
insulin biosynthesis and secretion, we employed 4μ8C and
analyzed insulin secretory pathways and XBP1u mRNA
splicing. We found that 4μ8C inhibited insulin exocytosis
independent of IRE1α RNase activity.

Materials and Methods

Cell culture

MIN6 (Ire1αflo/flo) cells were established in our laboratory from
pancreatic β-cells of IRE1α conditional knockout mouse (Tsuchiya
Y., Saito M., Kadokura H., Miyazaki J., Tashiro F., Imagawa Y.,
Iwawaki T., Kohno K. submitted). Briefly, IRE1α RNase domain-
floxed mice (Iwawaki et al., 2009) were crossed with IT-6 mice,
which expressed the SV 40 large T antigen under the control of an
insulin promoter (Miyazaki et al., 1990, 2010). IRE1α RNase
domain-floxed mice whose pancreatic β-cells were immortalized
by the large T antigen were obtained and pancreatic islets were
isolated to establish MIN6 (Ire1αflox/flox) cell lines. MIN6 (Ire1αflox/flox
IRE1α RNase domain-deficient cells derived from
MIN6 (Ire1αflox/flox) cells transfected with Cre by adenovirus for 4
days. GFP-transfected MIN6 (Ire1αflox/flox) cells were used as con-
trols. MIN6 (Ire1αflox/flox) and MIN6 (Ire1αflox/flox) cells were main-
tained in culture medium (DMEM, 4.5 g/l glucose (Nacalai,
Kyoto, Japan) supplemented with 15% FBS (Corning, lot.
35010117), 55 μM 2-mercaptoethanol, 100 U/ml penicillin, and
100 μg/ml streptomycin) at 37°C in a 5% CO2 incubator.

Animals

All experimental protocols involving animals were approved by the
Committee on Animal Research at Nara Institute of Science and
Technology (NAIST) and were carried out in accordance with
the institutional guidelines of NAIST.

Plasmids and primers

To generate the plasmids pcDNA3.1(+)-mXBP1 unspliced form
and pcDNA3.1(+)-mXBP1 spliced form, the unspliced form of
mouse XBP1 (accession number: NM013842) and the spliced
form of mouse XBP1 (accession number: AF443192) cDNAs
were constructed by RT-PCR using total RNA prepared from the
mouse liver treated with tunicamycin for 6 hr. They were digested
with HindIII and XbaI and ligated with the pcDNA3.1(+) vector
digested with HindIII and XbaI. Two primers were used to deter-
mine XBP1u mRNA splicing by PCR, 5'-GAAGATGTTCTGGGGAGGTGAC-3'
and 5'-GAAGATGTCTGAGGAGGTGAC-3'.

XBP1u mRNA splicing

A total of 4.0×106 MIN6 (Ire1αflox/flox) cells were plated in a 6-well
plate and incubated in culture medium for 4 days. The cells were
treated with DMSO or 4μ8C (TOCRIS, Ellisville, MO, USA) at
the indicated concentration for 5 hr. After 1 hr, they were treated
with 1 mM DTT and either DMSO or 4μ8C. RNA was extracted
run on a 5% acrylamide gel and stained with ethidium bromide.

Images were obtained using ImageQuant LAS 4000 (GE Health-
care, Chicago, IL, USA).

To analyze the time-course of XBP1u mRNA splicing after high
glucose stimulation, 4.0×106 MIN6 (Ire1αflox/flox) cells were plated in a
6-well plate and incubated in culture medium for 4 days. The cells were
washed 3 times with KRBB buffer (115 mM NaCl, 5.9
mM KCl, 1.2 mM MgCl2, 6H2O, 1.2 mM NaH2PO4, 2.5 mM
CaCl2, 25 mM NaHCO3, 10 mM HEPES, and 2 mg/ml BSA) and
incubated in low glucose medium (1.67 mM glucose/KRBB) for 2
hr. The medium was changed to low or high glucose medium (16.7 mM glucose/KRBH) with DMSO or 64 μM 4μ8C and the cells were incubated for indicated times. XBP1 mRNA splicing was examined as described above. The PCR products were run on a 7.5% acrylamide gel and stained with ethidium bromide; images were obtained using Gel Doc XR+ (Bio-Rad, Hercules, CA, USA).

