ER stress increases store-operated Ca\(^{2+}\) entry (SOCE) and augments basal insulin secretion in pancreatic β cells

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Running title: ER stress increases SOCE and β cell insulin secretion

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is characterized by impaired glucose-stimulated insulin secretion and increased peripheral insulin resistance. Unremitting ER stress can lead to β-cell apoptosis and has been linked to type 2 diabetes. Although many studies have attempted to link ER stress and T2DM, the specific effects of ER stress on β-cell function remain incompletely understood. To determine the interrelationship between ER stress and β-cell function, here we treated insulin-secreting INS-1(832/13) cells or isolated mouse islets with the ER stress inducer tunicamycin (TM). TM-induced ER stress as expected, as evidenced by activation of the unfolded protein response (UPR). β Cells treated with TM also exhibited concomitant alterations in their electrical activity and cytosolic free Ca\(^{2+}\) oscillations. As ER stress is known to reduce ER Ca\(^{2+}\) levels, we tested the hypothesis that the observed increase in Ca\(^{2+}\) oscillations occurred because of reduced ER Ca\(^{2+}\) levels and, in turn, increased store-operated Ca\(^{2+}\) entry (or SOCE). TM-induced cytosolic Ca\(^{2+}\) and membrane electrical oscillations were acutely inhibited by YM58483, which blocks store-operated Ca\(^{2+}\) channels. Significantly, TM-treated cells secreted increased insulin under conditions normally associated with only minimal release, e.g. 5 mM glucose, YM58483 blocked this secretion. Taken together, these results support a critical role for ER Ca\(^{2+}\) depletion-activated Ca\(^{2+}\) current in mediating Ca\(^{2+}\)-induced insulin
secretion in response to ER stress.

Type 2 diabetes mellitus (T2D) is characterized by impaired glucose-stimulated insulin secretion in the setting of insulin resistance (1–3). Insulin secretion from pancreatic beta cells is triggered by glucose-induced Ca\(^{2+}\) entry triggered by the closure of K(\(\text{ATP}\)) channels (4–6). In many preparations, Ca\(^{2+}\) entry is manifested by regular oscillations in cytosolic Ca\(^{2+}\), where each oscillation in turn provokes the release of insulin granules (4, 7–10). Maintaining intracellular Ca\(^{2+}\) homeostasis is critical for proper insulin secretion and for retaining beta cell fitness. In mammalian cells, such as the pancreatic beta cell, the ER is the intracellular organelle where proteins of the secretory pathway are synthesized and initially packaged for export (11). In addition, the ER maintains protein quality control (12), and serves as a Ca\(^{2+}\) reservoir that sequesters but also can release free Ca\(^{2+}\) into the cytosol to generate a physiological signal (13–15). Ca\(^{2+}\) is pumped into the ER lumen via sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA pumps) and released to the cytosol through the triggered activation of inositol trisphosphate (IP3) and/or ryanodine receptors in the ER membrane (16–21).

β Cells undergo apoptosis after sustained exposure to the ER stress inducers tunicamycin (TM), thapsigargin, dithiothreitol (DTT), high-glucose or saturated fatty acid (22–27). These conditions activate the unfolded protein response (UPR) through various mechanisms to restore normal proteostasis and preserve beta cell function and viability (22, 23). For instance, TM inhibits N-acetylglucosamine phosphotransferase, the key enzyme involved in the N-glycosylation of proteins, which in turn leads to the misfolding of glycoproteins in the ER (28). The resulting ER stress causes UPR activation which in turn may restore proper protein folding and trafficking, increase the protein-folding capacity of the cell, and causes the degradation of misfolded proteins. In addition, further activation of the UPR inhibits new protein synthesis to reduce the protein load of the ER during times of increased stress.

Disrupted ER homeostasis has been proposed to be a potential cause of T2DM (14) and increasing evidence has emerged suggesting that the ER stress cascade is activated in islets from T2DM patients and from animal models of diabetes (23, 29, 30). We have recently discussed the potential links between disrupted ER homeostasis and altered beta cell function in a review article (31). Other groups have also proposed the relevance of UPR signaling to beta cell loss and the pathology of diabetes (32). We wished to advance the study of ER stress in disrupting specific beta cell function, such as ER Ca\(^{2+}\) handling, cytosolic Ca\(^{2+}\) oscillations and insulin secretion, and we also wanted to determine how these changes in turn affected long term beta cell survival.

In our study, we used TM to experimentally induce ER stress in insulin-secreting INS-1(832/13) cells or isolated mouse islets. ER stress responses in the form of UPR endpoints, ER and cytosolic Ca\(^{2+}\) levels, insulin secretion and beta cell death were measured at various time points after exposing islets or cells to TM to determine the timeline of these events. TM treatment increased cytosolic Ca\(^{2+}\) and insulin secretion, even in 5
mM glucose, a level that is below the normal glucose threshold of insulin secretion and the triggering of cytosolic Ca^{2+} oscillations. We further found that this abnormal Ca^{2+} signaling resulted from the activation of store-operated Ca^{2+} entry (SOCE), most likely due to a stress-induced reduction of ER Ca^{2+} concentration. The possible significance of this novel mechanism for augmenting insulin secretion for patients with T2DM is discussed.

**Results**

**Tunicamycin induced the ER stress response and apoptosis**

Tunicamycin, a commonly used pharmacological inducer of ER stress in beta cells, inhibits protein glycosylation (22, 33–35). To investigate the relationship between ER stress, ER Ca^{2+} and cytosolic Ca^{2+}, we systematically measured the concentration of Ca^{2+} in the cytosol and ER in parallel with UPR markers to establish their respective time courses following TM treatment. Changes in the three canonical ER stress response markers, spliced XBP1, CHOP and BiP were determined at the mRNA or protein level. Mouse islets or insulin secreting INS-1(832/13) cells were treated with vehicle (DMSO) as a control or TM for 6, 12 or 16 hours in 11 mM glucose-containing medium prior to extracting total cell mRNA and making whole cell protein lysates.

As shown in Figures 1A and 1B, XBP1 splicing increased after 6 hours of TM treatment in both INS-1(832/13) cells and mouse islets, while total XBP1 levels were unchanged. Similarly, as shown in Figure 1C, CHOP increased after 6 hours of TM treatment in INS-1(832/13) cells. In contrast, as shown in Figures 1D through 1G, levels of BiP protein only increased after 12 hours of exposure to TM. XBP1 splicing is known to be an early event in the UPR, while the upregulation of BiP expression has been reported to be more delayed (22, 23, 36, 37).

