The Antipsychotic Olanzapine Induces Apoptosis in Insulin-secreting Pancreatic β Cells by Blocking PERK-mediated Translational Attenuation

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ABSTRACT. Patients with schizophrenia receive medication to alleviate various symptoms, but some efficacious second generation antipsychotics, particularly olanzapine, can cause obesity, dyslipidemia, and diabetes mellitus. It has been generally considered that olanzapine contributes to the development of diabetes by inducing obesity and subsequent insulin resistance. In this study, we examined the effect of olanzapine and risperidone, another second generation antipsychotic, on a hamster pancreatic β cell line, and found that both evoked mild endoplasmic reticulum (ER) stress, as evidenced by mild activation of the ER stress sensor molecule PERK. Surprisingly, only olanzapine induced marked apoptosis. Phosphorylation of the α subunit of eukaryotic initiation factor 2, an event immediately downstream of PERK activation, was not observed in cells treated with olanzapine, protein synthesis continued despite PERK activation, and ER stress was thereby sustained. Secretion of insulin was markedly inhibited, and both proinsulin and insulin accumulated inside olanzapine-treated cells. Inhibition of protein synthesis and knockdown of insulin mRNA, which result in less unfolded protein burden, both attenuated subsequent olanzapine-induced apoptosis. Given clinical observations that some patients taking olanzapine exhibit hyperlipidemia and hyperglycemia without gaining weight, our observations suggest that damage to pancreatic β cells may contribute to the undesirable metabolic consequences of olanzapine treatment in some cases.

Key words: apoptosis, diabetes, endoplasmic reticulum stress, schizophrenia, unfolded protein response

Introduction

Patients with schizophrenia, a common mental disorder, receive antipsychotic medication to alleviate various symptoms. Second generation antipsychotics such as olanzapine and risperidone were developed to overcome the severe movement disorders and extrapyramidal side effects caused by first-generation antipsychotics such as haloperidol. However, it has been recognized that second generation antipsychotics, particularly olanzapine, may cause obesity, dyslipidemia and diabetes mellitus in some patients (American Diabetes Association et al., 2004). Although the mechanisms underlying these metabolic side effects are poorly understood, insulin appears to play a key role as it is a hormone involved in the regulation of body weight, lipid metabolism and blood glucose level (Olefsky, 1997; Woods et al., 1997).

Diabetes develops when insulin production by the beta (β) cells of pancreatic islets fails to keep up with the demand imposed by hepatic or peripheral insulin target tissues, such as skeletal muscle, heart muscle and adipocytes (Olefsky, 1997). It was reported that neither olanzapine nor risperidone altered pancreatic β cell function in vivo in hyperglycemic clamp experiments conducted in healthy volunteers (Sowell et al., 2002). A prospective study conducted with schizophrenia patients and a control group
also showed no change in β cell function (Ebenbichler et al., 2003). Instead, schizophrenia patients treated with olanzapine showed a significant increase in the index representing peripheral insulin resistance (Ebenbichler et al., 2003). Based on these and other results, it is generally considered that olanzapine contributes to the development of diabetes by inducing obesity and subsequent insulin resistance (American Diabetes Association et al., 2004).

However, it has been reported that some patients treated with olanzapine or another diabetes-inducing antipsychotic, clozapine, developed hyperglycemia without gaining weight and that some hyperglycemic patients required insulin or oral hypoglycemic treatment even after the discontinuation of treatment with olanzapine or clozapine (Mir and Taylor, 2001). One of us (T.N.) has observed clinically that some Japanese patients taking olanzapine also exhibit hyperlipidemia and hyperglycemia without gaining weight (Nagamine, 2006, 2007, 2008). In these patients, the metabolic side effects of olanzapine do not appear to result primarily from olanzapine-induced obesity and insulin resistance.

Here, we have examined the effects of olanzapine and risperidone on the viability of hamster pancreatic β cells, as a simple system to learn if damage to pancreatic β cells may also contribute to the metabolic effects of olanzapine. We chose HIT-T15 cells which are known to synthesise and secrete insulin (Santerre et al., 1981), because the hamster genome apparently encodes only one insulin gene, facilitating knockdown insulin mRNA in HIT-T15 cells compared with β cells from other mammalian species that have two insulin genes.