**ELISA**

8.0×10⁶ MIN6 (Ire1α<sup>fl/fl</sup>) cells were plated in a 6-cm dish and incubated in culture medium for 4 days. The cells were washed 3 times with KRBH buffer and incubated in low glucose medium with DMSO or 4μ8C for 2 hr. The medium was changed to low or high glucose medium with DMSO or 4μ8C and the cells were incubated for indicated times. The insulin concentration in the medium was determined using the Mouse Insulin ELISA KIT (AKRIN-011T; Shibayagi, Gunma, Japan). Total insulin in the medium was determined by multiplying the insulin concentration by the total volume of the medium. The cells were washed twice with PBS, lysed with lysis buffer (2% SDS, 100 mM Tris-HCl, pH 6.8), and centrifuged at the maximum speed of a microcentrifuge for 10 min. The cell lysate was prepared from the supernatant and its protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The total protein amount was determined by multiplying the protein concentration of the cell lysate by the total volume of the cell lysate. Insulin secretion was calculated by dividing the total insulin amount in the medium by the total protein amount in the cell lysate. To analyze the effect of 4μ8C on insulin secretion in a short time period, the experiments were performed with following modifications. A total of 4.0×10⁶ MIN6 (Ire1α<sup>fl/fl</sup>) cells were plated in a 6-well plate and incubated in culture medium for 4 days. The cells were washed 3 times with KRBH buffer and incubated in low glucose medium for 2 hr without 4μ8C. The cells were incubated for indicated times with or without 4μ8C and insulin secretion was quantitatively evaluated by ELISA.

**SEAP secretion**

A total of 4.8×10⁶ pancreatic β-cells were transfected with a plasmid carrying secretory alkaline phosphatase (SEAP, kindly provided by Dr. Paul Randazzo, National Cancer Institute, Bethesda, MD, USA) with Lipofectamine 2000 by reverse transfection, following the manufacturer’s protocol (Thermo Fisher Scientific). They were plated on a 6-well plate. Two days after transfection, the cells were washed twice and incubated with 1 ml of culture medium for times indicated in Fig. 1F at 37°C with either DMSO or 4μ8C. Then, 30 μl of culture medium was collected to determine the amount of secreted SEAP. The cells were washed twice with PBS and lysed in 200 μl of lysis buffer (0.1% Triton X-100, 1× dilution buffer). Then, 10 μl of lysate was obtained to determine the amount of SEAP in the cell lysate. The medium and the lysate were processed as per the manufacturer’s protocol (Phospha-Light<sup>TM</sup> SEAP Reporter Gene Assay System; Thermo Fisher Scientific). Chemiluminescence was measured using the TriStar2 LB 942 (Berthold, Bad Wildbad, Germany). SEAP secretion was calculated by dividing the amount of SEAP in the medium by the total amount of SEAP in the medium and the lysate.

**Pulse experiments**

A total of 4.0×10⁶ MIN6 (Ire1α<sup>fl/fl</sup>) cells were plated in a 6-well plate and incubated in culture medium for 4 days. To examine the effect of 4μ8C under low glucose treatment, cells were washed 3 times with KRBH buffer and incubated in low glucose medium with or without 4μ8C for 1 hr. The medium was changed to low glucose medium including 110 μCi/ml [35S]-Cys/Met (Perkin-Elmer, NEG772) with or without 4μ8C and incubated for 30 min. To examine the effect of 4μ8C under high glucose treatment, the cells were washed 3 times with KRBH buffer and incubated in low glucose medium with or without 4μ8C for 2 hr. The medium was changed to high glucose medium including 110 μCi/ml [35S]-Cys/Met with or without 4μ8C and incubated for 30 min. The cells were washed twice with PBS, lysed with lysis buffer (2% SDS, 100 mM Tris-HCl, pH 6.8), and centrifuged at the maximum speed of the microcentrifuge for 10 min. The cell lysate was prepared from the supernatant and its protein concentration was determined using the Pierce BCA Protein Assay Kit. Then, 4× sample buffer was added to the cell lysate, followed by treatment with 10% 2-mercaptoethanol. The samples were boiled at 98°C for 10 min and run on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). Labeled proteins were detected using the BAS-2500 (FujiFilm, Tokyo, Japan).

**Pulse-chase experiments**

The pulse experiments were performed using the methods described above with the following modifications. Cells were pretreated with low glucose medium for 2 hr with or without 4μ8C. The medium was changed to high glucose medium containing 110 μCi/ml [35S]-Cys/Met with or without 4μ8C and the cells were labeled for 15 min. After pulse labeling, the medium was changed to high glucose medium with or without 4μ8C and the cells were chased for indicated times.