Apoptosis occurs in a variety of cell types as a consequence of prolonged ER stress (23) and previous studies have shown that TM induces cell death in INS-1(832/13) cells and other cell lines (35, 38–42). To determine the presence of apoptosis, we assayed the level of cleaved PARP protein, an established marker of apoptosis (43, 44) by western blotting. As shown in Figure 2A and 2B, a band corresponding to cleaved PARP was visible at 89 kDa in lysates obtained from INS-1(832/13) cells exposed to TM for 12 hours or more. Cleaved PARP was barely detected in any of our cell samples under control conditions or if TM exposure was for 6 hours or less. It thus appeared that TM only triggered significant apoptosis after 12 hours. The percentage of cleaved versus total PARP was monitored and is shown in Figure 2B to rule out the effect of uneven protein loading. In addition, the percentage of INS-1(832/13) cells that take up propidium iodide (PI), a dye that is indicative of cell death, only increased after 16 hours of TM treatment, compared to DMSO-treated controls (Figure 2C). Cell death as assessed using this marker was not observed at any of the earlier time points studied. Figure 2D shows there was a four-fold increase in the number of cells in the sub-G1 phase following 24 hours exposure to TM, indicating they were late stage apoptotic cells compared to DMSO treated controls.

Taken together, TM triggered a classic ER stress response in INS-1(832/13) cells after 6
hours, while apoptosis was only seen after 12 hours. Frank, quantitative beta cell death, in turn, was evident much later, after about 16 hours of TM treatment, as evidenced by increased propidium iodide uptake.

**Tunicamycin led to ER Ca\(^{2+}\) loss**

As mentioned, TM has been used to induce ER stress in several studies of beta cells (22, 23, 33, 35). The ER plays an important role in beta cell function since it is the site where proteins of the secretory pathway are folded and processed in preparation for transport to the Golgi apparatus (15, 45), and it is the location where proteostasis occurs (45, 46). In terms of cellular Ca\(^{2+}\) homeostasis, the ER also has a central role in this process due to its ability to sequester and buffer cytosolic Ca\(^{2+}\), serve as a releasable Ca\(^{2+}\) source in response to surface membrane GPCR signaling, and it supplies Ca\(^{2+}\) to Ca\(^{2+}\)-binding ER-resident protein chaperones that act to ensure proper protein folding (47, 48).

To test whether TM altered ER Ca\(^{2+}\) level in our system, the ER Ca\(^{2+}\) probe D4ER was transiently expressed in islet beta cells using an adenovirus delivering the D4ER gene placed behind the rat insulin promoter, RIP2. Islets were then treated with vehicle control (DMSO) or TM (10 µg/ml) for 6, 12 or 16 hours and then ER Ca\(^{2+}\) was measured. Figure 3A shows ER Ca\(^{2+}\) normalized to the initial FRET ratio (F0), expressed in relative units, as a function of time, and the effect of thapsigargin (TG, 1 µM) is shown for both control and TM-treated islets. TG is a SERCA blocker that is well known to deplete ER Ca\(^{2+}\) by blocking Ca\(^{2+}\) uptake into the ER (49). Only beta cells that responded to TG are shown in Fig. 3A; these constituted approximately 50% of the beta cells tested. As shown in Figure 3B, TM caused a decline of steady state ER Ca\(^{2+}\) in islets compared to DMSO after 6, 12 and 16h of treatment.

**Tunicamycin increased cytosolic free Ca\(^{2+}\) under sub-threshold glucose conditions**

Mouse islets do not typically show oscillations in cytosolic Ca\(^{2+}\) or electrical activity when acutely exposed to glucose concentrations < 7 mM (2, 50–52). To determine the relationship between ER stress and cytosolic free Ca\(^{2+}\) in our experimental system, mouse islets were exposed to TM or vehicle control (DMSO) in standard RPMI medium for 6,12 or 16 hours. Following this treatment, cytosolic free Ca\(^{2+}\) and islet electrical activity were recorded in parallel studies using an extracellular recording solution containing 5 mM glucose.

As shown in Figure 4A, cytosolic free Ca\(^{2+}\) in control islets did not display oscillatory activity in 5 mM glucose solution, as expected (2, 50–52). In contrast, islets treated with TM exhibited islet Ca\(^{2+}\) oscillations or Ca\(^{2+}\) transients when exposed to the TM for 6 hours or more. 40% of islets treated with TM for 6 hours displayed free Ca\(^{2+}\) oscillations compared to those treated with DMSO (Figure 4B). Treatment with TM for 12 or 16 hours resulted in a greater % of oscillating islets.

The plateau fraction, frequency and amplitude of oscillating islets as well as their baseline Ca\(^{2+}\) levels were analyzed and plotted in Figure 5. Plateau fraction, oscillation frequency and amplitude were not plotted for control islets as they did not exhibit oscillations. Statistically significant increases in baseline...
Ca\textsuperscript{2+} levels were observed after 6 and 12 hours of TM exposure (Figures 5B).

**Changes in electrical activity occurred in parallel with changes in Ca\textsuperscript{2+} oscillations**

Our observation that islets treated with TM exhibited cytosolic Ca\textsuperscript{2+} oscillations (Figure 6) was next confirmed by separate measurements of islet electrical activity, obtained using perforated patch clamp recording. TM-treated beta cells thus exhibited oscillations in islet membrane potential in 5 mM glucose, which was rarely observed in control islets exposed to the same glucose concentration, as was found for Ca\textsuperscript{2+}. However, the occurrence of oscillations was related to the duration of TM treatment. As shown in Figure 6, islets subjected to TM for 6 hours showed occasional oscillations in 5 mM glucose, while islets treated for 12 or 16 hours showed regular oscillations having an average period of 5 – 8 minutes. Importantly, the oscillations we observed in TM-treated islets in 5 mM glucose strongly resembled those of normal islets exposed to glucose concentrations >7-8 mM (53).

**Tunicamycin increased insulin secretion under sub-threshold glucose conditions**

When beta cells are depolarized, Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels leads to a rise in cytosolic Ca\textsuperscript{2+} concentration that triggers the release of insulin granules from the cell (4, 10, 51, 54). To test whether the changes we observed in islet electrical and cytosolic Ca\textsuperscript{2+} activity in response to TM treatment were sufficient to elicit insulin secretion even under normally subthreshold conditions, islets were pretreated with DMSO or TM in standard RPMI medium (including 11 mM glucose) for 6, 12 or 16 hours. After treatment, islets were thoroughly washed, and a static incubation protocol was used to measure insulin secretion in 5 mM glucose. As shown in Figure 7A, insulin secreted into the medium was significantly increased after 12 hours or more of TM exposure, while islet insulin content was unchanged. Expressed another way, TM exposure for 12 hours or more resulted in greater insulin secretion as a percent of insulin content, compared to controls (Figure 7B). The time course of increased secretion closely paralleled the increase in cytosolic Ca\textsuperscript{2+} or electrical activity depicted in Figures 4-6. They support the hypothesis that the activation of cytosolic Ca\textsuperscript{2+} activity by ER stress in 5 mM glucose was triggered by increased islet electrical activity and was sufficient to release more insulin from the beta cell. Secreted insulin and percent insulin content were both higher after 6 hours of TM compared to DMSO exposure, but the differences were not statistically significant. The unique aspect of the 6-hour time point will be addressed in the Discussion. We also point out that the magnitude of the secretion response of TM-treated islets in 5 mM glucose is still much lower than that seen in response to 11 mM or more glucose.

