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Stress-induced dissociations between intracellular calcium signaling and insulin secretion in pancreatic islets

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Abstract

In healthy pancreatic islets, glucose-stimulated changes in intracellular calcium ($[Ca^{2+}]_i$) provide a reasonable reflection of the patterns and relative amounts of insulin secretion. We report that $[Ca^{2+}]_i$ in islets under stress, however, dissociates with insulin release in different ways for different stressors. Islets were exposed for 48-hours to a variety of stressors: cytokines (low-grade inflammation), 28mM glucose (28G, glucotoxicity), free fatty acids (FFAs, lipotoxicity), thapsigargin (ER stress), or rotenone (mitochondrial stress). We then measured $[Ca^{2+}]_i$ and insulin release in parallel studies. Islets exposed to all stressors except rotenone displayed significantly elevated $[Ca^{2+}]_i$ in low glucose, however, increased insulin secretion was only observed for 28G due to increased nifedipine-sensitive calcium-channel flux. Following 3-to-11mM glucose stimulation, all stressors substantially reduced the peak glucose-stimulated $[Ca^{2+}]_i$ response (first phase). Thapsigargin and cytokines also substantially impacted aspects of calcium influx and ER calcium handling. Stressors did not significantly impact insulin secretion in 11mM glucose for any stressor, although FFAs showed a borderline reduction, which contributed to a significant decrease in the stimulation index (11mM:3mM glucose) observed for FFAs and also for 28G. We also clamped $[Ca^{2+}]_i$ using 30mM KCl + 250uM diazoxide to test the amplifying pathway. Only rotenone-treated islets showed a robust increase in 3-to-11mM glucose-stimulated insulin secretion under clamped conditions, suggesting that low-level mitochondrial stress might activate the metabolic amplifying pathway. We conclude that different stressors dissociate $[Ca^{2+}]_i$ from insulin secretion differently: ER stressors (thapsigargin, cytokines) primarily affect $[Ca^{2+}]_i$ but not

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Eden A. Dejene: wrote manuscript, acquired and analyzed data, edited manuscript

Kathryn L. Corbin: acquired data, edited manuscript

Craig S. Nunemaker: wrote manuscript, analyzed data, edited manuscript, provided funding. C.S.N. is guarantor of this article and, as such, has full access to all the data in the study and takes responsibility for the integrity and accuracy of the data.

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conventional insulin secretion and ‘metabolic’ stressors (FFAs, 28G, rotenone) impacted insulin secretion.

GRAPHICAL ABSTRACT

Keywords

islets; beta-cells; cytokines; calcium; insulin; low-grade inflammation; diabetes; chronic; free fatty acid; palmitate; oleate; linoleate; glucotoxicity; high glucose; endoplasmic reticulum; thapsigargin; unfolded protein response; ER stress; oxidative stress; rotenone; interleukin; IL-1beta; IL-6

1. Introduction

The primary function of beta cells is to synthesize and secrete insulin, a critical regulator of blood glucose. At the level of the individual beta-cell, the ‘Consensus Model’ provides a detailed description of the cellular response to glucose stimulation as summarized in [1,2]. In this model, the beta cell is electrically silent at low glucose concentrations (<~5 mM, representing fasting conditions), secreting insulin at a low, basal rate. In response to sharp increases in blood glucose, beta cells take up glucose through glucose transporters. During this time, the endoplasmic reticulum (ER) and mitochondria take up [Ca²⁺]_i in response to increased glucose metabolism, which causes an overall dip in [Ca²⁺]_i. The resulting increase in the ATP to ADP ratio closes ATP-sensitive potassium channels (K_{ATP}-channels). As the dominant resting conductance of the beta cell, K_{ATP}-channels normally hyperpolarize the beta-cell membrane under basal glucose conditions. The closure of K_{ATP}-channels in response to increased glucose metabolism depolarizes the cell membrane. This initiates the repetitive firing of calcium-dependent action potentials and the influx of calcium into the beta cell, resulting in increased calcium in the mitochondria, ER, and nucleus. A large spike in calcium influx leads to the exocytosis of a readily releasable pool of docked insulin granules, which is termed first phase insulin release. Following first phase insulin release, [Ca²⁺]_i and insulin secretion remain elevated throughout the second phase response for as long as glucose remains elevated.

Because [Ca²⁺]_i is a strong trigger of exocytosis, both glucose-stimulated [Ca²⁺]_i (GSCa) and glucose-stimulated insulin secretion (GSIS) show similar trajectories under these conditions. GSCa can thus be used to assess the physiological response of islets to glucose stimulation. [Ca²⁺]_i imaging is advantageous because it provides high temporal precision of real-time changes in response to stimuli at the level of the individual islet [3]. Changes in the

latency, trajectory, and amplitude of the triphasic GSCa response may indicate specific defects in stimulus-secretion coupling or other aspects of islet dysfunction. However, there are also amplifying processes that operate in parallel with the pathways of the Consensus Model to couple glucose uptake and metabolism with insulin exocytosis [4-6]. The amplifying pathway allows additional insulin release to occur independently of changes in $[Ca^{2+}]_i$ and thus provide a means by which the processes of insulin release and $[Ca^{2+}]_i$ signaling can dissociate from one another.

