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Evidence that low-grade systemic inflammation can induce islet dysfunction as measured by impaired calcium handling

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Summary

In obesity and the early stages of type 2 diabetes (T2D), pro-inflammatory cytokines are mildly elevated in the systemic circulation. This low-grade systemic inflammation exposes pancreatic islets to these circulating cytokines, but at levels ~100–1000x lower than seen within the islet during insulinitis, which have not been well described. We examined mouse islets treated overnight with a low-dose cytokine combination commonly associated with inflammation (TNF-alpha, IL-1 beta, and IFN-gamma). We then examined islet function primarily using intracellular calcium ($[Ca^{2+}]_i$), a key component of insulin secretion and cytokine signaling. Cytokine-treated islets demonstrated several features that suggested dysfunction including excess $[Ca^{2+}]_i$ in low physiological glucose (3mM), reduced responses to glucose stimulation, and disrupted $[Ca^{2+}]_i$ oscillations. Interestingly, islets taken from young db/db mice showed similar disruptions in $[Ca^{2+}]_i$ dynamics as cytokine-treated islets. Additional studies of control islets showed that the cytokine-induced elevation in basal $[Ca^{2+}]_i$ was due to both greater calcium influx through L-type-calcium-channels and reduced endoplasmic reticulum (ER) calcium storage. Many of these cytokine-induced disruptions could be reproduced by SERCA blockade. Our data suggest that chronic low-grade inflammation produces circulating cytokine levels that are sufficient to induce beta-cell dysfunction and may play a contributing role in beta-cell failure in early T2D.

Keywords

oscillations; islets; cytokines; transplantation; inflammation; calcium; insulin; biphasic; beta cells; endoplasmic reticulum; ER stress

1. Introduction

Proinflammatory cytokines play a prominent role in the pathophysiology of type 1 diabetes (T1D) [1]. Tumor necrosis factor alpha (TNF-a), interleukin (IL)-1B, and interferon-gamma (IFN-g) are the most abundant pro-inflammatory cytokines from the infiltrating immune cells, and these cytokines act synergistically to inflict direct inhibitory and cytotoxic effects on pancreatic beta cells [1–4]. Increasing evidence suggests that inflammatory mediators

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The authors have nothing to disclose.

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also play a significant role in the loss of beta-cell mass during the latter stages of type 2 diabetes (T2D) [5,6] and may arguably link the pathologies of the T1D and T2D [6–8].

Prior to the onset of T2D, the metabolic stress of obesity causes adipocytes to begin to secrete low levels of TNF- α [9,10]. This is thought to stimulate endothelial cells and preadipocytes to secrete monocyte chemoattracting protein-1 (MCP-1), which in turn attracts macrophages to the adipose tissue [9,10]. Macrophages are the source of several additional proinflammatory cytokines that include TNF- α , IL-1 β , and IFN- γ [1–4]. This results in a low-grade, chronic, systemic inflammation that elevates certain proinflammatory cytokines to several fold over basal levels into the low pg/ml range (100–1000x less than observed in direct islet infiltration). Recent clinical studies have identified several cytokines, including IL-1 β and TNF- α , as risk factors for subjects developing T2D based on elevated blood serum levels [11–13]. Whether cytokines at these low concentrations are sufficient to directly impact islet function is still unknown [14].

Intracellular calcium ($[Ca^{2+}]_i$) plays an important role in the signaling cascades of proinflammatory cytokines [15–17]. Cytokines can induce cell death by raising cytosolic calcium either through depletion of ER calcium [18–20] or by disrupting plasma membrane calcium flux [21]. In the NOD mouse model of T1D, increased expression of the low-voltage activated (LVA) T-type calcium channel was observed in beta-cells, resulting in elevated basal $[Ca^{2+}]_i$ [22]. In addition, chronic treatment of beta cells from control mice with a combination of cytokines upregulated expression of this channel [22]. Also, exposing beta cells to serum isolated from T1D patients induced apoptosis, but not when the L-type channel blocker nifedipine was included in the media [23,24].