**Tunicamycin increased cytosolic Ca\textsuperscript{2+}, membrane potential oscillations and insulin secretion through store-operated Ca\textsuperscript{2+} entry**

Store-operated Ca\textsuperscript{2+} entry (SOCE) links reduced ER Ca\textsuperscript{2+} concentration to the activation of voltage-independent, plasma membrane Ca\textsuperscript{2+} channels that can replenish the depleted ER, with Ca\textsuperscript{2+} entering the cell from the
extracellular space (55–57). To determine whether SOCE played a role in mediating the oscillations we observed following chronic ER stress and ER Ca²⁺ lowering, we tested whether YM58483, a selective blocker of membrane SOCE channels, interfered with our physiological endpoints (58–60). As shown in Figures 8A and 8B, both cytosolic Ca²⁺ oscillations and the electrical activity observed in TM-treated islets in 5 mM glucose were abruptly abolished by YM58483 treatment. These results show that SOCE, which normally plays little or no role in the genesis of glucose-induced islet electrical oscillations (61), was here facilitated by TM-induced ER stress in beta cells, presumably because TM reduced ER Ca²⁺. Importantly, YM58483 also blocked TM-induced insulin secretion in islets bathed in 5 mM glucose (Figures 8C and 8D). In contrast, the addition of YM58483 had no effect on insulin secretion, islet electrical activity, or intracellular Ca²⁺ in control islets (Figures 8A-D).

At the molecular level, main components of SOCE are stromal interaction molecule-1 (STIM1) and Ca²⁺ release-activated Ca²⁺ channel protein 1 (ORAI1). STIM1 is an ER Ca²⁺ sensor while ORAI1, which is found on the plasma membrane, is the pore-forming subunit of functional SOCE. When STIM1 senses ER Ca²⁺ depletion, STIM1 molecules aggregate and interact with ORAI1 at ER-PM junctions, and this complex mediates Ca²⁺ influx through SOCE (62, 63). To confirm the results, we obtained at the molecular level, siRNA was used to knockdown STIM1 in INS-1(832/13) cells. Transfecting INS-1(832/13) cells with siRNA-STIM1 (siSTIM1) reduced STIM1 mRNA by ~ 80-90% (Figure 9A) and STIM1 protein by ~70-75% (Figure 9B and 9C) compared to treatment with control siRNA (siCon). STIM1 reduction did not result in significant upregulation of ORAI1, suggesting the cells did not compensate for the loss of STIM1 (Figure 9B and 9C). The percentage of cells showing cytosolic Ca²⁺ transients in 5 mM glucose was decreased in siSTIM-transfected cells (~40%) compared to siCon-transfected control cells (~10%) following 16 hour TM exposure (Figure 9D and 9E). Even after 16 hours of DMSO exposure, controls showed no change in their Ca²⁺ activities.

As an alternative to blocking SOCE channels with YM, we tested whether removing extracellular Ca²⁺ was similarly able to abolish the cytosolic Ca²⁺ oscillations we observed in TM-treated islets in 5 mM glucose. Removing extracellular Ca²⁺ confirmed the results we obtained with YM, supporting the hypothesis that the oscillations seen after TM treatment indeed require increased influx of extracellular Ca²⁺ (Figure S1A). On the other hand, applying other SOCE channel blockers, 2-Aminoethoxydiphenyl borate (2APB) or SKF96365 (SKF) acutely at the end of a Ca²⁺ imaging experiment surprisingly increased cytosolic Ca²⁺ levels in both control and experimental groups (Figure S1B and S1C) (64, 65). 2APB and SKF are nonselective SOCE inhibitors as they also inhibit other channels over a similar concentration range (66).

While ER Ca²⁺ decreased in TM-treated islets compared to controls, blocking SOCE with YM58483 had little or no measurable effect on the ER Ca²⁺ levels of either control or TM-treated beta cells (Figure 10A). This finding was unexpected, but will be addressed further in the Discussion. Blocking SOCE with
YM58483 also did not affect any of the TM-induced UPR endpoints we measured (Figure 10B).

Previous reports have shown that elevated cytosolic Ca\textsuperscript{2+} is detrimental to beta cells (67). Thus, preventing excessive cytosolic Ca\textsuperscript{2+} elevation due to overactive SOCE might have at least partly protected beta cells from cell death induced by prolonged exposure to TM. However, as shown in Figure 10C, 24 hour treatment with TM increased cell death in INS-1(832/13) cells, but we found no protection afforded by the inclusion of YM58483.

**Tunicamycin did not affect cytosolic free Ca\textsuperscript{2+} under above-threshold glucose conditions**

To maintain glucose homeostasis, beta cells secrete insulin when blood glucose concentration rises. Islets exhibit oscillations in cytosolic free Ca\textsuperscript{2+} when exposed to 7 mM or more glucose (52). After isolated mouse islets were exposed to TM or vehicle control (DMSO), free Ca\textsuperscript{2+} and insulin secretion were measured in parallel in 11 mM glucose. As shown in Figure 11A, both control and experimental groups showed Ca\textsuperscript{2+} oscillations in 11 mM glucose. The percentages of oscillating islets we observed were very similar between the two groups (70–80%), while the remaining islets tended to go to a plateau (Figure 11B). The frequency of the oscillations observed in TM-treated islets was higher than for controls, while no significant change was observed in plateau fraction, baseline Ca\textsuperscript{2+} or oscillation amplitude (Figure 11C-F). In addition, we found no significant change in insulin secretion between experimental and control groups after they were stimulated with 11 mM glucose for 30 minutes (Figure 11G and 11H).

**Other ER stress inducers also increased cytosolic free Ca\textsuperscript{2+} under sub-threshold glucose conditions**

Besides tunicamycin, ER stress can be induced by treating islets with thapsigargin or high glucose (22, 23). As shown in Figure 12A, mouse islets exposed to thapsigargin (200 nM) for 16 hours exhibited oscillatory cytosolic Ca\textsuperscript{2+} levels despite being in 5 mM glucose. Similarly, mouse islets cultured in medium containing 25 mM glucose to induce stress also exhibited cytosolic Ca\textsuperscript{2+} oscillations (Figure 12B). These oscillations were also abruptly abolished by YM58483 treatment. DMSO treated or 11 mM glucose cultured islets did not exhibit Ca\textsuperscript{2+} oscillations in 5 mM glucose solution, however, as expected.