Pancreatic β cells synthesize large amounts of proinsulin molecules, which represent a major burden on the protein folding machinery in the endoplasmic reticulum (ER) (Liu et al., 2010). Accumulation of unfolded or misfolded proteins in the ER is counteracted by cellular homeostatic responses collectively termed the unfolded protein response (UPR) (Mori, 2000). Three signaling pathways operate in the mammalian UPR, namely IRE1, PERK and ATF6 (Walter and Ron, 2011). IRE1 is a type I transmembrane protein with protein kinase and endoribonuclease activities whose activation in response to ER stress initiates unconventional splicing of XBP1 mRNA to produce the active form of its downstream transcription factor, XBP1. This pXBP1(S) induces transcription of a variety of genes to maintain the homeostasis of the ER. PERK is a type I transmembrane protein with protein kinase activity whose activation in response to ER stress phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α). The resulting general translational attenuation not only decreases the burden on the ER but also paradoxically induces translation of the downstream transcription factor ATF4 to induce its target genes, including the proapoptotic transcription factor CHOP. ATF6 is a type II transmembrane protein with a transcription factor domain facing the cytoplasm whose activation by ER stress-induced proteolysis results in transcriptional induction of ER-localized molecular chaperones and folding enzymes. Interestingly, it was reported that approximately half of PERK knockout mice are born alive but eventually develop diabetes mellitus due to apoptosis of pancreatic β cells (Harding et al., 2001). Various experimental data to date have demonstrated that translational control of proinsulin through phosphorylation of eIF2α is required to coordinate proinsulin synthesis with proinsulin folding to maintain the homeostasis of β cells, and that XBP1 is also required for insulin maturation and secretion (Wang and Kaufman, 2012). Thus, we paid particular attention to which pathway is involved in olanzapine-induced apoptosis of HIT-T15 cells.

**Materials and Methods**

**Cell culture, transfection and materials**

Simian virus 40-transformed hamster pancreatic β cell line HIT-T15 (ATCC, CRL-1777) (Santerre et al., 1981) and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, glucose at 4.5 g/liter) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate) in a 5% CO2, 95% air incubator at 37°C. HepG2 cells were grown in the above DMEM medium containing non-essential amino acids using poly-L-lysine-coated dishes (SIGMA-ALDRICH). DMEM/F12 medium was obtained from SIGMA-ALDRICH. The mouse pancreatic β cell line MIN6 (Miyazaki et al., 1990) was grown in the above DMEM medium containing β-mercaptoethanol (5 μ/liter). Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfection efficiency of HIT-T15 cells was estimated to be 30–40% by determination using green fluorescent protein. In the case of reporter assay, transfection of HIT-T15 cells was done using the standard calcium phosphate method (Sambrook et al., 1989). Firefly and Renilla luciferase activities were determined as described previously (Yoshida et al., 1998). Olanzapine and risperidone were purchased from Toronto Research Chemicals.

**Construction of plasmids**

Recombinant DNA techniques were performed according to standard procedures (Sambrook et al., 1989) and the integrity of all constructed plasmids was confirmed by extensive sequencing analyses. Insulin cDNA was amplified from total RNA isolated from HIT-T15 cells using a pair of primers, 5’-CCCAAGCTTGGAC-CAGCTATAATCAGAAGC-3’ and 5’-CCGGAATTCGGTTT-TATTCGCTGCAGGAA-3’, and inserted between the HindIII and EcoRI sites of pcDNA3.1 (Invitrogen) to create pcDNA-insulin. For knockdown experiments, double stranded oligonucleotides corresponding to 5’-GATCCCATGTCCTGACACAGCAT-
AGCGAGCAGATGCTGGTTTTTA-3’ and 5’-GATCCCCCGACATCTGCTGGCTCTATTTCAAGAGATAG-AGCGAGCAGATGCTGGTTTTTA-3’ were inserted between the BglII and HindIII sites of the shRNA expression vector pSUPER (Oligo engine) to create KD-1 and KD-2, respectively.

**Apoptosis assay**

Terminal deoxytransferase-mediated dUTP-X nick end labeling (TUNEL) assay was performed using an *in situ* Cell Death Detection Kit, Fluorescein (Roche). Cells cultured on slide glasses in 24-well plates were appropriately treated, then washed, fixed, permeabilized, and labeled according to the manufacturer’s instructions. DNA fragmentation assay was carried out as previously described (Yoshida et al., 2006).