The exposure of islets to stress can further dissociate calcium signaling and insulin release. In this study, we systematically examined the effects of five putative triggers of islet stress in type 2 diabetes, each of which affected a different target: 1) rotenone to partially inhibit mitochondrial complex I [7,8], 2) thapsigargin to disrupt ER function by blocking sarco(endo)plasmic calcium ATPase (SERCA) pump activity [9,10], 3) cytokines to target the inflammatory response [11,12], 4) high glucose to mimic a state of hyperglycemia and induce hypersecretion of insulin [13,14], or 5) a combination of palmitate, oleate, and linoleate to target free fatty acid metabolism [15]. At much higher concentrations, each of these treatments is associated with toxicity and cell death, but we chose concentrations that approximated physiological levels (or subpharmacological levels) and did not induce cell death. Following 48-hr treatment with these various stressors, we measured $[Ca^{2+}]_i$ and insulin secretion in low (3mM) and stimulatory (11mM) glucose to examine calcium entry through voltage-gated calcium channels, $[Ca^{2+}]_i$ handling by the ER, and the consensus pathway and amplifying pathways of insulin secretion. We show that all stressors reduced the first phase calcium response to glucose stimulation, an established marker of early beta-cell decline for insulin in type 1 diabetes [16] and type 2 diabetes [17,18], to a similar degree, yet glucose-stimulated insulin secretion was not affected in all cases. Our data suggest that stressors related to nutrient metabolism (FFAs, glucose, and rotenone) have greater impact on insulin secretion, whereas stressors associated with the ER (cytokines and thapsigargin) produced substantial changes in $[Ca^{2+}]_i$ handling without affecting insulin secretion.

2. Materials and methods

2.1 Mice

Studies were conducted using outbred CD-1 mice at ages of 8-12 weeks (Charles River Laboratories, MA). All animal procedures were approved by the University of Virginia (UVA) Institutional Animal Care and Use Committee.

2.2 Islet isolation and treatment

Pancreatic islets were isolated by collagenase-P digestion (Roche Diagnostics, Indianapolis, IN) followed by centrifugation with Histopaque 1100 (Sigma-Aldrich, St. Louis, MO) as previously described [19]. Islets were incubated overnight in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin to allow recovery from collagenase digestion before further treatment. Various islet stressors were prepared as follows: Rotenone and thapsigargin were purchased from Sigma-Aldrich and prepared in stocks of DMSO to final concentrations less than 0.1%. Glucose-free RPMI

medium (10% FBS and 1% penicillin/streptomycin) was supplemented with 1M glucose stock to produce the 28G condition. Stocks of the murine forms of IL-1B and IL-6 (purchased from R&D Systems, Inc., Minneapolis, MN) were prepared in PBS with 0.1% BSA. Palmitate, oleate, and linoleate were purchased from Sigma-Aldrich and were prepared in 100mM stock concentrations in methanol and stored in -80 degrees C. To treat the islets, the required amount of each fatty acid was taken in a glass tube and dried under a stream of nitrogen to remove methanol. The dried mixture of fatty acids was then resuspended in the RPMI medium with 0.1% BSA followed by vortexing and sonication. Islets were exposed to final concentrations of each stressor as follows: 28G/glucotoxicity (28mM glucose), free fatty acids (50 uM palmitate + 100 uM oleate + 50 uM linoleate), ER stress (100nM thapsigargin), low-grade inflammation (10 pg/ml IL-1 β + 20 pg/ml IL-6), or mitochondrial stress (20nM rotenone). Following 48-hr exposure, islets were removed from the treatment conditions and tested for function by measuring glucose-stimulated changes in $[Ca^{2+}]_i$ and insulin release. These doses had effects on islet function but did not produce cell death as measured by propidium iodide fluorescence ($P > 0.38$ for each condition compared to basal cell death in untreated control islets; $n = 18-37$ islets/condition).

2.3 Glucose-stimulated calcium measurements

$[Ca^{2+}]_i$ was measured using the ratiometric $[Ca^{2+}]_i$ indicator fura-2 AM as previously described [20,21]. Briefly, Cell Tracker Red CMTPX (CTR, Invitrogen), a membrane penetrating fluorescent probe, was used in order to distinguish stressor-treated islets from control islets by selectively labeling only one of the two groups, thus enabling a simultaneous comparison of the two treatment groups (see [21] for additional details). All islets were loaded with 1 uM fura-2 AM in KRB solutions containing 3mM glucose for 30 minutes (with one of two groups for each recording also loaded with 200 nM CTR and the other not), washed, and then transferred to a recording chamber. Islets were washed of excess surface fura-2AM by perfusing islets with KRB solution containing 3mM glucose for ~15-min at ~400ul/min using a peristaltic pump (Minipuls 2, Gilson, Middleton, WI). Islets were recorded with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Japan) attached to an Olympus BX51WI fluorescence microscope (Olympus, Tokyo, Japan) using 340 and 380 nm excitation light and 510 nm emission as previously described [22]. Data were recorded and analyzed with IP Lab software Version 4.0 (Scanalytics, Rockville, MD).