**The effect of tunicamycin on gene expression**

As SOCE activated in response to TM treatment in our study, we also assayed the level of STIM1 and ORAI1 expression under these same experimental conditions. As shown in Figure 13A, we observed a protein band corresponding to STIM1 as expected, and an additional, smaller molecular weight band in lysates from TM-treated INS-1(832/13) cells after 16 hours of treatment. The intensity of the upper band for STIM1 was not significantly altered in response to TM compared to controls (Figure 13B). ORAI1 protein was also unchanged by TM treatment (Figure 13A and 13B), as reported in another recent study (68). GLUT2 protein was also measured in INS-1(832/13) cells after 6 hours of TM treatment compared to control, and no change in protein expression was found (Figure S2A and S2B).
Discussion

In this study, we sought to delineate the temporal relationship between the induction of ER stress, altered beta cell function, and altered beta cell viability, focusing on the role of ER and cytosolic Ca\(^{2+}\) in these processes. Studies were carried out by exposing mouse islets or INS-1(832/13) cells to the glycosylation inhibitor tunicamycin for up to 24 hours. We found that UPR activation appeared to be linked to a reduction in ER Ca\(^{2+}\) and a phase of increased extracellular Ca\(^{2+}\) influx linked to ER Ca\(^{2+}\) unloading. The Ca\(^{2+}\) oscillations that were triggered by store-operated Ca\(^{2+}\) influx were sufficient to trigger the release of insulin, even in normally sub-threshold glucose. Cell death was found to occur much later, e.g. after 16 hours post-treatment and appeared to be independent of the early phase of SOCE-mediated Ca\(^{2+}\) influx and concomitant insulin secretion.

Previous research carried out using many types of cells has shown that thapsigargin, a SERCA blocker, which prevents ATP-dependent Ca\(^{2+}\) sequestration by the ER, unloads the ER Ca\(^{2+}\) store, triggering SOCE (16, 60). Activated SOCE results in increased cytosolic Ca\(^{2+}\), which serves to replenish the ER Ca\(^{2+}\) pool (69). The recent findings reported by Yamamoto et al. indicate that tunicamycin decreases ER Ca\(^{2+}\) by increasing ryanodine receptor 2 activity which in turn elicits spontaneous cytosolic Ca\(^{2+}\) transients that are seen after raising extracellular Ca\(^{2+}\) concentration (70). We agree with Yamamoto et al. that ryanodine receptors (RyRs) are likely involved in ER stress induced ER Ca\(^{2+}\) lowering, as we observed inhibitory effects of the RyR blocker ryanodine (data not shown). However, we propose a very different interpretation in this paper. Our data that ER stress conditions activate a Ca\(^{2+}\) current mediated by SOCE channels under low glucose conditions, which likely occurs secondary to ER Ca\(^{2+}\) depletion by tunicamycin.

The normal glucose threshold for islet oscillations in our hands is near 7 mM (2, 50), which means that TM-induced ER stress in a sense increased the sensitivity of islets to glucose concentration. In our view, glucose-induced islet Ca\(^{2+}\) oscillations are induced despite the low level of glucose by the activation of SOCE-mediated Ca\(^{2+}\) current, which depolarizes the beta cell membrane to threshold despite incomplete closure of beta cell K\(_{ATP}\) channels. The evidence for this interpretation is that (1) the Ca\(^{2+}\) oscillations we observed strongly resemble those of control islets exposed to glucose >7 mM, suggesting a common origin; (2) the Ca\(^{2+}\) oscillations of stressed islets were completely blocked by the selective SOCE blocker YM58483 (58, 59); and notably this drug had no effect on untreated control islets; (3) patch clamp electrophysiology confirmed the electrical nature of the ER stress-induced oscillations and, as for the Ca\(^{2+}\) oscillations, the electrical bursting we observed in 5 mM glucose was similarly abolished by YM58483; and (4) the percentage of Ca\(^{2+}\) oscillations were decreased in TM-treated siSTIM1-knockdown INS-1(832/13) cells compared to controls. Taken together these data are in strong support of a plasma-membrane delimited mechanism, and they rule out intracellular store Ca\(^{2+}\) release as the proximal cause of the Ca\(^{2+}\) oscillations we observed in TM-treated islets, although we
believe ER Ca\textsuperscript{2+} depletion by ER stress indirectly caused the oscillations by triggering SOCE.

Physiologically, when blood glucose rises, \(K_{\text{ATP}}\) channel closure mediates plasma membrane depolarization, which in turn increases cytosolic Ca\textsuperscript{2+}, which then drives insulin secretion (2, 5, 8). Membrane potential changes in mouse beta cells have been shown to precede changes in cytosolic Ca\textsuperscript{2+} under physiological conditions (5). The cytosolic Ca\textsuperscript{2+} oscillations shown in Figures 4A occurred in parallel with membrane potential oscillations in 5 mM glucose saline in response to TM treatment, shown in Figure 6. In simultaneous measurements of cytosolic Ca\textsuperscript{2+} and insulin secretion, each oscillation in islet Ca\textsuperscript{2+} has been shown to be well synchronized with a pulse of insulin secretion (4, 5, 10).

Although cytosolic Ca\textsuperscript{2+} was increased after 6 hours of TM treatment, the change in insulin secretion and percent insulin (Figure 7) we measured at this time point were not statistically significant compared to controls, although the means we obtained were greater than controls. This may be explained by our observation that less than 40% of islets displayed elevated cytosolic Ca\textsuperscript{2+} within 6 hours of TM treatment (Figure 4B). Our results at the 6-hour time point may thus underestimate the amount of insulin secretion seen in response to TM because it included both responding and non-responding islets.

YM58483 did not affect the extent of ER Ca\textsuperscript{2+} depletion that followed TM treatment (Figure 10A), which was surprising. This could be due to several possible factors: (1) the influx of Ca\textsuperscript{2+} due to SOCE may have been too small to cause a detectable change in ER Ca\textsuperscript{2+} due to limits in the Ca\textsuperscript{2+} sensitivity of the D4ER Ca\textsuperscript{2+} probe; (2) SERCA expression and/or function might also be reduced by TM treatment, such that under these pathophysiological conditions, SOCE is capable of mediating an electrical current and Ca\textsuperscript{2+} oscillations but not significant ER store refilling. ER stress has in fact been reported to cause reduced SERCA2b expression in beta cells, which supports this idea (18, 71, 72); (3) the ER may become so leaky to Ca\textsuperscript{2+} after TM treatment that a modest activation of SOCE is unable to do enough to measurably refill the ER, like turning on a small hose to refill a very leaky barrel.

Our results support the hypothesis that TM-triggered beta cell death occurs as a consequence of ER Ca\textsuperscript{2+} depletion, and that SOCE activation is a separate action that is unrelated to the ultimate fate of the cell, as shown in Figure 14. Similar observations and conclusions were made in studies of thapsigargin-treated LNCaP, PC3 and MCF7 cells (49). Thapsigargin caused the unloading of ER Ca\textsuperscript{2+} and resulted in cell death despite genetic knockdown of the SOCE components STIM1 and/or ORAI1 in this case. Therefore, ER Ca\textsuperscript{2+} depletion due to ER stress appeared to be an important contributor to thapsigargin-induced cell death, instead of SOCE activation and increased cytosolic Ca\textsuperscript{2+} (49).