**Immunological techniques**

Immunoblotting analysis was carried out according to the standard procedure (Sambrook et al., 1989) as described previously (Okada et al., 2002) using Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Chemiluminescence was detected using an LAS-3000mini LuminolImage analyzer (Fuji Film). Anti-insulin, anti-eIF2α, anti-ATF4, anti-CHOP anti-ribophorin antibodies were purchased from Santa Cruz: anti-phosphorylated eIF2α and anti-phosphorylated PERK antibodies from Cell Signaling; anti-PERK antibody from Rockland; anti-GADD34 antibody from Proteintech; anti-GAPDH antibody from Trevigen.

**Metabolic labeling**

HIT-T15 cells untreated or treated with chemicals were labeled for 10 min with 5 μl (2.05 MBq) of Easy Tag EXPRE5/S/5’S protein labeling mix (PerkinElmer) without prestarvation and without removing reagents such as olanzapine and risperidone, washed with PBS, dissolved in 70 μl of Laemml’s sample buffer, boiled for 7 min, and subjected to SDS-PAGE. Radiolabeled protein bands were visualized and quantified using an FLA-3000 fluorescent image analyzer (Fuji Film).

**Northern blot hybridization and RT-PCR**

Total RNA was extracted from HIT-T15 cells by the acid guanidinium/phenol/chloroform method using Isogen (Nippon Gene). Northern blot hybridization was carried out according to the standard procedure (Sambrook et al., 1989). RT-PCR to detect ER stress-induced splicing of hamster and human XBP1 mRNA was carried out as described previously (Nadanaka et al., 2006; Yoshida et al., 2001).

**ELISA assay for insulin**

After appropriate treatment of HIT-T15 cells, the medium was removed and clarified by centrifugation, while cells were washed once with PBS, lysed with 200 μl of 1% Triton X-100 buffer containing a protease inhibitor cocktail (Nacalai Tesque) and 10 μM MG132, and then clarified by centrifugation. Insulin content in the medium was determined using an ELISA insulin kit (Morinaga Seikagaku) according to the manufacturer’s instructions. Protein concentration in cell lysates was measured using the BCA protein assay reagent kit (PIERCE).

**Results**

**Olanzapine induces apoptosis in HIT-T15 cells**

We first examined the effects of olanzapine and risperidone, two second-generation antipsychotics, on the viability of HIT-T15 cells. Both TUNEL (Fig. 1A) and DNA fragmentation (Fig. 1B) assays revealed that apoptosis was markedly induced in HIT-T15 cells from 7 h after treatment with 100 μM olanzapine but not with 100 μM risperidone; apoptosis was induced by olanzapine at the concentration of more than 50 μM (see Discussion for the significance of death observed at these high concentrations). This detrimental effect appeared to be specific to HIT-T15 cells as apoptosis was not induced in HEK293 or HepG2 cells after treatment with the same concentration of olanzapine (Fig. 1C and 1D). Thus, olanzapine damages HIT-T15 cells, and investigation of the underlying mechanism might therefore provide new insights into how some patients taking olanzapine develop metabolic side effects consistent with defective insulin secretion.

**Olanzapine blocks phosphorylation of eIF2α by PERK**

ER stress is a known inducer of β cell death (Eizirik et al., 2008). Consistent with this, tunicamycin, an agent that evokes ER stress potently by inhibiting protein N-glycosylation (Kaufman, 1999), also induced apoptosis in HIT-T15 cells (Fig. 1B, lanes 8 and 9) and activated an UPR reporter gene, namely the luciferase gene under the control of BiP promoter (Fig. 1E). Olanzapine and risperidone similarly enhanced luciferase expression from the BiP promoter-luciferase reporter gene transfected into HIT-T15 cells, which is indicative of activation of the ATF6 pathway (Yamamoto et al., 2007; Yoshida et al., 1998), but the effects were weak compared with that of tunicamycin (Fig. 1E), and cleavage of ATF6 was not detected in these cells in contrast to tunicamycin-treated cells (our unpublished observation). These results suggest that both olanzapine and risperidone causes mild ER stress in HIT-T15 cells.