2.4 Glucose-stimulated insulin secretion

Islets were tested for glucose-stimulated insulin secretion as described previously [20]. Briefly, islets were pre-incubated at 37 °C and 5% CO₂ for one hour in a standard KRB solution containing no glucose in order to reduce initial variance in insulin release rates among islets. Islets were then washed and incubated in KRB supplemented with 3 mM glucose for one hour followed by treatment with KRB containing 11 mM glucose for one hour. The supernatant was collected after each treatment and insulin concentration in the supernatant was measured by an ELISA method (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

2.5 Data analysis

All calcium studies were conducted in triplicate, with each trial including multiple islets (typically $n=5-10$ islets) from a stressor condition paired with untreated controls that were recorded simultaneously. Mean fura-2 ratios $\pm$ SEM from all islets from each stress condition were analyzed by one-way ANOVA; this required that islets from 5 untreated controls paired to each of the 5 stressors for all three trials were combined together (a total of 107 untreated islets from 14 recordings; one trial from the cytokine treatment was removed as an outlier). In addition, islets from each stress condition were analyzed against their matching untreated controls using a two-tailed t test to confirm the same significant effects for basal calcium, phase 0, phase 1, and phase 2 calcium as found by ANOVA. For data presentation purposes, fura-2 ratios were converted to estimated nanomolar calcium concentrations as described previously [12] using methods originally described in [23]. Insulin data were analyzed by one-way ANOVA and two-tailed t test. The less stringent t test was used to identify 'borderline' effects on insulin secretion ($P<0.05$) that were not considered significant by ANOVA. A one-tailed t test was used to show reversal of effects of 28G with nifedipine treatment since this was a unidirectional hypothesis (Figure 3C). For all experiments, a p-value of <0.05 was used as an indication of statistical significance, with # indicating a p-value between 0.05 and 0.10 as borderline significant.

3. Results

3.1 $[Ca^{2+}]_i$ measurements provide details on the kinetics of glucose stimulation in islets

We first examined the effects of each stressor on GSCa responses to examine detailed changes in the kinetics of glucose stimulation in islets. As shown in Figure 1, this technique measures the amplitude, timing, and duration of the triphasic response to glucose stimulation: phase 0, a small dip in $[Ca^{2+}]_i$ due primarily to the ER taking up $[Ca^{2+}]_i$ in response to glucose; phase 1, a rapid spike in calcium influx that triggers insulin release caused by the closure of K_{ATP} -channels and consequent opening of voltage-gated calcium channels in response to a shift in ATP/ADP; phase 2, a plateau in $[Ca^{2+}]_i$ associated with ongoing insulin release. These phases are labeled for the untreated controls in Figure 1A. Each panel in Figure 1 displays the average $[Ca^{2+}]_i$ at 5-sec intervals for five or more islets treated with a specific stressor (solid lines) paired with five or more untreated controls (dotted line) that were recorded simultaneously. Several stress conditions elevated $[Ca^{2+}]_i$ in low glucose, including 48-hour treatment with cytokines (Figure 1A), 28mM glucose (Figure 1B), free fatty acids (Figure 1C), and thapsigargin (Figure 1D). Only rotenone had no significant effect on basal $[Ca^{2+}]_i$ (Figure 1E). In 11mM glucose, each stressor had inhibited peak $[Ca^{2+}]_i$ responses to glucose stimulation (phase 1) to a similar degree, and each stressor inhibited phase 2 $[Ca^{2+}]_i$ responses to varying degrees.

3.2 Elevated $[Ca^{2+}]_i$ in low glucose does not lead to increased insulin release for most stress conditions

The data presented in Figure 1 were from one of three trials. Values for mean $[Ca^{2+}]_i$ for all three trials in low glucose are quantified in Figure 2A, which totaled 107 untreated islets and 115 stress-treated islets (see Figure Legend for additional details). Cytokines, thapsigargin, free fatty acids, and high glucose treatments all produced a significant elevation in basal

[Ca²⁺]_i. For insulin release in 3 mM glucose, however, only the islets treated with high glucose (28G) showed significantly increased insulin secretion compared to untreated controls as shown in Figure 2B (FFAs showed a mild increase by t test, P<0.05). Rotenone had no significant effect on basal [Ca²⁺]_i or insulin secretion.