Even without causing cell death, cytokines can negatively impact beta-cell function. Cytokines directly reduce insulin secretion in response to glucose [1], in part, through their effects on $[Ca^{2+}]_i$ handling since calcium is a key component of the insulin secretory pathway [25]. Reduced insulin release can exacerbate T2D in the face of greater insulin demand and can also exacerbate T1D in the face of progressive deterioration of beta-cell mass and function. Calcium is thus a potentially sensitive marker for assessing the effects of cytokine action on beta-cell function.

In this study, we examined the effects of cytokines at progressively lower concentrations to within the range of serum levels estimated from studies of patients with type 2 diabetes [11–13] in order to determine if mild cytokine exposure would disrupt islet function and/or induce islet damage. We subjected mouse islets overnight to the cytokines TNF- α , IL-1 β , and IFN- γ , and then examined several aspects of islet calcium handling as measures of islet function. We also compared these cytokine-induced effects with the changes in islet function that occur during the early stages in disease progression in the db/db mouse model of T2D. Our findings suggest that low-dose cytokine treatment is sufficient to disrupt normal islet function, which may contribute to beta-cell loss under conditions of chronic low-grade systemic inflammation.

2. Materials and methods

2.1 Mice and islet isolation

All mice were housed in a pathogen-free facility in the Center for Comparative Medicine at the University of Virginia (UVA) for use in all studies. Male CD-1 mice at ages of 8–12 weeks were used for studies involving cytokines (Charles River Laboratories, MA). Additional studies were conducted using male Cg-m $^{+}/+$ Lepr $^{db}/J$ (db/db) mice as a model of T2D, with age-matched with heterozygous db/db mice as controls (Jackson Laboratories,

Bar Harbor, ME). Studies were also repeated using age-matched C57BLKS/J (BKS) mice as controls.

Mice were euthanized according to IACUC approved protocol, and their pancreatic islets were isolated by collagenase digestion as previously described in detail [26]. Briefly, the pancreas was perfused through the common bile duct with 5 mL of 1.4 mg/mL collagenase P (Roche Diagnostics, Indianapolis, IN), then removed and incubated at 37 °C for 8–11 minutes in 1 mL HBSS solution. Following incubation, pancreatic tissue was centrifuged and resuspended in Histopaque 1100 (Sigma-Aldrich, St. Louis, MO) and centrifuged again to separate islets from acinar tissue. All islets were incubated overnight in RPMI-1640 medium (Invitrogen) to allow sufficient recovery time from collagenase digestion before any experiments were performed.

2.2 Cytokines and drug treatments

The cytokines chosen for this study are used widely as a means of inducing inflammatory responses in islets [2,4,27,28]. Mouse cytokines (R&D Systems, Inc., Minneapolis, MN) were used at fractional concentrations of the following full dose: 10 ng/ml for TNF-alpha, 5 ng/ml for IL-1beta, and 100 ng/ml for IFN-gamma in PBS [29]. Islets were treated overnight with cytokines following a full day of recovery from the isolation procedure. Thapsigargin and cyclopiazonic acid, which promote the release of ER calcium, and nifedipine, used to block L-type voltage-gated calcium channels, were purchased from Sigma-Aldrich (St. Louis, MO) and prepared in 1000x stock concentration in DMSO (final DMSO <0.1%). All experimental tests were performed 1–2 days after isolation.

2.3 Intracellular calcium ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured using the ratiometric $[Ca^{2+}]_i$ indicator fura-2 AM using previously described methods [29,30]. Briefly, Cell Tracker Red CMTPX (Invitrogen), a membrane penetrating fluorescent probe, was used in order to distinguish cytokine-treated islets from control islets, thus enabling a simultaneous comparison of the two treatment groups [30]. Islets were loaded with 1 μ M fura-2 AM and 0.2 μ M CTR (30–40 min), washed, and then 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. During each recording, islets were perfused in a small volume chamber (Warner Instruments, Hamden, CT) with KRB solution using a peristaltic pump (Gilson, France) at ~35°C by an in-line heater (Warner Instruments, Hamden, CT). Islets were incubated in 3 mM glucose for 15-min and then recorded to determine islet response to 28 mM glucose stimulation. Glucose-stimulated $[Ca^{2+}]_i$ response (GSCa) was calculated as the change in islet $[Ca^{2+}]_i$ as measured by fura-2 ratio (340/380 nm fluorescence) during the switch from 3 to 28 mM glucose. Data were analyzed with IP Lab software Version 4.0 (Scanalytics, Rockville, MD).