We then monitored the activation status of the PERK pathway in HIT-T15 cells treated with olanzapine or risperidone by immunoblotting using anti-phosphorylated PERK (Fig. 2A) and anti-PERK (Fig. 2B) antibodies. As expected, PERK was highly autophosphorylated in HIT-T15 cells treated with tunicamycin (Fig. 2A and 2B, lanes 11–14), resulting in strong phosphorylation of eIF2α (Fig. 2B, lanes 11–14), and 1C and 1D). Thus, olanzapine damages HIT-T15 cells, and investigation of the underlying mechanism might therefore provide new insights into how some patients taking olanzapine develop metabolic side effects consistent with defective insulin secretion.
Fig. 1. Effects of olanzapine and risperidone on viability of various cells. (A) HIT-T15 cells were untreated (CON) or treated with 100 μM olanzapine (OLA) or risperidone (RIS) for 7 h, and then fixed. Nuclei were stained with DAPI (blue), whereas fragmented chromosomal DNA was labeled with FITC (green) using the TUNEL method. The number of TUNEL-positive, apoptotic cells was measured in 500–600 cells, and their percentages are presented as the average±standard error of four independent experiments at bottom. ** P<0.01. (B) HIT-T15 cells were treated with 100 μM olanzapine (OLA), 100 μM risperidone (RIS) or 2 μg/ml tunicamycin (TM) for the indicated periods. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis. DNA was stained with Gel Red. The migration positions of DNA size markers are shown at left. (C) HEK293 cells were treated and analyzed as in (A). (D) HepG2 cells were treated and analyzed as in (A). (E) Twenty-four hours after transfection with the reporter plasmid pGL3-GRP78P-luc and the reference plasmid pRLUC, HIT-T15 cells were treated with 100 μM olanzapine (OLA), 100 μM risperidone (RIS) or 2 μg/ml tunicamycin (TM) for 24 h. Firefly luciferase activity relative to Renilla luciferase activity in transfected cells was determined and presented as the average±standard error of two independent experiments.
Fig. 2. Effects of olanzapine and risperidone on the PERK pathway. (A) HIT-T15 cells were treated with 100 μM olanzapine (OLA), 100 μM risperidone (RIS) or 2 μg/ml tunicamycin (TM) for the indicated periods. Cell lysates were prepared and analyzed by immunoblotting using anti-phosphorylated PERK and anti-ribophorin antibody. (B) Cell lysates prepared as in (A) were analyzed by immunoblotting using anti-PERK, anti-eIF2α, anti-phosphorylated eIF2α and anti-GAPDH antibodies. (C) Cell lysates prepared as in (A) were analyzed by immunoblotting using anti-ATF4, anti-CHOP and anti-GAPDH antibodies. (D) HEK293 cells were treated with 100 μM olanzapine (OLA) or 100 μM risperidone (RIS) for the indicated periods. Total RNA was extracted and subjected to RT-PCR analysis to detect splicing of XBP1 mRNA. PCR products from pcDNA-XBP1(unspliced) and pcDNA-XBP1(spliced) were electrophoresed for comparison. (E) Lysates were prepared from HEK293 cells treated as in (D) and analyzed by immunoblotting using anti-phosphorylated eIF2α and anti-GAPDH antibodies.
11–14) and strong induction of its downstream effectors, ATF4 and CHOP (Fig. 2C, lanes 13 and 14). Low levels of PERK autophosphorylation were also noted in risperidone-treated HIT-T15 cells (Fig. 2A and 2B, lanes 6–10), consistent with the results shown in Fig. 1E, resulting in mild phosphorylation of eIF2α (Fig. 2B, lanes 6–10) and mild induction of ATF4 and CHOP (Fig. 2C, lanes 7–12). In marked contrast, although PERK was mildly autophosphorylated in HIT-T15 cells treated with olanzapine (Fig. 2A and 2B, lanes 1–5), similarly to the case of treatment with risperidone, eIF2α was barely phosphorylated in HIT-T15 cells treated with olanzapine (Fig. 2B, lanes 1–5), and ATF4 and CHOP were barely induced (Fig. 2C, lanes 1–6). These differential effects of olanzapine and risperidone were also observed in HEK293 cells. Although both olanzapine and risperidone similarly albeit slightly induced splicing of XBP1 mRNA, which is indicative of activation of the IRE1 pathway (Fig. 2D), phosphorylation of eIF2α was observed only in cells treated with risperidone (Fig. 2E). Thus, olanzapine-induced mild activation of PERK somehow did not result in phosphorylation of eIF2α. This raised several possibilities: eIF2α phosphorylated by PERK may be rapidly dephosphorylated in cells treated with olanzapine; eIF2α may no longer be phosphorylated in cells treated with olanzapine; and PERK can phosphorylate itself but may be unable to phosphorylate its substrate eIF2α in cells treated with olanzapine. To determine which of these, we then conducted serial experiments.