**Islets**

Pancreatic islets from 8-week-old C57BL/6J male mice were plated in a 6-well transwell plate with 40–50 islets/well and incubated overnight in the culture medium (DMEM, 4.5 g/l glucose (Nacalai) supplemented with 15% FBS, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin). Transwells were used to wash islets to minimize physical stimulation. When islets were washed, the medium was added to both the transwell and the plate, and the medium was aspirated from the plate. The islets were washed 3 times with KRBH buffer and incubated in low glucose medium for 2 hr. The medium was changed to low or high glucose medium and the islets were incubated for times indicated with 4μ8C and 4μ8C and incubated for indicated times. Islets were washed 3 times with KRBH buffer and incubated in low glucose medium for 2 hr. The medium was changed to low glucose medium with or without 4μ8C for 1 hr. The medium was changed to low glucose medium including 110 μCi/ml [35S]-Cys/Met (Perkin-Elmer, NEG772) with or without 4μ8C and incubated for 30 min. To examine the effect of 4μ8C under high glucose treatment, the medium was changed to high glucose medium including 110 μCi/ml [35S]-Cys/Met with or without 4μ8C and incubated for 30 min. The cells were washed twice with PBS, lysed with lysis buffer (2% SDS, 100 mM Tris-HCl, pH 6.8), and centrifuged at the maximum speed of the microcentrifuge for 10 min. The cell lysate was prepared from the supernatant and its protein concentration was determined using the Pierce BCA Protein Assay Kit. Then, 4× sample buffer was added to the cell lysate, followed by treatment with 10% 2-mercaptoethanol. The samples were boiled at 98°C for 10 min and run on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). Labeled proteins were detected using the BAS-2500 (FujiFilm, Tokyo, Japan).
Fig. 1. 4μ8C inhibits insulin secretion within 2 hr after high glucose treatment. A. MIN6 (Ire1α<sup>fl/fl</sup>) cells were treated with the indicated concentration of 4μ8C for 6 hr and 1 mM DTT for 1 hr with 4μ8C. XBP1<sub>u</sub> mRNA splicing was determined by RT-PCR with XBP1 primers. B. Cells were stimulated by high glucose (16.7 mM) and treated with 64 μM 4μ8C for the indicated times and analyzed as in A. The panel shows representative results of three independent experiments. C. The graph shows the ratio between XBP1<sub>s</sub> and total XBP1<sub>(s+u)</sub>. Data are presented as averages of three independent experiments with S.D. *p < 0.05, **p < 0.01, n.s.; not significant. D. Cells were incubated with low (1.67 mM) or high glucose (16.7 mM) medium for the indicated times with or without 64 μM 4μ8C. The secreted insulin level in the medium was measured by ELISA and normalized by the total protein amount. Data are presented as averages of three independent experiments with S.D. E. The graph shows a comparison of secreted insulin levels between control and 4μ8C-treated cells under high glucose condition. Student’s t-test was used to analyze statistical significance. *p < 0.05, n.s.; not significant. F. SEAP secretion was determined by measuring secreted SEAP in the medium and normalized by total SEAP in the medium and lysate. The mean of three experiments is shown. n.s.; not significant.
bated with DMSO or 4μ8C. The islets were incubated for indicated times and insulin secretion was quantitatively evaluated by ELISA. In these experiments, the medium was changed every time samples were obtained for ELISA.

**Results**

4μ8C inhibits insulin secretion within 2 hr

To examine the effects of 4μ8C in our pancreatic β-cells (i.e., MIN6 (Ire1αβ/)) cells, we first determined the 4μ8C concentration that inhibits XBP1u mRNA splicing in these cells. We treated pancreatic β-cells with 4μ8C at concentrations of 16, 32, and 64 μM for 6 hr and examined if XBP1u mRNA splicing induced by 1 mM DTT was inhibited. As shown in Fig. 1A, 64 μM 4μ8C inhibited 87.1% of XBP1u mRNA splicing without DTT and 72.1% with DTT. Next, we analyzed the time-course of XBP1u mRNA splicing after high (16.7 mM) glucose stimulation with or without 4μ8C. As shown in Fig. 1B, high glucose stimulation increased XBP1u mRNA splicing 2.4-fold at 15 and 30 min compared with that at 0 min. By treatment of 64 μM 4μ8C, XBP1u mRNA splicing was inhibited by 69.5% at 15 min and 79.7% at 30 min compared with that observed in DMSO-treated cells (Fig. 1B and 1C). These results indicate that 64 μM 4μ8C inhibits XBP1u mRNA splicing by more than ~70% in our cells.