2.4 Cell death measurements

Islets were treated with 20 μ g/ml concentration PI and incubated for 10 min. Islets were imaged once under brightfield illumination to determine the islet borders and imaged again to measure PI fluorescence using 535 nm excitation and 617 nm emission. It should be noted that PI is a fairly blunt indicator of cell death that does not distinguish between apoptosis and necrosis, so it is possible for islets to be apoptotic but not positive for PI staining. However, PI staining was used in this context to simply guide the selection of cytokine doses to induce islet dysfunction for these studies.

2.5 Islet insulin secretion

After overnight incubation, islets were tested for insulin secretion as described previously [29,30]. Briefly, islets were preincubated at 37 °C and 5% CO₂ for 1 hour in a standard KRB solution, then washed and incubated in KRB supplemented with 3 mM glucose for 1 hour followed by a 1-hour treatment with KRB containing 28 mM glucose. The supernatant was collected after each treatment and insulin concentration in the supernatant was measured by an ELISA method (Merckodia, Uppsala, Sweden) according to the manufacturer's instructions. The intra-assay variation was 3.6% and inter-assay variation was <10%.

2.6 Data analysis and statistics

[Ca²⁺]_i patterns were smoothed with a 3-point moving average and analyzed initially by the pulse detection algorithm CLUSTER8, as used previously [29,31] using the following parameters: 10-sec minimum peak and minimum nadir size (2 points), 2.0 for t-score to detect peaks and nadirs, and no minimum value for peak amplitude. False positives were kept below 5% by using point-to-point noise in [Ca²⁺]_i during 3 mM glucose (non-oscillatory glucose concentration) as a standard deviation for pulse detection. To improve the detection of possible false negatives, the data were reanalyzed using a t-score of 1, producing the same results. In a subset of recordings, we observed no significant difference between CLUSTER8 calculations of period and direct measurement from the start of one cycle to the start of the next, so the direct method was used. Oscillatory capacity was calculated as the percentage of islets with oscillatory activity among all islets recorded. Fisher's exact test was used to compare oscillatory and non-oscillatory islets among db/db and het mice. A two-tailed t-test was used for all other two-group comparisons, with a p-value of p < 0.05 used as an indication of statistical significance. Statistical analysis was performed using Prism version 4 software.

3. Results

3.1 Dose-response curve for cytokine-induced cell death

To first establish the effective dose range to induce islet dysfunction, islets were treated with a combination of cytokines at several different fractions of the following dose: 10 ng/ml TNF-alpha 5 ng/ml IL-1beta + 100 ng/ml IFN-gamma [29,30]. As shown in Figure 1A, untreated islets excluded propidium iodide (PI), a dye used to estimate cell death, whereas cytokine-treated islets showed substantial PI uptake, particularly at higher cytokine concentrations (Figure 1B). Cytokine-induced PI fluorescence increased in a dose-dependent manner as shown in Figure 1C with an EC₅₀ of 0.0097 (~1/100 of the full dose). For subsequent experiments, we limited the cytokine concentrations to doses that were above (1/50), at (1/100), and below (1/500) the EC₅₀ (see also Methods for caveats).