As shown in Figures 13A–D, we found two bands corresponding to STIM1 protein. The upper band of STIM1 expression at 77 kDa and ORAI1 expression remained unchanged. Both STIM1 and ORAI1 are known to be N-linked glycosylated proteins (62, 63, 73, 74). Other investigators also observed no change in ORAI1 in response to induced ER stress, while STIM1 responded to TM treatment. The
slightly smaller molecular weight STIM1 species, representing non-glycosylated STIM1, were reported. Blocking STIM1 glycosylation led to diminished SOCE (73, 74). Evans-Molina and colleagues have recently reported that STIM1 was downregulated in a diabetes model, while overexpressing STIM1 restored SOCE under high glucose conditions (68). However, Evans-Molina and colleagues propose that SOCE is an essential driver of glucose-induced Ca\textsuperscript{2+} oscillations (15 mM glucose) under normal conditions and that SOCE is impaired in response to proinflammatory cytokines or palmitate mediated stress conditions. In contrast, we propose that SOCE is not involved in the triggering or modulation of glucose-induced Ca\textsuperscript{2+} oscillations in untreated control islets, but is activated by ER stress, resulting in the appearance of Ca\textsuperscript{2+} oscillations under subthreshold glucose conditions (5 mM glucose) by virtue of this abnormal triggering mechanism, which in essence shifts the glucose sensitivity of the islet to the left where islet Ca\textsuperscript{2+} activity could then contribute to the production of high basal insulin release. Different glucose conditions may account for the different interpretations.

The justification for our use of insulin secreting INS cells in addition to mouse islets in the present paper relates to the small amount of tissue available for biochemical and molecular studies if just islets were used. For example, analyzing propidium iodide levels with flow cytometry in order to quantify cell death is extremely challenging if primary beta cells are used. INS-1(832/13) cells are one of the most commonly used insulin-secreting cell lines that display many important characteristics of primary beta cells. Importantly, INS-1(832/13) cells are very responsive to glucose (75). According to Figures 1A and 1B, INS-1(832/13) cells had identical UPR responses as isolated islets. Thus, we believe that the molecular studies done in INS-1(832/13) cells while not perfectly reflecting what we might expect if islets or primary beta cells were used in their place, are reasonable surrogates for the primary cells with regards to UPR activation and cell death. This is not likely to be true regarding physiology where our methods are well attuned to studying primary islets and their oscillatory and secretory characteristics.

In summary, as shown in Figure 14, we propose that TM induced beta cell death occurs through ER Ca\textsuperscript{2+} depletion, whereas SOCE and concomitant increased cytosolic Ca\textsuperscript{2+} were required for our finding increased insulin secretion under stress conditions. During prediabetes, which is associated with insulin resistance, the pancreatic beta cell is thought to compensate for rising levels of glucose by increasing insulin secretion and, if that fails, increasing beta cell mass, provided the cells are capable of doing so (76). However, long-term hyperinsulinemia, and the increased metabolic workload it represents, can potentially exhaust the beta cell and promote beta cell death (77). In our results, TM-induced ER stress resulted in increased beta cell electrical activity, cytosolic Ca\textsuperscript{2+} oscillations and insulin secretion by activating SOCE. Blocking SOCE by applying YM58483 to stressed but not control islets abolished ER stress-triggered increases in electrical activity, cytosolic Ca\textsuperscript{2+} oscillations and insulin secretion (Figures 8A-D). Therefore, the increased insulin secretion data
not only confirmed that TM increased cytosolic Ca\textsuperscript{2+} oscillations, but it also established SOCE as the key mechanism.

This report shows that SOCE is a key player in ER stress-induced cytosolic Ca\textsuperscript{2+} oscillations and insulin secretion, and we suggest that this pathway must work in parallel with the UPR and cell death pathways. The cytosolic Ca\textsuperscript{2+} oscillations we observed clearly resulted from electrical oscillations and not Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from the ER. Thus, these results suggest the possibility that in T2DM or under pre-diabetic conditions increased secretion due to SOCE activation may contribute to the increased basal insulin secretion that is a hallmark of type 2 diabetes. Combining our findings with more detailed mechanistic and pharmacological studies on SOCE activity in prediabetes may disclose additional valuable information and perhaps novel treatment strategies.

Experimental procedures

Materials

Tunicamycin (TM), YM58483 (YM), thapsigargin (TG), 2-Aminoethoxydiphenyl borate (2APB) and SKF96365 (SKF) were all obtained from Cayman chemical. Small interfering RNAs (siRNAs) were purchased from ThermoFisher scientific. Supplementary Tables 1A and 1B contain a complete list of PCR primers and antibodies, respectively. ECL reagents was obtained from Bio-rad.

Isolation of pancreatic islets and islet pretreatments

Pancreatic islets were isolated from male Swiss-Webster mice (3 months of age; 25-35 g) according to the regulations of the University of Michigan Committee on the Use and Care of Animals (UCUCA), using previously described methods (78) and with an approved protocol. Isolated islets from a given mouse were divided into control and experimental groups, and both were cultured in standard RPMI 1640 medium containing 11 mM glucose, 10% fetal bovine serum (FBS), 10 mM HEPES, 1% penicillin/streptomycin and 1% sodium pyruvate. Control islets were incubated with DMSO, while test islets were pretreated with 10 µg/mL tunicamycin.

Cell culture and transfection

INS-1(832/13) cells were grown in RPMI 1640 containing 11 mM glucose, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 10 mM HEPES and 1% sodium pyruvate. INS-1(832/13) cells were grown in 10 cm culture dishes, 6-well plates or T25 flasks at 37\textdegree C in a 5% CO\textsubscript{2} humidified atmosphere. Cells obtained ∼70% confluence prior to the initiation of experimentation. INS-1(832/13) cells were transfected with STIM1-specific siRNA or negative control siRNA using lipofectamine RNAiMAX reagent as described in the manufacturer’s protocol (Invitrogen). The treated cells were assessed by real-time PCR and western blot.

Real-Time PCR

Total RNA was extracted from INS-1(832/13) cells or islets using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. One µg of total RNA from INS-1(832/13) cells or 0.4 µg of total islet RNA was reverse-transcribed using Superscript RT II (Invitrogen) according to the manufacturer’s instructions. Real-time experiments were
carried out using an SYBR green PCR master mix (Applied Biosystems) with the primers shown in Table 1A. Raw threshold-cycle (CT) values were obtained using Step One software, and mean CT values were calculated from triplicate PCR reactions for each sample. Data were presented as RQ values (2^ΔΔCT) with expression presented relative to an endogenous control, HPRT1.

**Western blotting**

Total protein was obtained by treating INS-1(832/13) cells or mouse islets with KHEN lysis buffer (50 mM KCl, 50 mM HEPES, 10 mM EGTA, 1.92 mM MgCl2; pH 7.2) and then separating proteins using 4-12% SDS-PAGE and transferring them to nitrocellulose membranes. Membranes were blocked in 5% w/v nonfat dry milk or 5% BSA in 1X TBST containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20. Blots were incubated overnight with primary antibodies diluted in 5% nonfat dry milk in 1X TBST at 4 °C as described in Table 1B. Blots were incubated with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit antibodies or goat anti-mouse antibodies and these were visualized using ECL reagents.