3.3 Calcium influx through nifedipine-sensitive channels drives low-glucose insulin release in islets exposed to 28 mM glucose

We examined whether calcium influx through voltage-gated calcium-channels links the elevated basal [Ca²⁺]_i with increased insulin release observed in 28G but not others. Islets were treated with the voltage-gated calcium channel blocker nifedipine in 3mM glucose to identify possible differences in calcium influx through the plasma membrane. As shown in the representative example in Figure 3A, islets exposed to 28 mM glucose for 48 hours had clearly elevated basal [Ca²⁺]_i compared to untreated control islets. Acute treatment with 10uM nifedipine substantially reduced [Ca²⁺]_i levels to near normal in these islets. Note that the islets were removed from 28mM glucose (as all islets were removed from their respective conditions) and incubated in 3mM glucose for ~45 min before the start of the recording. Control islets that were not exposed to 28mM glucose showed no change in [Ca²⁺]_i when exposed to nifedipine.

The same experiment utilizing nifedipine treatment was used for all other stress conditions. As shown in Figure 3B, the 28 mM glucose treatment had by far the greatest effect of nifedipine on [Ca²⁺]_i. To further show that calcium influx is responsible for increased insulin release in the 28G group, nifedipine was included in GSIS studies. As shown in Figure 3C, the elevated insulin release brought about by culturing islets in 28 mM glucose was eliminated in the presence of nifedipine.

For other stress conditions, these experiments highlight a dissociation between [Ca²⁺]_i and insulin release. Cytokine-treated islets showed increased voltage-gated calcium influx that did not lead to increased insulin release (compare Figure 3B with Figure 2B). In contrast, FFA-treated islets showed borderline increased insulin release in 3 mM glucose (significant by t test, but not by ANOVA), but no corresponding increase in nifedipine-sensitive calcium influx (compare Figure 2B with Figure 3B).

3.4 ER calcium contributions to islet stress responses

We also examined possible contributions of the ER to [Ca²⁺]_i in low glucose for each stress condition. Following 48-hour treatments, islets were removed from their respective stressors and placed in KRB solution with 3mM glucose for 30-min for fura-2 loading and then transferred to the recording chamber. Islets were treated with nifedipine throughout the recording to remove any contributions of calcium influx through voltage-gated calcium channels in the plasma membrane. Following 3-min of baseline readings, islets were treated with cyclopiazonic acid (CPA), which causes ER calcium to drain by blocking the SERCA pumps responsible for ER calcium uptake. As shown from one trial in Figure 4A, the CPA-induced increase in [Ca²⁺]_i reflects the release of ER calcium into the cytosol, which was enhanced among islets exposed to 28 mM glucose for 48 hours. In contrast, thapsigargin pretreatment nearly abolished the CPA-induced [Ca²⁺]_i increase, as shown in Figure 4B. As

quantified in Figure 4C, CPA-induced ER calcium release was inhibited to the greatest degree among islets exposed to thapsigargin for 48 hours (n=3 trials); this indicates lasting effects on ER calcium handling even after removal of thapsigargin and is consistent with established effects of thapsigargin-induced ER stress [24,25]. ER calcium storage was augmented in islets treated with 28G. No effects were observed for cytokines, FFAs, or rotenone.

We also examined the phase 0 response to glucose stimulation, another estimate of ER calcium handling by measuring the transient dip in $[Ca^{2+}]_i$ accompanying increased ER calcium uptake in response to glucose stimulation [9]. As shown in Figure 4D, thapsigargin-pretreated islets had the smallest phase 0 response to glucose stimulation, which is consistent with reduced ER calcium handling [9,22]. Cytokines also reduced the phase 0 response, which is consistent with previous observations demonstrating impaired ER function [3,12]. Rotenone, 28G, and FFAs showed little difference from controls, suggesting relatively normal ER calcium responses to glucose stimulation.

3.5 $[Ca^{2+}]_i$ vs. insulin in stimulatory glucose

We also compared GSCa and GSIS in 11 mM glucose. As quantified in Figure 5A, each stressor produced similar inhibitory effects on the peak $[Ca^{2+}]_i$ (phase 1) response to glucose stimulation. The phase 2 $[Ca^{2+}]_i$ response that is associated with ongoing insulin release was also mildly reduced by most stressors, with FFAs and rotenone showing the greatest inhibition. As shown in Figure 5C, these same stressors had the largest inhibitory effect on insulin release as well, although this finding was only borderline significant by t test ($P=0.03$ for FFAs and $P<0.06$ for rotenone). Islets treated with cytokines or with 28G actually displayed a mild increase in insulin release (not significant), which contradicts the inhibitory effects of these stressors on $[Ca^{2+}]_i$ responses. These findings again highlight dissociations between these two readouts of the stimulus-secretion coupling pathway for some stressors.

3.6 The role of the amplifying pathway in insulin and calcium differences

We hypothesized that these divergences between $[Ca^{2+}]_i$ and insulin might be due to the metabolic amplifying pathway, which can elicit additional insulin secretion even while $[Ca^{2+}]_i$ is kept constant [6]. To investigate this pathway, $[Ca^{2+}]_i$ was clamped at an elevated level by adding 30mM KCl to all solutions in order maintain a high state of plasma membrane depolarization and calcium influx. To prevent conventional K_{ATP} -channel-dependent changes in membrane potential in response to glucose stimulation, the K_{ATP} -channel opener diazoxide (250uM) was also included in all solutions. Under these conditions, any increase in insulin release between 3mM and 11mM glucose is thus outside of the normal K_{ATP} -channel-dependent and calcium-dependent pathway [6,26].