Next, we analyzed the effects of 4μ8C on insulin secretion. We treated pancreatic β-cells with either DMSO (control) or 64 μM 4μ8C in low (1.67 mM) or high (16.7 mM) glucose medium and examined insulin secretion. In control cells, insulin secretion was stimulated in high glucose medium, but not in low glucose medium (Fig. 1D, left panel). Surprisingly, 64 μM 4μ8C inhibited 95.8% of insulin secretion under high glucose within 2 hr (Fig. 1D, right panel and Fig. 1E). We also analyzed constitutive secretion by SEAP (secretory alkaline phosphatase) secretion. We transfected pancreatic β-cells with SEAP cDNA and examined SEAP secretion with or without 4μ8C (Fig. 1F). SEAP secretion was not perturbed by 4μ8C. Our results indicate that 4μ8C specifically impaired insulin secretion, but not constitutive secretion.

4μ8C does not inhibit insulin biosynthesis and maturation

To determine the process by which 4μ8C inhibits insulin secretion, we first investigated insulin protein synthesis after treatment with 4μ8C. As shown in Fig. 2A, pancreatic β-cells were labeled with [35S]-Cys/Met and treated with 4μ8C for 30 min, and cell lysates were prepared for autoradiography. Although the amount of synthesized insulin increased by high glucose stimulation, the amount of total synthesized protein also increased (Fig. 2A). We quantified proinsulin, insulin, and total synthesized protein, as indicated in Fig. 2B, and calculated insulin synthesis as described in the Materials and Methods (Fig. 2C). There was no significant difference in insulin synthesis between cells treated with or without 4μ8C. Second, we investigated insulin maturation after treatment with 4μ8C. Pancreatic β-cells were labeled with [35S]-Cys/Met for 15 min in high glucose and chased for 30, 60, and 120 min with or without 4μ8C. The medium and the cell lysates were prepared for autoradiography (Fig. 2D). We quantified proinsulin and insulin amounts in the medium and the cell lysates, as shown in Fig. 2E, and determined insulin maturation as described in Fig. 2F and the Materials and Methods. Our results showed that insulin maturation was not significantly affected by 4μ8C (Fig. 2F). We concluded that 4μ8C does not inhibit insulin protein synthesis and maturation in our conditions.

4μ8C inhibited insulin secretion independent of the RNase domain of IRE1α

We found that 4μ8C inhibited insulin secretion at 5 min after high glucose treatment in our cells and in mouse islets. At this time point, pre-formed insulin granules can fuse with the plasma membrane upon high glucose stimulation, whereas newly synthesized proinsulin requires a longer time period for secretion (Sugawara et al., 2009; Wang et al., 2011; Xie et al., 2015; Zhu et al., 2013). Our data imply that 4μ8C inhibits insulin exocytosis. However, at 5
Fig. 2. 4μ8C does not affect insulin biosynthesis and maturation. A. Cells were incubated with low or high glucose medium containing 110 μCi/ml with or without 4μ8C and incubated for 30 min. Radiolabeled proteins from whole cell lysates are shown. The panel shows representative results of three independent experiments. B. The results shown in A were quantified to determine total protein, proinsulin, or insulin, as indicated. C. The graph shows synthesized insulin normalized by synthesized total protein. Insulin synthesis in low glucose was set as 1. The graph shows the average of three independent experiments with S.D. n.s.; not significant. D. Cells were incubated with high glucose medium including 110 μCi/ml [35S]-Cys/Met with or without 4μ8C for 15 min. After labeling, the medium was changed to high glucose medium with or without 4μ8C and the cells were chased for the indicated times. Radiolabeled proteins from the medium and the whole cell lysates are shown. The panel shows representative results of three independent experiments. E. The results shown in D were quantified as indicated to determine proinsulin and insulin. F. The graph shows a comparison of insulin maturation between control and 4μ8C-treated cells. The equation to calculate insulin maturation is indicated above. The graph shows the average of three independent experiments with S.D. n.s.; not significant.