We determined the level of GADD34, which recruits protein phosphatase 1c to eIF2α (Novoa et al., 2001), by immunoblotting and found that, in contrast to the case of treatment with tunicamycin, treatment with neither olanzapine nor risperidone induced GADD34 (Fig. 3A), indicating that suppression of eIF2α phosphorylation in cells treated with olanzapine was unlikely due to the hyperactivation of eIF2α dephosphorylation. To determine whether eIF2α can be phosphorylated in cells treated with olanzapine, we exposed cells to amino acid starvation, which activates an alternative eIF2α kinase, GCN2 (Harding et al., 2000a). Leucine starvation of HIT-T15 cell culture did not enhance splicing of XBP1 mRNA and thereby did not enhance ER stress (Fig. 3B), and leucine-starvation-induced phosphorylation of eIF2α was not mitigated by the presence of olanzapine (Fig. 3C), indicating that olanzapine treatment did not interfere with eIF2α phosphorylation by other kinases.

We then determined whether olanzapine is able to inhibit the phosphorylation of eIF2α by PERK in vitro. To this end, a recombinant eIF2α (1–182 fragment) was incubated with various concentrations of a recombinant cytosolic portion of mouse PERK fused to glutathione S-transferase (GST-PERK) in the presence of 1 mM ATP as well as in the absence or presence of 100 μM olanzapine or risperidone. As shown in Fig. 3D, neither olanzapine nor risperidone showed a significant effect on phosphorylation of eIF2α by PERK in vitro. Because GST-PERK represents fully activated PERK, such as PERK activated by tunicamycin treatment (see Fig. 2A) via constitutive dimerization through the GST portion, we next examined the effect of olanzapine treatment on tunicamycin-induced phosphorylation of eIF2α. Pretreatment of HIT-T15 cells with olanzapine for 30 min was able to significantly suppress phosphorylation of eIF2α induced by the subsequent addition of tunicamycin to HIT-T15 cells at the 30 min time point (Fig. 3E, compare lane 2 with lane 6) but this suppression was lost at the 60 and 120 min time points (Fig. 3E, compare lanes 3 and 4 with lanes 7 and 8). These results suggest that olanzapine may block activation-dependent recruitment of eIF2α by PERK in vivo, but not in vitro (see Discussion).

**Continuous protein synthesis in the presence of olanzapine-induced mild ER stress causes apoptosis**

Both olanzapine and risperidone mildly activated PERK, whereas phosphorylation of eIF2α was observed in cells treated with risperidone but not olanzapine (Fig. 2). The inability to attenuate translation in response to mild ER stress that occurred in HIT-T15 cells treated with olanzapine was confirmed by pulse labeling with 35S-methionine and cysteine. As shown in Fig. 4A, global protein synthesis was attenuated in HIT-T15 cells treated with risperidone to the level of ~30% of control (lanes 5, 8, and 11); however, such attenuation was barely observed in HIT-T15 cells treated with olanzapine (lanes 4, 7, and 10). RT-PCR analysis revealed that the XBP1 mRNA splicing initially induced by risperidone treatment eventually subsided (Fig. 4B, lanes 6–9), whereas sustained XBP1 mRNA splicing was observed in olanzapine-treated cells (Fig. 4B, lanes 2–5). These observations suggested that continuous protein synthesis in the presence of mild ER stress induced by olanzapine may cause apoptosis. To probe this notion, HIT-T15 cells were simultaneously treated with olanzapine and cycloheximide. As shown in Fig. 4C, inhibition of new protein synthesis mitigated splicing of XBP1 mRNA and thus reduced ER stress. Consistent with this notion, we further found that brief pretreatment of HIT-T15 cells with cycloheximide followed by wash out was able to significantly suppress subsequent olanzapine-induced cell death, as determined by DNA fragmentation (Fig. 4D) and TUNEL (Fig. 4E) assays.