As shown in Figure 6, islets exposed to 28G had significantly higher insulin secretion in $[Ca^{2+}]_i$ -clamped conditions in 3 mM glucose. Cytokine exposure produced a similar increase in insulin release over untreated controls, although this was not significant by ANOVA ($P<0.10$ by t test). These were also the only two treatments to show significant nifedipine-sensitive calcium influx in low glucose when $[Ca^{2+}]_i$ was not clamped (see Figure 3B), suggesting a possible effects of 28G or cytokine exposure on permeability,

gating, or other aspect of calcium-channel activity. Alternatively, these results are also consistent with a left shifting of glucose sensitivity following prolonged exposure to hyperglycemia [27,28]. In contrast, insulin release was reduced compared to controls for thapsigargin and rotenone (significant by t test only, $P<0.05$), which is consistent with reduced insulin-secreting capacity of the consensus pathway.

In 11 mM glucose with $[Ca^{2+}]_i$ -clamped conditions, 48-hour treatment with 28 mM glucose again augmented insulin release compared to untreated controls ($P<0.05$) as did cytokines to a lesser extent ($P<0.10$ by t test only); FFAs, thapsigargin, and rotenone did not differ significantly from untreated islets (Figure 6B). The most intriguing result, however, was the relative increase in insulin release in the rotenone condition. As shown in Figure 6C, islets treated with rotenone showed the greatest increase in the ratio of insulin release between 3 and 11mM glucose (11G:3G), suggesting a substantial activation of the amplifying pathway. The effect of rotenone suggests a possible role for reactive oxygen species in amplifying-pathway-mediated insulin secretion [29]. In contrast, the 28G condition showed a reduction in the ratio of insulin release between 3 and 11mM glucose (significant by t test only, $P<0.05$), possibly due to maximal upregulation of insulin secretory pathways in low glucose.

3.7 Summary of findings

Findings of this study are summarized in Table 1 for all measurements of $[Ca^{2+}]_i$ and insulin secretion. Arrows indicate direction and the degree of statistical significance in comparison to untreated controls (1 arrow = $P<0.05$, 2 arrows = $P<0.01$, 3 arrows = $P<0.001$, dash = not significant by one-way ANOVA). As shown in Table 1, the only uniform effect across all five stressors was the phase 1 $[Ca^{2+}]_i$ response to glucose stimulation, which was strongly reduced for all stressors compared to untreated controls. Of interest, loss of first phase insulin secretion is a well-known marker for beta-cell decline in type 1 and type 2 diabetes [16-18]. However, the decreased response of $[Ca^{2+}]_i$ to glucose did not lead to significant changes in insulin secretion for any stressors. As also shown in Table 1, several similarities between stressors were observed among these 12 categories of $[Ca^{2+}]_i$ and insulin measurements. For example, rotenone and FFAs showed nearly identical $[Ca^{2+}]_i$ profiles to one another (except for slightly elevated basal $[Ca^{2+}]_i$ for FFAs but not rotenone). The overall key finding is that ER stressors (thapsigargin, cytokines) affect $[Ca^{2+}]_i$ without significantly affecting insulin secretion, whereas each metabolic stressor (FFAs, 28G, rotenone) had significant effects on various aspects of insulin secretion.

4. Discussion

The consensus model of GSIS includes calcium influx as a requisite step linking the increase in the ATP-to-ADP ratio that drives K_{ATP} -channel closure with the machinery of insulin exocytosis. Thus, GSCa patterns mirror GSIS under normal physiological conditions. Certain stress conditions can disconnect these processes, resulting in changes in $[Ca^{2+}]_i$ that are not accompanied by changes in insulin release, as well as changes in insulin release that are not accompanied by changes in $[Ca^{2+}]_i$. As detailed below, despite having overlapping mechanisms of action [30-33], each stressor seems to uniquely alter the cellular pathways involved with $[Ca^{2+}]_i$ regulation and insulin release.

4.1 Cytokines (low-grade inflammation)

Cytokines have long been associated with islet dysfunction and death in type 1 diabetes [34], but recently, increased circulating levels of proinflammatory cytokines have been implicated with obesity-induced chronic low-grade inflammation as a possible trigger of beta-cell decline in type 2 diabetes [35,36]. As we have shown previously, exposure to low-dose cytokines elevates basal $[Ca^{2+}]_i$ due to both depletion of ER calcium and increased calcium influx in basal glucose, as well as reduces phase 1 and phase 2 $[Ca^{2+}]_i$ responses to glucose stimulation [3,12]; findings that the present study also support. These changes in $[Ca^{2+}]_i$ handling did not result in any significant effect on insulin release in low or high glucose in this or previous studies [3,12], suggesting that another pathway of insulin release may compensate for these mild inhibitory effects of cytokines on $[Ca^{2+}]_i$ or that the $[Ca^{2+}]_i$ changes are not associated with insulin release. Interestingly, cytokine exposure increased insulin release when $[Ca^{2+}]_i$ levels were clamped high. It is possible that enhanced amplifying pathway activity coupled with reduced activity of conventional calcium-driven pathways offset one another to result in little change in insulin release overall. There is evidence that the amplifying pathway is dysfunctional in type 2 diabetes in the GK rat [37,38] and possibly in humans [6]. A dysfunctional amplifying pathway could explain our previous observation that cytokines reduce GSIS in islets from diabetes-prone mice but not healthy controls [12].