**Fura-2/AM imaging**

Islets were loaded with fura-2/AM (2.5 µM) for 45 min. in medium containing 5 mM glucose prior to imaging. Islets were then transferred to a 1 mL perfusion chamber containing 5 mM glucose imaging buffer for 6 min, followed by 10 to 30 min perfusion with this solution at approximately 1 mL/min. Imaging buffer contained (in mM): 140 NaCl, 3CaCl2, 5 KCl, 2 MgCl2, 10 HEPES and 5 glucose. Ratiometric fura-2 imaging was carried out using 340/380 nm excitation and collecting 502 nm emission, as previously described (78). The fluorescence data were acquired using Metafluor.

**FRET measurements**

To measure ER Ca^{2+}, we utilized a previously described ER-localized FRET biosensor, D4ER (79). The sensor was selectively expressed in the beta cells of intact mouse islets using an adenovirus and under the control of the rat insulin promoter, as previously described (79). The same system described above for Fura-2/AM imaging was employed here. D4ER imaging was carried out using 430 nm excitation, and 470/535 nm ratiometric emission. The imaging solution used contained (in mM): 140 NaCl, 3CaCl2, 5 KCl, 2 MgCl2, 10 HEPES, 5 glucose and 0.2 diazoxide (Dz). Dz was included to keep the K\textsubscript{ATP} channel in its open state to prevent oscillatory Ca^{2+} activity and improve the signal/noise ratio and stability of the ER Ca^{2+} recordings. FRET ratios were acquired using Metafluor, and mean values were calculated using Prism.

**Analysis of cytosolic Ca^{2+} recordings**

Traces containing cytosolic Ca^{2+} oscillations were analyzed using MATLAB (Mathworks) to obtain the plateau fraction (PF), periods, baseline ratios and relative amplitudes of Ca^{2+} oscillations, as described (50). PF was calculated as the active phase duration divided by the period of each oscillation (50). Only islets displaying oscillations were assigned a PF, and those exhibiting a persistent plateau phase were assigned a PF value of 1.0.
Electrophysiology

Islet membrane potential was measured using perforated patch whole cell current clamp as described (53). Electrophysiological recordings were made from single beta cells in intact islets treated with TM for 6, 12 and 16 hours, respectively. Islets treated with vehicle medium were used to make control recordings. Only one beta cell in each intact islet was patched. Membrane potential of each beta cell in an intact islet was recorded in current-clamp mode after perforated patch configuration was established. The external recording solution contained (in mM): 140 NaCl, 3 CaCl2, 5 KCl, 2 MgCl2, 10 HEPES and 5 glucose.

Assays of cell death

INS-1(832/13) cells were dislodged from T25 flasks with 0.05% trypsin and after gentle shaking, and propidium iodide (PI) was applied to label dead cells, as described in the manufacturer’s protocol (Sigma). The percentage of PI-positive cells was determined using a flow cytometer provided by the Flow Cytometry Core of the University of Michigan.

Assays of apoptosis

INS-1(832/13) cells were harvested as described in above cell death assay and fixed in cold 70% ethanol and stored in 4°C. Before measurement, PI was added as described in the manufacturer’s protocol (Sigma), and the percentage apoptotic cells was determined by calculating the percentage of sub-G1 cells in the DNA content histogram using a flow cytometer provided by the Flow Cytometry Core of the University of Michigan.

Glucose-stimulated insulin secretion assay (GSIS)

Islets were washed with glucose free KRB buffer (115 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4\textsubscript{7}H\textsubscript{2}O, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 20 mM NaHCO\textsubscript{3}, 16mM HEPES, 2.56 mM CaCl\textsubscript{2}-2H\textsubscript{2}O, 0.2% BSA) for 30 min at 37°C, and then incubated with KRB buffer containing 5 mM or more glucose for an additional 30 min. The supernatant and islets were then collected separately to determine insulin content using a mouse insulin ELISA kit according to the manufacturer’s instructions (Crystal Chem).

Statistical analysis

Data were expressed as means +/- SEM, unless specified, and were analyzed using an unpaired Student’s t test (Prism, GraphPad Software Solutions) when comparing two groups. Differences between two or more groups were analyzed using two-way ANOVA (Prism) with post hoc multiple comparison by Tukey’s procedure. Values of p< 0.05 were considered statistically significant.

Data availability

All data are contained within the manuscript.
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FOOTNOTES

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Figure Legends

**Figure 1. Tunicamycin induced the ER stress response.** INS-1(832/13) cells and isolated mouse pancreatic islets were treated with vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for the indicated length of time shown. 1A: Expression levels of spliced XBP1 and total XBP1 mRNA in INS-1(832/13) cells and 1B: Same but in islets. 1C: Expression level of CHOP in INS-1(832/13) cells. 1D: Representative western blot showing the level of BiP in INS-1(832/13) cells and 1F: Same but in islets. GAPDH and tubulin are loading controls. Quantitative protein levels are shown graphically in 1E and 1G respectively. 1E: Protein levels are all normalized to DMSO 16h. All values shown are means ± SEM. #, p< 0.05, ##, p< 0.01, ###, p< 0.0005, ####, p< 0.0001 compared with control conditions; n= at least 3 times repeated per condition.

**Figure 2. Tunicamycin triggered apoptosis.** INS-1(832/13) cells were treated with vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for the indicated length of time shown. 2A: Representative western blot showing the level of total PARP at 116 kDa and cleaved PARP at 89 kDa in INS-1(832/13) cells. Tubulin is shown as loading control. 2B: Quantitative percentage of cleaved PARP out of total PARP are shown graphically. Protein levels are normalized to DMSO 16h. 2C: After various hours of DMSO or TM treatment, cell death in INS-1(832/13) cells is shown by propidium iodide (PI) staining and was quantified using flow cytometry. Fold change was derived by comparing to untreated group. 2D: After 24 hours of DMSO or TM treatment, late stage apoptotic INS-1(832/13) cells is shown using the sub-G1 assay measured by flow cytometry. Fold change was derived by comparing to DMSO group. All values shown are means ± SEM. #, p< 0.05, ##, p< 0.01, ###, p< 0.0005, ####, p< 0.0001 compared with control conditions; n= at least 3 times repeated per condition.

**Figure 3. Tunicamycin treatment decreased basal ER Ca²⁺ level.** Mouse pancreatic islets were infected with an adenovirus expressing a beta cell directed D4ER probe for three hours followed by a 48 hour recovery period. Islets were then treated with vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for 6, 12 and 16 hours in 11 mM glucose islet culture medium (RPMI, see Methods). 3A: Basal ER Ca²⁺ (normalized to the initial intensity) traces for each condition obtained in 5 mM glucose solution before and after thapsigargin (TG, 1 µM). 3B: The raw data showing resting ER Ca²⁺ level from mouse islets in 5 mM glucose solution with 200 uM diazoxide present. Each data point shown was a D4ER ratio obtained for one selected region of interest, a single cell or small group of cells. All values shown are means ± SD. ####, p< 0.0001; n= at least 5 mice.