3.2 Low-dose cytokine effects on glucose-stimulated [Ca²⁺]_i and insulin secretion

We investigated cytokine effects on islet function initially by measuring changes in [Ca²⁺]_i in response to glucose stimulation. Typical responses to glucose stimulation are shown in Figure 2A for islets treated with a cytokine dose of 1/100, 1/500 or 0. Cytokines substantially elevated basal [Ca²⁺]_i in 3 mM glucose and reduced peak stimulation during 28 mM glucose in these examples. We chose 28 mM glucose stimulation in order to elicit a maximal calcium response [32,33], which is needed to determine if cytokines reduce the full capacity to respond to glucose. Distinguishing between a decrease in stimulatory capacity and a shift in glucose sensitivity is more difficult using more physiological concentrations, such as 11 mM. As summarized in Figure 2B, these effects were consistent with mean values observed with 1/50, 1/100, and 1/500 cytokine doses, which ultimately reduced the overall [Ca²⁺]_i response to glucose stimulation (Figure 2C).

In parallel studies using islets from the same mice, glucose stimulated insulin secretion (GSIS) was measured using the same glucose concentrations as for GSCa. As shown in Figure 2D, GSIS gradually declined but did not reach significance until the 1/50 dose, a ten-fold higher concentration than needed to detect significant changes in GSCa. Similarly, the total stimulated insulin release (calculated by subtracting stimulated insulin secretion from basal insulin secretion, Figure 2E) and the ratio between stimulated and basal insulin (also called the stimulation index, Figure 2F) did not differ significantly until the 1/50 dose. It should be noted that the inherently greater variability in measuring insulin and the smaller number of replicates could obscure a possible trend toward reduced GSIS correlated with increasing cytokine dose. Beta-cell insulin exocytosis therefore maybe impaired by low-dose cytokine treatment; the effects of cytokines on $[Ca^{2+}]_i$, however, were much more readily detectable by comparison.

3.3 ER and ion-channel contributions to cytokine effects on basal $[Ca^{2+}]_i$

Cytokine effects were most striking on basal $[Ca^{2+}]_i$, even at the lowest cytokine dose used. To determine the mechanism underlying this effect, we investigated the two largest sources of $[Ca^{2+}]_i$: 1) plasma membrane calcium channels and 2) the endoplasmic reticulum (ER). As shown in Figure 3A, islets treated overnight with a 1/100 cytokine dose had substantially higher basal $[Ca^{2+}]_i$ in 3 mM glucose than untreated islets. However, this gap narrowed due to a substantial rise in $[Ca^{2+}]_i$ among untreated islets following thapsigargin treatment to promote ER calcium release. When L-type voltage-gated calcium-channels were additionally blocked by nifedipine treatment, the $[Ca^{2+}]_i$ gap was largely eliminated due to a much larger drop in $[Ca^{2+}]_i$ among cytokine-treated islets compared to untreated islets. Figure 3B shows that both ER (blocked by thapsigargin) and calcium influx (blocked by nifedipine) contribute to cytokine-induced increases in basal $[Ca^{2+}]_i$.

3.4 Cytokines disrupt islet $[Ca^{2+}]_i$ oscillations

$[Ca^{2+}]_i$ oscillations are endogenously produced in the presence of stimulatory (11 mM) glucose and are concurrent with pulses of insulin secretion [34]. Impaired pulsatile insulin secretion has been linked with T2D patients [35] and their non-diabetic relatives [36]. In our studies, untreated islets were typically oscillatory (34 of 49, 70%). As shown in the representative example in Figure 4A, all but one untreated islet (#5) showed robust and regular oscillatory activity. Overnight treatment with pro-inflammatory cytokines, however, reduced or eliminated oscillatory activity. As shown in the representative examples in Figure 4B, only 50% of islets showed any oscillatory activity at a 1/500 cytokine dose, and of those, only #5 showed the slow oscillations characteristic of the untreated islets. Islet #19 showed only rapid ~15 sec oscillations characteristic of electrically-driven bursts [33,37]. Oscillatory capacity was further degraded at the 1/100 cytokine dose, as shown by representative example in Figure 4C, in which only ~25% of islets displayed $[Ca^{2+}]_i$ oscillations (represented by #17). These findings are summarized in Figure 4D-F for the various cytokine doses. Percent of oscillatory islets (Figure 4D), oscillatory amplitude (Figure 4E), and oscillatory period (Figure 4F) all declined in a dose-dependent manner.