4.2. 28mM glucose (glucotoxicity)

Previous reports have shown that exposing islets to hyperglycemic conditions results in elevated calcium and increased insulin release in low glucose, thus reducing or eliminating additional increases in insulin secretion in response to higher glucose levels [27,28]. This left-shifting of glucose sensitivity can lead to chronic overstimulation and beta-cell failure marked by an overall reduction in insulin release [39,40], which is also observed in type 2 diabetes [41,42]. Our study demonstrated a similar left shifting in glucose sensitivity. In low glucose, islets from the 28G group had uniquely large increases in both insulin release and elevation in $[Ca^{2+}]_i$, among the stressors.

Chronic glucose exposure produced more complex effects in stimulatory glucose. We observed significant decreases in phase 1 and phase 2 $[Ca^{2+}]_i$ in the 28G group, yet insulin levels were elevated on average (although this effect was not significant due to high variability). When $[Ca^{2+}]_i$ was clamped at high levels using KCl + diazoxide, insulin release rates were more than 4-fold greater than controls for both the 3 and 11 mM glucose conditions compared to controls. The net effect of this substantial increase was a loss of glucose stimulation, as shown by the decrease in insulin release between 3 and 11 mM glucose (see Figure 6C). This complex set of results has been described and explained previously in a study of rat islets exposed to high glucose for one week [43]. Their results demonstrated two important effects of chronic glucose overstimulation: 1) Left-shifted (increased) glucose sensitivity and 2) Reduced calcium influx and/or degranulation, resulting in reduced peak insulin secretion in response to glucose stimulation. Our comparatively shorter treatment period may have prevented us from seeing the more deleterious effects of chronic hyperglycemia that results in greatly reduced insulin secretion and increased cell death [39,40,43].

4.3 Free Fatty Acids (lipotoxicity)

The effects of FFAs on $[Ca^{2+}]_i$ homeostasis and insulin release are well-documented but differ depending on the dose, duration, and type or types of free fatty acid used [44-46]. Individual free fatty acids are often used to investigate their physiological effects on cells, but FFAs are not found in isolation in the body and can even confound the effects of one another [47,48]. We thus used a 1:2:1 mixture of palmitate, oleate, and linoleate to represent the approximate physiological make-up and approximate levels that might be found within the blood stream in obesity [15].

We found that this mixture of FFAs produced the closest association between changes in $[Ca^{2+}]_i$ and insulin release of any stressors. Specifically, FFA treatment in 11mM glucose had the greatest inhibitory effect on phase 2 $[Ca^{2+}]_i$ and insulin release among stressors. Possible mechanisms for this effect include GPR40 activation leading to increased K_{ATP} -channel activity [49] or reduced cAMP signaling as shown for long-term exposure to palmitate [50]. We also observed increases in both $[Ca^{2+}]_i$ and insulin release in 3mM glucose. In addition, FFAs did not significantly affect the amplifying pathway, which is largely independent of changes in $[Ca^{2+}]_i$. Our results are consistent with other studies of chronic FFA exposure and suggest the effects on insulin release are calcium-mediated [51-54]. Oddly, the increased $[Ca^{2+}]_i$ in low glucose could not be explained by either a significant change in influx through nifedipine-sensitive calcium channels or a change in ER calcium handling despite these being known FFA targets [46,55,56]. Because FFA action on $[Ca^{2+}]_i$ handling is complex and multifaceted, the observed increase in global basal $[Ca^{2+}]_i$ could involve direct, indirect, or even opposing effects of the three FFAs used in this study. Future studies targeting calcium changes in microdomains in isolated beta-cells will be needed to determine how FFA-induced changes in $[Ca^{2+}]_i$ appear to reflect patterns of insulin release.

4.4 Thapsigargin (ER stress)

The ER is a key source and sink for $[Ca^{2+}]_i$, particularly with regard to stimulus-secretion coupling [57]. Problems with ER calcium handling have long been associated with islet dysfunction, as first shown by chronic thapsigargin treatment [9] and observations of islet $[Ca^{2+}]_i$ handling from diabetic (db/db) mice [12,58]. In low glucose, chronic thapsigargin exposure elevated basal $[Ca^{2+}]_i$ and attenuated the phase 0 $[Ca^{2+}]_i$ response to glucose due to ER calcium depletion, as shown in Figure 4 and previously [9]. Chronic thapsigargin exposure has been shown to inhibit insulin release as much as 90% [9] and to induce apoptosis [24,59]. In our studies, we observed milder effects by using 100nM thapsigargin instead of the typical 1μM concentration. Thapsigargin pretreatment mildly reduced the first and second phases of the GSCa, accompanied by a slight drop in insulin release (not significant). Our data suggest that unfolded protein response compensated for the relatively low-levels of thapsigargin-induced ER stress to maintain fairly normal secretory function. However, thapsigargin treatment substantially reduced insulin release in low glucose when $[Ca^{2+}]_i$ levels were clamped high by KCl + diazoxide but showed no difference from controls in higher glucose. This suggests that relatively small perturbations in ER calcium have greater impact in low-glucose conditions, but that most aspects of insulin secretion are maintained in conditions of mild ER stress. Of interest, islets from diabetes-prone mice