**Figure 4. Tunicamycin increased cytosolic free Ca²⁺ under sub-threshold glucose conditions.** Isolated pancreatic mouse islets were treated with a vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for 6, 12 and 16 hours in 11 mM glucose. 3A: The responses of cytosolic free Ca²⁺ to solution containing 5 mM glucose under the indicated conditions. 3B: Percentage of oscillating
Figure 5. **Cytosolic free Ca^{2+} imaging analysis.** Summary findings for the cytosolic free Ca^{2+} traces shown in 4A. 5A: Plateau fraction. 5B: Baseline values. 5C: oscillation frequency. 5D: oscillation amplitude. All values shown are means ± SEM. #, p< 0.05, ##, p< 0.01, ###, p< 0.0005, ####, p< 0.0001; n= at least 3 mice.

Figure 6. **Tunicamycin treatment resulted in the appearance of electrical activity under sub-threshold glucose conditions.** Isolated mouse islets were treated with vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for 6, 12 and 16 hours in 11 mM glucose. The acute responses of islet membrane potential to 5 mM glucose solution under the conditions indicated are shown. Details are provided in the text. Consistent results were observed in at least 3 mice.

Figure 7. **Tunicamycin increased the amount of insulin secreted under sub-threshold glucose conditions.** Isolated mouse islets were treated with vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for 6, 12 and 16 hours in 11 mM glucose. Insulin secretion was measured by acutely exposing 10 islets to 5 mM glucose for 30 minutes for each experimental condition. 7A: Both secreted insulin and insulin remaining in the extracted islets were quantified in triplicate by ELISA and normalized to total protein concentration (BCA protein assay). 7B: The percent insulin that was secreted was obtained by dividing the secreted insulin by total insulin (the sum of secreted insulin and insulin in the lysate). Values shown are means ± SEM. #, p< 0.05 compared with control conditions; n= 3 mice.

Figure 8. **Increased cytosolic Ca^{2+}, membrane potential oscillations and insulin secretion observed after tunicamycin treatment were mediated by store-operated Ca^{2+} entry (SOCE).** Islets were treated with vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for 16 hours, and were then acutely exposed to 5 mM glucose containing solution with or without YM58483 (YM, 10 µM). 8A: Cytosolic free Ca^{2+} changes. 8B: Membrane potential changes. 8C: Insulin secretion. Row Factor F(1, 12)= 24.25, p= 0.0004, Column Factor F(1, 12)= 8.923, p=0.0113, Interaction F(1, 12)= 3.170, p= 0.1003, by two-way ANOVA. 8D: Percentage insulin secreted. Row Factor F(1, 12)= 24.75, p= 0.0003, Column Factor F(1, 12)= 9.572, p=0.0093, Interaction F(1, 12)= 3.446, p= 0.0881, by two-way ANOVA. All values shown are means ± SEM. #, p< 0.05, ##, p< 0.01; n= at least 3 mice, by two-way ANOVA with post hoc multiple comparison by Tukey’s procedure.

Figure 9. **STIM1 knockdown inhibited TM-triggered cytosolic Ca^{2+} transients.** 9A: STIM1 siRNA knockdown in INS-1(832/13) cells was assessed by qPCR 48 hours after siRNA transfection. 9B: Representative western blots showing the expression of STIM1 and ORAI1 48 hours after transfection with STIM1 siRNA compared to the negative control siRNA. 9C: Quantitative protein
levels of STIM1 and ORAI1 are shown graphically. INS-1(832/13) cells were treated with vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for 16 hours after transfecting with STIM1 siRNA or negative control siRNA for 48 hours. 9D: The responses of cytosolic free Ca\(^{2+}\) to solution containing 5 mM glucose. 9E: Percentage of active INS-1(832/13) cells showing Ca\(^{2+}\) transients. All values shown are means ± SEM. #, p< 0.05, ##, p< 0.01, ###, p< 0.0005, ####, p< 0.0001; n= 3 times repeated per condition.

Figure 10. Increased beta cell death seen after tunicamycin treatment was not mediated by store-operated Ca\(^{2+}\) entry (SOCE). INS-1(832/13) cells or mouse islets were treated with vehicle control (DMSO), tunicamycin (TM, 10 µg/ml), DMSO+YM58483 (YM, 10 µM) or TM+YM58483 for 10A: 16 hours, 10B: 6 hours and 10C: 24 hours in 11 mM glucose containing culture medium. 10A: Summary of raw data showing basal ER Ca\(^{2+}\) ratios obtained from islets of 3 mice. Row Factor F(1, 82)= 69.54, p< 0.0001, Column Factor F(1, 82)= 0.1515, p=0.6981, Interaction F(1, 82)= 0.01884, p= 0.8912, by two-way ANOVA. 10B: Expression level of spliced XBP1 mRNA. Row Factor F(1, 8)= 23.75, p= 0.0012, Column Factor F(1, 8)= 0.3909, p=0.5493, Interaction F(1, 8)= 0.003133, p= 0.9567, by two-way ANOVA. 10C: Cell death observed in INS-1(832/13) cells stained with propidium iodide (PI) and quantified using flow cytometry. Row Factor F(1, 12)= 20.29, p= 0.0007, Column Factor F(1, 12)= 0.008395, p=0.9285, Interaction F(1, 12)= 0.1708, p= 0.6867, by two-way ANOVA. The results were obtained from 3 different batches of INS-1(832/13) cells. All values shown are means ± SEM. #, p< 0.05; n= 3 times repeated per condition, by two-way ANOVA with post hoc multiple comparison by Tukey’s procedure.

Figure 11. Tunicamycin did not affect cytosolic free Ca\(^{2+}\) under above-threshold glucose conditions. Isolated pancreatic mouse islets were treated with a vehicle control (DMSO) or tunicamycin (TM, 10 µg/ml) for 16 hours in 11 mM glucose. 11A: The responses of cytosolic free Ca\(^{2+}\) to solution containing 11 mM glucose under the indicated conditions. 11B: Percentage of oscillating islets. Summary findings for the data are shown in 11C-F. 11C: Plateau fraction. 11D: Baseline values. 11E: oscillation frequency. 11F: oscillation amplitude. 11G and 11H: Insulin secretion was measured by acutely exposing 10 islets to 11 mM glucose for 30 minutes for each experimental condition. 11G: Both secreted insulin and insulin content and 11H: the percent insulin were quantified as described in Figure 7. All values shown are means ± SEM. ##, p< 0.01; n= at least 3 mice.

Figure 12. Alternative ER stress inducers also increased cytosolic free Ca\(^{2+}\) under sub-threshold glucose conditions. 12A: Isolated pancreatic mouse islets were treated with a vehicle control (DMSO) or thapsigargin (TG, 200 nM) in 11 mM glucose. 12B: Mouse islets were cultured in 11 mM glucose (untreated control) or 25 mM glucose for 16 hours. The responses of cytosolic free Ca\(^{2+}\) to solution containing 5 mM glucose under the indicated conditions. n= at least 3 mice.
**Figure 13. The effect of tunicamycin on gene expression.** INS-1(832/13) cells were treated with a vehicle control (DMSO) or tunicamycin (TM, 10 μg/ml) for 16 hours in 11 mM glucose. 13A: Representative western blots show the level of STIM1 and ORAI1. GAPDH is shown as a loading control. 13B: Quantitative protein levels are shown graphically. All values shown are means ± SEM; n= 3 times repeated per condition.