3.5 The ER as a possible mediator of low-dose cytokine effects on $[Ca^{2+}]_i$ oscillations

We further analyzed these data by determining the percentage of ‘fast oscillators’ as a possible indicator of disrupted beta-cell metabolism. Fast oscillations in insulin release are considered too frequent to be detected by standard blood sampling intervals *in vivo* and would thus be interpreted as irregular or non-rhythmic [38]. A 2-min cutoff was used to distinguish between the two forms of oscillations based on a previously published bimodal distribution [38]. As shown in Figure 5A, the percentage of fast islets increased with each increased cytokine dose (untreated: 5 fast, 24 slow; 1/500 cyto: 11 fast, 10 slow; 1/100 cyto: 8 fast, 3 slow; 1/50 cyto: 1 fast, 0 slow). Since the ER is a substantial calcium-storing

organelle and a known cytokine target, we used thapsigargin to examine the ER as a possible mediator of the observed effects. Acute treatment of oscillatory islets with thapsigargin decreased the period of islet oscillations as shown by two representative examples in Figure 5B. Both fast (n=6) and slow (n=2) islets exhibited similar decreases in oscillatory period, as shown by others [39,40]. Among 8 islets treated with 1 μ M thapsigargin, all islets showed a decrease in oscillatory period (mean period: 75 ± 15 sec to 31 ± 12 sec, n=8, p<0.05), indicating that these cytokine-induced effects on oscillations are consistent with disruptions in ER calcium handling. We observed even more substantial disruptions using another SERCA blocker, cyclopiazonic acid (CPA, 50 μ M). Acute CPA treatment induced elevated $[Ca^{2+}]_i$ and a loss of slow oscillations as shown in Figure 5B (bottom panel, representative of n=8 islets).

We also examined long-term ER calcium deprivation as a possible mechanism for the reduced glucose sensitivity in cytokine-treated islets. As shown in Figure 5C, exposure to CPA for 48 hours impaired GSCa compared to untreated control islets. This effect was similar to overnight cytokine treatment, both in terms of elevated basal $[Ca^{2+}]_i$ and reduced response to stimulation, though not as severely. As summarized in Figure 5D, both CPA and thapsigargin significantly reduced the GSCa compared to their respective controls, providing further evidence that the ER plays a role in cytokine-mediated islet dysfunction.

3.6 Cytokines induce islet dysfunction at levels consistent with low-grade systemic inflammation

The cytokine concentrations typically found in the blood stream are somewhat lower than what has been presented thus far. Using estimates from the Handbook of Human Immunology as a guide [41] (see Table 17.1), we designed a cocktail containing 10 pg/ml TNF- α + 15 pg/ml IL-1B + 1 pg/ml IFN- γ or untreated. As shown by the representative examples in Figure 6, the magnitude of cytokine effects on calcium handling in response to 28 mM glucose were slightly diminished, but the key features were quite similar. Elevated basal calcium and reduced peak were observed with the low pg/ml cytokine treatments.

We also tested the individual effects of each of these low dose cytokine treatments. As shown in Figure 7A, 15 pg/ml of IL-1B elevated basal calcium levels and decreased peak glucose stimulation, as observed with the combination (see Figure 6). However, treatment with 10 pg/ml TNF- α (Figure 7B) and 1 pg/ml IFN- γ (Figure 7C) had no significant effects on calcium handling compared to untreated islets. These results suggest that IL-1B is the key cytokine affecting islet calcium handling, however, it should be noted that these cytokines also act synergistically to negatively impact islet function [4,16,21,28].