produce aberrant unfolded protein responses that associate with decreased insulin release and increased apoptosis, whereas normal islets do not [12].

4.5 Rotenone (mitochondrial stress)

Rotenone is a mitochondrial complex I inhibitor that partially blocks electron transfer in the mitochondrial respiratory chain, promoting the reaction with molecular O₂ and increasing superoxide formation [60]. There is evidence that ROS is necessary for normal insulin secretion [29,61], but excessive ROS can be detrimental [62]. There is some controversy about the effects of rotenone on ROS formation in pancreatic beta-cells, with studies showing substantial decreases [63-65] and others showing increases in ROS [29,66]. Although we did not measure ROS, effects of 20nM rotenone exposure for 48 hours produced no change in basal [Ca²⁺]_i or ER calcium handling in low glucose, but rotenone did substantially decrease both first and second phase GSCa and mildly decreased glucose-stimulated insulin secretion. These findings are consistent with a study of acute effects of rotenone on insulin secretion, which also showed reduced mitochondrial membrane potential, reduced ATP production, and reduced ROS levels [64]. Acute rotenone treatment has also been shown to directly stimulate insulin release and raise [Ca²⁺]_i by increasing ROS levels. Our finding is thus consistent with chronic low-dose rotenone leading to mild reductions in glucose-stimulated [Ca²⁺]_i and insulin secretion, possibly by decreasing ROS levels.

The intriguing effect of rotenone was its strong activation of the amplifying pathway, denoted by the nearly 5-fold increase over untreated controls in 11 vs 3 mM glucose in the presence of 30mM KCl + 250uM diazoxide. Although part of this effect was due to significantly lower insulin release levels in 3mM glucose (as also observed for thapsigargin), insulin release rates in 11mM glucose under [Ca²⁺]_i-clamped conditions was above that of untreated islets, suggesting a clear amplification of insulin release that is independent of changes in [Ca²⁺]_i. Rotenone, by blocking complex 1, may slightly reduce ATP production while increasing NAD(P)H concentration, which may act as a metabolic coupling factor that could potentially amplify the action of glucose in these islets [64].

4.6. Considerations and Conclusions

The intent of this study was to investigate several hypothesized mechanisms of islet decline in type 2 diabetes using concentrations that are sufficient to cause stress but not islet destruction. During chronic exposure, any of these “smoldering” stressors could ignite beta-cell failure. Considering the similarity in the net effect among stressors on GSCa, we were surprised to find such divergent effects from one stressor to the next on insulin secretion and some aspects of calcium handling. It should be noted that only a single dose was used for each treatment in these studies. However, each stressor was tested at multiple doses initially to determine a dose that was sufficient to impact islet calcium handling without causing cell death. Thus, the data in this paper represent effects on islet function, not effects on dying cells. Additionally, a single duration was chosen for this work. Many of these stressors have acute stimulatory effects, but inhibitory effects in longer-term treatments, so it is possible that some of our observed differences among stressors could be the result of different rates of progression along the same mechanism rather than unique mechanisms of action for each

stressor. This idea is consistent with observations of overlapping pathways related to cytokines, ER stress, oxidative stress, glucotoxicity, and free fatty acids [30-33,59]. Our results demonstrate that although all stressors affected $[Ca^{2+}]_i$ handling, metabolic stressors have greater lasting impact on glucose-stimulated insulin secretion compared to stressors that primarily target ER function. By describing how various forms of cellular stress play out in unique interactions and dissociations between $[Ca^{2+}]_i$ and insulin secretion, our study thus provides insights into normal islet stress responses and a foundation upon which to examine deficiencies in islet stress responses in conditions of obesity and/or type 2 diabetes.

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Highlights

- 48-hr exposure to different stressors impaired peak glucose-stimulated Ca^{2+} equally
- Thapsigargin & cytokines (ER stressors) affect Ca^{2+} ; minimal insulin release effect
- FFAs, 28G, & rotenone (metabolic stressors) affected Ca^{2+} -dependent insulin release
- Conclusion: Mild metabolic stress impacts insulin release more than mild ER stress

Figure 1.