**Continuous synthesis and accumulation of insulin cause apoptosis**

As insulin is a major protein synthesized and secreted by pancreatic β cells, we examined the effect of olanzapine treatment on insulin. Insulin secretion was blocked almost completely by olanzapine treatment but was little affected by risperidone (Fig. 5A), although neither olanzapine nor risperidone affected insulin mRNA level (Fig. 5B). Immunoblotting revealed that both proinsulin and insulin accumu-
Fig. 3. Effect of olanzapine on phosphorylation of eIF2α by PERK and GCN2. (A) HIT-T15 cells were treated with 100 μM olanzapine (OLA), 100 μM risperidone (RIS) or 2 μg/ml tunicamycin (TM) for the indicated periods. Cell lysates were prepared and analyzed by immunoblotting using anti-GADD34 antibody. (B) HIT-T15 cells were cultured in the absence of leucine [DMEM/F12(-leu)] for the indicated periods. The status of XBP1 mRNA splicing was determined as in Fig. 2D. (C) HIT-T15 cells were cultured in the presence [DMEM/F12(+leu)] or absence [DMEM/F12(–leu)] of leucine with (+) or without (−) simultaneous treatment with 100 μM olanzapine for the indicated periods. Cell lysates were prepared and analyzed by immunoblotting using anti-phosphorylated eIF2α and anti-GAPDH antibodies. (D) Five micrograms of bacterially expressed and purified eIF2α (1–182 fragment) were incubated for 30 min with various concentrations of bacterially expressed and purified cytosolic portion of mouse PERK fused to GST in the presence of 1 mM ATP as well as in the absence or presence of 100 μM olanzapine (OLA) or 100 μM risperidone (RIS), and then subjected to phos-tag gel electrophoresis together with a molecular weight marker (25 kDa). The percentages of phosphorylated eIF2α in total eIF2α were quantified and presented at bottom. (E) HIT-T15 cells were pretreated with or without 100 μM olanzapine (OLA) for 30 min, washed with PBS and then treated with 2 μg/ml tunicamycin (TM) for the indicated periods. Cell lysates were prepared and analyzed by immunoblotting using anti-eIF2α and anti-phosphorylated eIF2α antibodies.
Fig. 4. Effect of cycloheximide on olanzapine-induced apoptosis. (A) HIT-T15 cells were untreated (UNT), mock-treated (CON) or treated with 100 μM olanzapine (OLA) or 100 μM risperidone (RIS) for the indicated periods, and then pulse labeled for 10 min with 35S-methionine and cysteine without medium change. Cell lysates were prepared, subjected to SDS-PAGE and autoradiographed. Total radioactivity in each sample was determined, normalized with the value of UNT and presented as the average±standard error of five independent experiments on right. * P<0.05. (B) HIT-T15 cells were treated with 100 μM olanzapine (OLA), 100 μM risperidone (RIS) or 2 μg/ml tunicamycin (TM) for the indicated periods. The status of XBP1 mRNA splicing was determined as in Fig. 2D. (C) HIT-T15 cells were treated with 100 μM olanzapine (OLA) together with (+) or without (–) 20 μg/ml cycloheximide. The status of XBP1 mRNA splicing was determined as in Fig. 2D. (D) HIT-T15 cells were pretreated with (+) or without (–) 20 μg/ml cycloheximide for 15 min, washed out and then incubated in the absence (–) or presence (+) of 100 μM olanzapine (OLA) or 2 μg/ml tunicamycin (TM) for 24 h. DNA fragmentation assay was carried out as in Fig. 1B. (E) HIT-T15 cells treated as in (D) were subjected to TUNEL assay. The number of apoptotic cells was determined as in Fig. 1A, and their percentages are presented as the average±standard error of three independent experiments on right. * P<0.05.
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To further interrogate the role of unfolded protein load in the death of olanzapine-treated cells, we tried to knockdown insulin mRNA by transfecting shRNA-producing plasmids. We constructed four plasmids and found that two significantly decreased insulin mRNA level (Fig. 6A). RT-PCR analysis revealed that olanzapine-induced enhancement of XBP1 mRNA splicing was suppressed in cells transfected with the knockdown constructs (Fig. 6B). Most importantly, both DNA fragmentation (Fig. 6C) and TUNEL (Fig. 6D) assays showed that olanzapine-induced apoptosis was significantly decreased in cells expressing shRNAs specific to insulin mRNA. We concluded that the sustained synthesis and accumulation of proinsulin and insulin in cells treated with olanzapine causes subsequent apoptosis of HIT-T15 cells, at least in part.