min of treatment with 4μ8C, there was no apparent inhibition of XBP1u mRNA splicing (Fig. 1B). This result implies that 4μ8C inhibits insulin exocytosis independent of XBP1u mRNA splicing. We examined whether 4μ8C inhibits insulin secretion independent of the RNase domain of IRE1α using pancreatic β-cells that lack the RNase domain of IRE1α (MIN6 (Ire1αΔR/ΔR) cells), which were established by our group (see Materials and Methods). Control GFP-transfected MIN6 (Ire1αfl/fl) cells showed insulin secretion in high glucose medium, similar to non-transfected cells (Fig. 4A and 4B). In contrast, Cre-transfected MIN6 (Ire1αΔR/ΔR) cells showed more than 50% inhibition of insulin secretion at all time points after high glucose stimulation compared with secretion by GFP-transfected MIN6 (Ire1αfl/fl) cells (Fig. 4C). Our results support the idea that IRE1α RNase activity is required for insulin secretion (Hassler et al., 2015). Next, we treated MIN6 (Ire1αΔR/ΔR) cells with 4μ8C (Fig. 4D). We observed strong inhibition of insulin secretion, even in MIN6 (Ire1αΔR/ΔR) cells with 4μ8C (Fig. 4D). These results indicate that 4μ8C blocks insulin secretion, beyond the effect of the depletion of the RNase
Fig. 3. 4μ8C inhibits insulin secretion within 5 min after high glucose treatment. A. MIN6 (Ire1α<sup>fl/fl</sup>) cells were incubated with low or high glucose medium for indicated times. The insulin secretion was measured by ELISA as in Fig. 1D. Data are presented as the average of four independent experiments with S.D. B. MIN6 (Ire1α<sup>fl/fl</sup>) cells were incubated with low or high glucose medium for the indicated times with 4μ8C. Data are presented as the average of four independent experiments with S.D. C. The graph shows a comparison of insulin secretion in control and 4μ8C-treated cells under high glucose condition. Student’s t-test was used to analyze statistical significance. *p < 0.05, n.s.; not significant. D. Mice pancreatic islets were incubated with low or high glucose medium for indicated times with 4μ8C. The insulin secretion was measured as in A. Ex.1, 2, and 3 show the results of three independent experiments. E. The graph shows a comparison of secreted insulin levels under high glucose conditions between control and 4μ8C-treated islets. The graph shows the average of six independent experiments with S.D. Student’s t-test was used to analyze the statistical significance between control and 4μ8C treatment. *p < 0.05, **p < 0.01, n.s.; not significant.
Fig. 4. 4μ8C inhibits insulin secretion in cells deficient in the RNase domain of IRE1α. A. MIN6 (Ire1αfl/fl) cells were incubated with low or high glucose medium for the indicated times. The insulin secretion was measured by ELISA as in Fig. 1D. Data are presented as the average of three independent experiments with S.D. B. The graph shows insulin secretion in MIN6 (Ire1αfl/fl) cells transfected with GFP by adenovirus as a control. The cells were treated as in A. C. MIN6 (Ire1αfl/fl) cells were transfected with Cre by adenovirus to make the cells deficient in the RNase domain of IRE1α (MIN6 (Ire1αΔR/ΔR)). The graph shows insulin secretion in MIN6 (Ire1αΔR/ΔR) cells. The cells were treated as in A. D. MIN6 (Ire1αΔR/ΔR) cells were incubated with low or high glucose medium for the indicated times with 4μ8C. The insulin secretion was measured as in A. Data are presented as the average of three independent experiments with S.D. E. The graph shows a comparison of secreted insulin levels in 4μ8C-treated or DMSO-treated MIN6 (Ire1αΔR/ΔR) cells under high glucose conditions. The graph shows the average of three independent experiments with S.D. Student’s t-test was used to analyze the statistical significance. *p < 0.05, **p < 0.01, n.s.; not significant. F. Non-transfected and GFP-transfected MIN6 (Ire1αfl/fl) cells and MIN6 (Ire1αΔR/ΔR) cells were treated with 4μ8C for 6 hr and 1 mM DTT for 1 hr with 4μ8C. XBP1u mRNA splicing was determined by RT-PCR with XBP1 primers. The panel shows XBP1u and XBP1s mRNAs. Note that MIN6 (Ire1αΔR/ΔR) cells did not show any residual XBP1s mRNA without 4μ8C.
domain of IRE1α. To confirm that there is no residual XBP1u mRNA splicing activity in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells, we treated MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells with 1 mM DTT and examined XBP1u mRNA splicing (Fig. 4F). MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells did not show any XBP1u mRNA splicing, even under DTT treatment, supporting the idea that MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells do not have any residual XBP1u mRNA splicing activity. 4μ8C did not show further inhibition of XBP1u mRNA splicing in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells (Fig. 4F). These results indicate that 4μ8C inhibits insulin secretion independent of RNase activity of IRE1α.