3.7 Islets from db/db mice show similarities to low-dose cytokine-treated islets

The data presented thus far demonstrate that very low doses of cytokines disrupt islet function in terms of GSCa, ER calcium handling, and oscillations. In order to compare these effects with a well-known model of type 2 diabetes, we examined islets from the db/db mouse during the early stages of the disease at 6 and 12 weeks of age using heterozygous db/db mice as controls. Db/db mice have immune deficiencies [42] and are more sensitive to proinflammatory cytokine actions [43]. Body weight and blood glucose levels were normal at 6 weeks, but db/db mice displayed elevated body weight and blood glucose by 12 weeks as summarized in Table 1. IL-1B levels were measured in parallel studies of db/db mice that utilized age-matched BKS mice as controls. IL-1B levels were significantly elevated for db/db mice at 6 weeks of age and similarly elevated at 12 weeks of age, though not significantly due to a low n (see Table 1).

Using $[Ca^{2+}]_i$ as a marker of islet health, the most striking differences between the db/db islets and heterozygous controls were observed in their oscillatory activity, basal $[Ca^{2+}]_i$ levels, and response to glucose stimulation. Db/db islets at 6 weeks displayed either no oscillations or irregular patterns in comparison to control islets, as shown by the representative examples in Figure 7A. At 6 weeks of age, only 42% of db/db islets retained their oscillatory capacity in steady-state 11 mM glucose, whereas 92% of control islets displayed oscillations as shown in Table 1. By 12 weeks, oscillations were observed in only 17% of db/db islets compared to 92% of controls (see Table 1). These data suggest either a deficiency in the processes needed to generate oscillations or a shift in glucose sensitivity, such that normally oscillatory processes in the islet are saturated in 11 mM glucose, as suggested previously [44]. Despite the differences in oscillations, the GSCa among db/db islets was not significantly different from controls at 6 weeks of age (see Table 1). By 12 weeks of age, however, the GSCa among islets from db/db mice was significantly attenuated compared to controls and basal $[Ca^{2+}]_i$ levels were elevated, as shown in Figure 7B and Table 1. These results echo the decreased oscillatory capacity, elevated basal calcium levels, and reduced maximal peak that were observed among cytokine-treated islets. A comparison of cytokine-treated, db/db, and control islets in Table 2 summarizes these signs of islet dysfunction.

4. Discussion

Our findings suggest that cytokines at concentrations found circulating in the blood (low pg/ml) are sufficient in quantity to cause islet dysfunction by disrupting calcium handling in mouse islets. Calcium is a key component of the pathway that couples glucose stimulation with insulin secretion [25], but excess cytosolic calcium can lead to beta-cell dysfunction and cell death [15,16]. Exposure to serum from T1D patients has been shown to elevate basal calcium levels in clonal beta-cells [24] and to promote death in primary beta-cells in a calcium-dependent fashion [23]. Serum levels of proinflammatory cytokines are also elevated in the prediabetic stage of T1D and in first-degree relatives [45]. Among the many other well-established immunological factors involved with beta-cell destruction in T1D [46], these observations collectively suggest that circulating cytokines could contribute in a calcium-dependent manner to ongoing beta-cell death throughout the course of the disease [47] and following islet transplantation [48].

Low-grade systemic inflammation may also contribute to the pathology of T2D. Inflammation of the adipose tissue occurs prior to diabetes during the development of obesity. As adipocytes increase in size, they begin to secrete low levels of TNF- α , which can stimulate endothelial cells and preadipocytes to secrete monocyte chemoattracting protein-1 (MCP-1). This, in turn, attracts macrophages to the adipose tissue [6,9,10]. Macrophages are the sources of several additional proinflammatory cytokines that include TNF- α , IL-1 β , IFN- γ , IL-6, and others [6,9,10]. Due to infiltration of activated macrophages into adipose tissue, levels of these cytokines can increase in the systemic circulation several fold over basal levels [11–13]. Thus, cytokines produced by adipose and other tissues in response to obesity can reach the pancreatic islet by systemic circulation, albeit in low pg/ml concentrations, to potentially impact islet function.