**Discussion**

Diabetes is found when insulin secretion from pancreatic islets fails to meet the demands of the insulin responsive target tissues; thus both beta cell dysfunction and insulin resistance contribute to its development. A number of recent studies have documented the effects of olanzapine and related drugs on insulin secretion from isolated pancreatic islets or insulin-secreting cells (Johnson et al., 2005; Melkersson, 2004; Melkersson and Jansson, 2005) as well as the effect of insulin on peripheral target tissues (Engl et al., 2005; Heiser et al., 2006; Robinson et al., 2006). For example, it was reported that olanzapine but not risperidone increased basal insulin release when applied at 1 μM to isolated pancreatic islets and insulin-secreting INS-1 cells. It was also reported that olanzapine increased glucose transporter 5 mRNA level in human leukemic cell line U937 at 200 μg/ml (640 μM) (Heiser et al., 2006). These findings may explain the increased appetite and weight gain observed in olanzapine-treated patients (Heiser et al., 2006; Melkersson, 2004; Melkersson and Jansson, 2005). It was previously reported that glycogen synthesis and insulin signaling in L6 rat skeletal muscle cells were impaired after treatment with 100 μM olanzapine, which may explain the induction of insulin resistance observed in olanzapine-treated patients (Engl et al., 2005). These results support the general notion that olanzapine may cause diabetes by inducing obesity and subsequent insulin resistance (American Diabetes Association et al., 2004).

Here, we describe for the first time that treatment with 100 μM olanzapine, but not with 100 μM risperidone, directly damages insulin-secreting HIT-T15 cells (Fig. 1A and 1B). Importantly, 100 μM olanzapine-induced apoptosis was also observed in MIN6, a mouse pancreatic β cell
Fig. 6. Effect of insulin knockdown on olanzapine-induced apoptosis. (A) HIT-T15 cells were transfected with vector pSUPER alone or shRNA-producing plasmid (KD-1, KD-2). Two days after transfection, total RNA was prepared and analyzed by northern blot hybridization as in Fig. 5B. Band intensity was determined and normalized with the value of vector alone and is presented below. (B) Two days after transfection of HIT-T15 cells with pSUPER or KD-1, cells were treated with 100 μM olanzapine (OLA) for the indicated periods. The status of XBP1 mRNA splicing was determined as in Fig. 2D. (C) Two days after transfection of HIT-T15 cells with pSUPER or KD-1, cells were untreated (CON) or treated with 100 μM olanzapine (OLA), 100 μM risperidone (RIS) or 2 μg/ml tunicamycin (TM) for 24 h. DNA fragmentation assay was carried out as in Fig. 1B. (D) HIT-T15 cells were treated as in (C). TUNEL assay was carried out as in Fig. 1A. The data are presented as the average±standard error of two independent experiments. ** P<0.01.
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line that retains glucose-inducible insulin secretion (Miyazaki et al., 1990) (Fig. 7), implying that olanzapine induces β cell death generally. Although 100 μM risperidone showed much less effect on the viability of MIN6 cells compared with olanzapine, it also induced apoptosis in MIN6 cells significantly (Fig. 7), suggesting that the sensitivity to risperidone may vary depending on cell types. It has been reported that olanzapine concentrations in the serum of patients were in the range of 0.016–0.24 μM, while those in postmortem serum were in the range of 0.032–16 μM (Robertson and McMullin, 2000). However, it was also reported that olanzapine concentrations in various tissues were 4- to 46-fold higher than that in plasma in experiments with rats (Aravagiri et al., 1999). It is therefore possible that local concentrations of olanzapine might reach the 100 μM range, particularly in patients with potentially lower catabolic activity for olanzapine for genetic or other unknown reasons. In such individuals, which represent a minority of those receiving olanzapine, β cell toxicity may dominate and diabetes develops without weight gain or insulin resistance.