**Discussion**

In this study, we found a strong inhibitory effect of 4μ8C on insulin secretion (Fig. 1D and 1E), independent of insulin protein synthesis and maturation (Fig. 2). We further found that 4μ8C inhibited insulin secretion within 5 min of high glucose stimulation (Fig. 3), suggesting that 4μ8C inhibits insulin exocytosis. However, IRE1α is localized in the ER (Tirasophon et al., 1998), and 4μ8C took at least 15 min to inhibit XBP1u mRNA splicing induced by high glucose stimulation (Fig. 1B and 1C). Using MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells, we further found that the inhibition of insulin secretion by 4μ8C is not dependent on the RNase domain of IRE1α (Fig. 4C, 4D, and 4E). Our work revealed that 4μ8C has off-target effects, and might inhibit other molecule(s) in addition to the RNase domain of IRE1α in pancreatic β-cells to inhibit insulin exocytosis.

4μ8C is a widely-used inhibitor of IRE1α (Cross et al., 2012; Eletto et al., 2016; Harding et al., 2012; Sharma et al., 2015). However, its off-target effects have not been reported. Our work indicates that the existence of other targets of 4μ8C should be considered when using “crude” experimental systems, such as living cells or cell lysates. We tested the lower concentration of 4μ8C for XBP1u mRNA splicing and insulin secretion. We found that 32μM of 4μ8C inhibited 43% of XBP1u mRNA splicing and 16μM inhibited 26% compared with that in DMSO-treated cells in the presence of DTT (Fig. 1A). Whereas we observed 32μM 4μ8C still inhibited 84% of insulin secretion, and 16μM inhibited 71% (data not shown). That is, even if we used 16μM of 4μ8C that largely did not inhibit XBP1u mRNA splicing, 71% of insulin secretion was impaired. These results imply that the off-target effects are stronger than the effect of inhibition of RNase activity of IRE1α. We also tested STF-083010, another inhibitor of IRE1α RNase activity (Papandreou et al., 2011). STF-083010 also blocked insulin secretion within 5 min after high glucose stimulation (data not shown). STF-083010 could inhibit both the RNase activity of IRE1α and other target(s) in pancreatic β-cells, similar to 4μ8C, because these reagents have similar structures.

We did not detect any inhibitory effects of 4μ8C on insulin biosynthesis and the maturation process in our conditions (Fig. 2), whereas the overexpression of the K907A mutant of IRE1α has been reported to inhibit insulin biosynthesis (Hassler et al., 2015). In the latter case, the K907A mutant was overexpressed for 72 hr. This condition might be sufficient to change the gene expression profiles downstream of XBP1s expression, possibly leading to the inhibition of insulin biosynthesis. In contrast, we tested insulin biosynthesis and maturation within 2 hr of high glucose treatment with 4μ8C. We pretreated cells with 4μ8C in low glucose medium for 1 or 2 hr before high glucose treatment; the maximum treatment times with 4μ8C were 2 hr 30 min in the pulse experiment (Fig. 2A–2C) and 4 hr 15 min in the pulse-chase experiment (Fig. 2D and 2E). These durations are much shorter than 72 hr (in the K907A overexpression experiment). We speculate that 4μ8C enabled us to determine the effects that were independent of the changes in gene expression. The knockdown, knockout or overexpression method usually requires more than 12 hr to see its effects, therefore it is often difficult to distinguish the short-term effects (such as the structural changes of RNase domain) from the long-term effects (such as the change of gene expression profiles). Our experiments showed that 15 min is sufficient to inhibit IRE1α RNase activity with 4μ8C (Fig. 1B and 1C). Long-term treatment (e.g., 72 hr) with 4μ8C might inhibit insulin biosynthesis or maturation, similar to K907A overexpression.

The inhibitors are useful tools owing to their ability to inhibit IRE1α RNase activity in a short time period, without requiring genetic manipulations. They enable us to analyze the direct function of the IRE1α RNase domain, without considering changes in gene transcription and translation. The identification of more specific inhibitors of the IRE1α RNase domain that lack off-target effects is necessary for more precise analyses of IRE1α function.

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