**Figure 14. Scheme of beta cell death and increasing insulin secretion mediated by tunicamycin.**
**Figure 1**

**A)** INS 832/13 cells

- **XBP1 mRNA (fold change)**
  - Spliced xbp1 (TM)
  - Total xbp1 (TM)
  - Spliced xbp1 (DMSO)
  - Total xbp1 (DMSO)

**B)** mouse islets

- **XBP1 mRNA (fold change)**
  - Spliced XBP1
  - Total XBP1

**C)** INS 832/13 cells

- **CHOP mRNA (fold change)**
  - DMSO
  - TM

**D)**

- **BiP**
  - DMSO
  - TM
- **GAPDH**
- **Relative Intensity of BiP**
  - DMSO
  - TM
  - Tubulin

**E)**

- **Relative Intensity of BiP**
  - DMSO
  - TM

**F)**

- **BiP**
  - DMSO
  - TM
- **Tubulin**
  - DMSO
  - TM

**G)**

- **Relative Intensity of BiP**
  - DMSO
  - TM
Figure 2

A) 

|        | DMSO | TM   | DMSO |
|--------|------|------|------|
| 16     | 6    | 12   | 16   |
| 6      |      |      |      |
| 12     |      |      |      |
| 16     |      |      |      |

**PARP**

**Tubulin**

B) 

![Graph showing % PARP Cleavage](image)

C) 

![Graph showing PI positive cells](image)

D) 

![Graph showing Sub-G1 phase apoptotic cells](image)
Figure 3

A) D4ER (535/470) normalized to F0

- **DMSO 6h**
  - Time (min):
    - 0, 5, 10, 15, 20, 25, 30
  - D4ER values: 0.94, 0.96, 0.98, 1.00, 1.02, 1.04

- **DMSO 12h**
  - Time (min):
    - 0, 5, 10, 15, 20, 25, 30
  - D4ER values: 0.92, 0.94, 0.96, 0.98, 1.00, 1.02

- **DMSO 16h**
  - Time (min):
    - 0, 5, 10, 15, 20, 25, 30
  - D4ER values: 0.97, 0.98, 0.99, 1.00, 1.01

- **TM 6h**
  - Time (min):
    - 0, 5, 10, 15, 20, 25, 30
  - D4ER values: 0.92, 0.94, 0.96, 0.98, 1.00, 1.02

- **TM 12h**
  - Time (min):
    - 0, 5, 10, 15, 20, 25, 30
  - D4ER values: 0.94, 0.96, 0.98, 1.00, 1.02

- **TM 16h**
  - Time (min):
    - 0, 5, 10, 15, 20, 25, 30
  - D4ER values: 0.97, 0.98, 0.99, 1.00, 1.01

B) D4ER (535/470) by guest on May 5, 2020

- **DMSO 6h**
  - 6h of treatment
  - D4ER values: 1.00, 1.02, 1.04

- **TM 6h**
  - 6h of treatment
  - D4ER values: 1.00, 1.02, 1.04

- **DMSO 12h**
  - 12h of treatment
  - D4ER values: 1.00, 1.02, 1.04

- **TM 12h**
  - 12h of treatment
  - D4ER values: 1.00, 1.02, 1.04

- **DMSO 16h**
  - 16h of treatment
  - D4ER values: 1.00, 1.02, 1.04

- **TM 16h**
  - 16h of treatment
  - D4ER values: 1.00, 1.02, 1.04

### Statistical Significance

#### 6h of treatment
- **DMSO vs TM**: 1.00, 1.02, 1.04
- **DMSO vs TM**: 1.00, 1.02, 1.04

#### 12h of treatment
- **DMSO vs TM**: 1.00, 1.02, 1.04
- **DMSO vs TM**: 1.00, 1.02, 1.04

#### 16h of treatment
- **DMSO vs TM**: 1.00, 1.02, 1.04
- **DMSO vs TM**: 1.00, 1.02, 1.04

### Notes

- D4ER (535/470) values are normalized to F0.
- Time values are measured in minutes.
- DMSO and TM treatments are indicated.
- Statistical significance is denoted by symbols.

By guest on May 5, 2020
Figure 4

A) Graphs showing Fura-2 Ratio (340/380 nm) for different time points of treatment with DMSO and TM:
- 6h DMSO
- 12h DMSO
- 16h DMSO
- 6h TM
- 12h TM
- 16h TM

B) Bar chart showing percentage of oscillating and off/silent states for different hours of treatment:
- DMSO
- TM

Legend:
- Oscillating
- Off/Silent
Figure 6

- **DMSO 6h**
- **DMSO 12h**
- **DMSO 16h**
- **TM 6h**
- **TM 12h**
- **TM 16h**

**Membrane Potential (mV) vs. Time (min)**

- DMSO treatments at 6, 12, and 16 hours show decreasing membrane potential over time.
- TM treatments at 6, 12, and 16 hours show periodic fluctuations in membrane potential with time.

The graphs illustrate the effects of DMSO and TM on membrane potential over different time periods.
Figure 7

A)  

B)
Figure 9

A) STIM1 mRNA (fold change)

B) Fura-2 Ratio (340/380 nm)

C) Relative Intensity

D) Time (min)

E) %

siCon | DMSO | siSTIM1 | TM
--- | --- | --- | ---
Active | 20 | 40 | 60 | 80 | 100
Off/Silent | 0 | 0 | 0 | 0 | 0
Figure 11

A) DMSO vs. TM

B) % Oscillating vs. Plateau

C) Plateau fraction vs. Baseline

D) Frequency (peaks/min)

E) Amplitude

F) Insulin content vs. Secreted insulin

G) % Insulin
Figure 12

A) DMSO

B) untreated

25 mM glucose
Figure 13

A)

| Protein   | DMSO  | TM    |
|-----------|-------|-------|
| STIM1     | 100 kD| 75 kD |
| ORAI1     | 75 kD | 50 kD |
| GAPDH     | 37 kD | 25 kD |

B)

![Graph showing relative intensity of STIM1 and ORAI1](image)

- **STIM1 (77kD) --DMSO**
- **STIM1 (77kD) -TM**
- **STIM1 (<77kD) --DMSO**
- **STIM1 (<77kD) -TM**
- **ORAI1 --DMSO**
- **ORAI1 --TM**
Tunicamycin

Beta cell stress / ER Calcium

Beta cell death

SOCE

Cytosolic Calcium/
electrical oscillations

Insulin secretion
ER stress increases store-operated Ca\(^{2+}\) entry (SOCE) and augments basal insulin secretion in pancreatic β cells
Irina X. Zhang, Jianhua Ren, Suryakiran Vadrevu, Malini Raghavan and Leslie S. Satin

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