Both olanzapine and risperidone induced mild ER stress and activated PERK similarly in HIT-T15 cells (Fig. 2A), but PERK-mediated phosphorylation of eIF2α was attenuated in HIT-T15 cells treated with olanzapine, in contrast to those treated with risperidone (Fig. 2B). Accordingly, olanzapine-treated HIT-T15 cells showed phenotypes similar to those of PERK-deficient cells (Harding et al., 2000b); both were unable to attenuate translation in response to ER stress (Fig. 4A), and ER stress-induced activation of IRE1 was sustained in both (Fig. 4B). PERK-knockout and olanzapine-exposed cells were both sensitive to ER stress, and this sensitivity was decreased by suppression of new protein synthesis with cycloheximide treatment (Fig. 4D and 4E). We thus consider that olanzapine induces apoptosis in HIT-T15 cells by blocking PERK-mediated translational attenuation, although we are formally unable to exclude other possibilities.

There are several possible explanations how olanzapine blocks phosphorylation of eIF2α. Results in Fig. 3 showed that eIF2α dephosphorylation was not enhanced in cells treated with olanzapine and that eIF2α remained phosphorylatable by an alternative kinase GCN2, arguing for a PERK-specific defect. Interestingly, PERK has an unusually large kinase insert loop extending from the top of the N-terminal half of the catalytic site (Marciniak et al., 2006). It was previously reported that a recombinant cytoplasmic portion of PERK lacking this kinase insert loop (Δ-loop mutant) which is fused to GST is able to phosphorylate eIF2α present in cell lysate upon mixing as efficiently as wild-type PERK-GST but, unlike wild-type PERK-GST, is unable to pull down eIF2α from cell lysate, demonstrating that although dispensable to catalytic activity, PERK’s large kinase insert loop contributes to substrate recruitment (Marciniak et al., 2006). We observe that olanzapine does not block eIF2α phosphorylation in vitro by PERK-GST which, because of constitutive dimerization through the GST portion, represents highly activated PERK (Fig. 3D), but does block eIF2α phosphorylation by mildly activated PERK in HIT-15T cells (Fig. 2A and 2B). It is thus tempting to speculate that olanzapine interferes with recruitment of eIF2α by interacting with PERK’s large kinase insert loop, which is
particularly important when PERK is only mildly activated, without affecting PERK’s intrinsic kinase activity. This scenario may also explain olanzapine’s effect in tunicamycin-treated cells (Fig. 3E): tunicamycin-induced phosphorylation of eIF2α was suppressed by pretreatment with olanzapine at early time point, at which time PERK was only mildly activated, but this suppression was no longer apparent at later time points, when PERK was highly activated by tunicamycin (see Fig. 2A).

How olanzapine and risperidone induce mild ER stress is not clear yet. Because they induced mild ER stress in both HIT-T15 and HEK 293 cells (Fig. 2A and 2D), they probably cause misfolding of proteins generally. Blockage of eIF2α phosphorylation by olanzapine but not risperidone was observed not only in HIT-T15 cells but also HEK293 cells (Fig. 2B and 2E). Nonetheless, the consequence of the inability to attenuate protein synthesis in response to mild ER stress was remarkably different between HIT-T15 and HEK293 cells: apoptosis was markedly induced in HIT-T15 cells but not in HEK293 cells (Fig. 1A and 1C). As the proapoptotic CHOP was not induced in olanzapine-treated HIT-T15 cells (Fig. 2C), this apoptosis should proceed in a CHOP independent manner; several CHOP-independent mechanisms are known for ER stress-induced apoptosis (Szegedi et al., 2006; Tabas and Ron, 2011). Because insulin secretion was blocked almost completely (Fig. 5A) and both proinsulin and insulin accumulated in HIT-T15 cells treated with olanzapine (Fig. 5C), olanzapine might not only cause misfolding of proinsulin in the ER but might also interfere with proinsulin or insulin progress through the secretory pathway. Importantly, pancreatic β cells are apparently vulnerable to such accumulation of proinsulin as inhibition of protein synthesis by cycloheximide treatment (Fig. 4D and 4E) and knockdown of insulin mRNA (Fig. 6C and 6D) both mitigated olanzapine-induced apoptosis of HIT-T15 cells.

Olanzapine and the other diabetes-inducing antipsychotics clozapine and quetiapine, all belong to the benzodiazepine family, and contain a three-ring structure which is not present in risperidone. This structural difference may be key to the effect on eIF2α, and may explain the development of diabetes without obesity in some patients treated with olanzapine. This in turn indicates that, in certain cases, the metabolic side effects of medicating patients with schizophrenia can be significantly decreased by careful selection of the most appropriate antipsychotic.

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