Examples of calcium responses to glucose stimulation for normal untreated islets vs. islets exposed for 48 hours to (A) 28mM glucose, (B) free fatty acids (50 uM palmitate + 100 uM oleate + 50 uM linoleate), (C) 100nM thapsigargin, (D) cytokines (10 pg/ml IL-1 β + 20 pg/ml IL-6), or (E) 20nM rotenone. Each panel displays traces of the average $[Ca^{2+}]_i \pm$ SEM at 5-sec intervals from n=5-10 islets treated with the stressor described above and their n=5-10 untreated controls that were recorded simultaneously. Islets were stimulated from 3 to 11mM glucose at the 3-min mark in each panel. Note that oscillations appear in some panels but not others; this effect was unrelated to the stress condition involved and was due to similar overlapping oscillatory patterns among islets occurring in some trials.

Figure 2.

Differences in calcium levels and insulin release in low glucose (3 mM) for islets exposed to various stressors. (A) Basal calcium following 48-hour incubation with various stressors. A total of three trials were conducted for each stressor paired with untreated controls recorded simultaneously. Cytos = cytokines (n=14 untreated vs. n=17 cytos), 28G = 28mM glucose (n=24 untreated vs. n=28 28G), FFA = free-fatty acids (n=23 untreated vs. n=22 FFAs), thaps = thapsigargin (n=26 untreated vs. n=25 thaps), rotenone (n=20 untreated vs. n=23 rotenone). The same abbreviations are used in subsequent figures. Each column shows mean $[Ca^{2+}]_i \pm SEM$ in 3mM glucose (first 3-min of the recording). (B) Insulin release in low glucose for islets exposed to various stressors (N=6 sets of 20 islets/group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3.

Nifedipine blocks effects of chronic high glucose treatment. (A) Representative example of nifedipine effects in 3mM glucose for islets treated with high (28mM) glucose for 48 hours or untreated. (B) Relative changes in calcium in response to nifedipine as an estimate voltage-gated calcium channel activity levels for each stress condition (N=14 islets/condition or greater). (C) Effects of nifedipine on insulin secretion in low (3mM) glucose for islets exposed to 28 mM glucose for 48 hours (N=6 sets of 20 islets/group). *P<0.05, **P<0.01, #P<0.10.

Figure 4.

Comparative measurements of ER calcium. (A-B) Calcium traces showing the effects of 48-hour exposure to high glucose (A) or thapsigargin (B) on cyclopiazonic-acid-induced changes in $[Ca^{2+}]_i$. (C) Mean values for cyclopiazonic-acid-induced changes in $[Ca^{2+}]_i$ as an estimate of ER calcium storage for each stress condition. (D) Phase 0 ER calcium dip in response to glucose stimulation for islets exposed to various stressors. * $P < 0.05$, *** $P < 0.001$. $N = 12$ islets/condition or greater.

Figure 5.

Differences in calcium levels and insulin release in stimulatory glucose (11 mM) for islets exposed to various stressors. (A) Peak $[Ca^{2+}]_i$ (phase 1) response to glucose stimulation under each stress condition. (B) Plateau $[Ca^{2+}]_i$ (phase 2) response to glucose stimulation under each stress condition representing ongoing insulin secretion. (C-D) Insulin release in 11 mM glucose and the ratio of insulin release for 11mM:3mM glucose for islets exposed to various stressors (N=6 sets of 20 islets/group). Data for insulin secretion in 3mM glucose can be found in Figure 2B. *P<0.05, **P<0.01, ***P<0.001.

Figure 6.

Insulin release with calcium clamped to examine the amplifying pathway among islets exposed to various stressors. Following 48-hour treatment with various stressors, islets were incubated in solutions containing 30 mM KCl + 250 uM diazoxide to measure insulin secretion in either 3 mM glucose (A) or 11 mM glucose (B). (C) Insulin secretion due to the amplifying pathway as calculated by the ratio of 11G to 3G (N=7 sets of 20 islets/group). *P<0.05, **P<0.01.

Table 1

Summary of stressor effects on $[Ca^{2+}]_i$ and insulin secretion

Arrows indicate direction and the degree of statistical significance in comparison to untreated controls (1 arrow = $P < 0.05$, 2 arrows = $P < 0.01$, 3 arrows = $P < 0.001$).

	measurement	cytokines	28mM glucose	free fatty acids	thapsigargin	rotenone
$[Ca^{2+}]_i$	basal	↑	↑↑↑	↑	↑↑↑	—
	3 mM glucose	↑↑	↑↑↑	—	—	—
	nifedipine	—	↑↑↑	—	↑↓	—
	CPA	—	↑↑↑	—	↑↓	—
	phase 0	↓	—	—	↑↓	—
	11 mM glucose	↑↓	↑↓	↑↓	↑↓	↑↓
Insulin	phase 2	—	↑↓	↑↓	↑	↑↓
	3G	—	↑↑	—	—	—
	11G	—	—	—	—	—
	Glucose stimulated insulin secretion	—	↑	↑↓	—	—

	measurement	cytokines	28mM glucose	free fatty acids	thapsigargin	rotenone
	3G	—	↑↑	—	—	—
Amplifying pathway	11G	—	↑	—	—	—
	11G:3G amp	—	—	—	—	↑