We also show that low-grade inflammation in the db/db model of T2D correlates with reduced islet function. Our db/db mice also showed some signs of dysfunction (loss of oscillatory capacity) at least as early as 6-weeks of age, at which time body weight and blood glucose were still considered normal, with much greater declines in calcium handling observed by 12 weeks. Note that previous work by Roe et. al established similar declines in calcium handling as we report [44], although the disease progression was more rapid in their study. Our data also show that IL-1 β levels are significantly elevated by 6 weeks of age in

the db/db strain, so it is thus reasonable to suspect that these elevated cytokines can contribute to the deterioration of islet function between 6 and 12 weeks in db/db mice. Whether the cytokines are derived from adipose tissue or from other sources remains an open question. Regardless of the source, our data suggest that circulating levels of cytokines are sufficient to alter normal islet function and perhaps cause long-term damage under conditions of chronic inflammation in T2D. Of interest, recent work has shown that increasing levels of IL-1 receptor antagonist (also called anakinra) can reduce the deleterious effects of obesity in mice [49] and can reduce markers of systemic inflammation in patients with T2D [50].

We also found that cytokines elevated basal $[Ca^{2+}]_i$ without concurrently increasing basal insulin secretion, suggesting a decoupling of the normal stimulus-secretion coupling. Although there are several circumstances in which insulin secretion can be stimulated without changes in $[Ca^{2+}]_i$ [32,51] an increase in $[Ca^{2+}]_i$ is normally coupled to an increase in insulin secretion [25]. Calcium microdomains are thought to play a role in regulating important cellular processes including insulin secretion, metabolism, and maintaining cell viability [52]. If cytokines are misdirecting calcium to inappropriate subcellular regions, then this could lead to problems with organelles that rely on dynamic changes in calcium such as the ER [17,19], nucleus [53,54] and mitochondria [55–58]. The mitochondria represent a particularly intriguing target since ATP produced in the mitochondria triggers KATP-channel mediated depolarization of the beta cell by increasing the ATP/ADP. Since cytokines disrupt mitochondrial function and promote oxidative stress [55–58], this may directly impact intracellular calcium and insulin release. Although not the focus of the present study, determining whether the mitochondrial effects of low-dose cytokines are linked with disruptions in calcium handling merits further investigation.

Another potential early warning of cytokine-mediated islet dysfunction is the progressive deterioration of endogenous $[Ca^{2+}]_i$ oscillations. The loss of pulsatile secretion appears to be an early stage of T2D, with reduction in the amplitude and possibly frequency of the insulin pulses being linked to diabetic patients [59,60]. The close relatives of diabetic patients also demonstrate considerable degradation of pulsatile insulin secretion despite reporting clinically normal responses to glucose challenges and normal levels of insulin resistance [35,36]. Interestingly, diabetic patients were found to respond more effectively to pulsatile insulin delivery than to continuous delivery [61–63]. This is the first report to our knowledge describing detailed effects of cytokines on islet $[Ca^{2+}]_i$ oscillations. The observed signs of dysfunction include: (a) fewer islets displaying oscillations, (b) reduced amplitude, and (c) decreased period (increased frequency) among the remaining oscillatory islets. These effects were caused, at least in part, by the disruption of slow oscillations, which resulted in small amplitude and high frequency rhythms (also known as ‘fast bursting’). The preferential disruption in the slow oscillations suggests disruptions in some aspect of beta-cell glucose metabolism/sensitivity [64], disruption in communication among cells within the islet [65–67], or possibly changes in other islet cell types like glucagon-secreting alpha cells [68,69]. The shift in oscillatory period is also consistent with the depletion of ER calcium [39,40]. Since normal healthy islets often display fast, slow, or mixed oscillations [33,37], it is premature to assume that fast oscillating islets are inherently dysfunctional. Oscillations are only present within a range of glucose concentrations [33], so changes in glucose sensitivity could also alter or eliminate oscillations. Our data suggest, however, that the slow form of oscillation is particularly sensitive to cytokine effects, which may be an early indicator islet stress.

It should be noted that cytokine signaling is complex and can result in markedly different effects depending on the dose, duration, and combination of cytokines involved. For example, short-term treatment with low-dose IL-1B improves the function of rat islets,

whereas long-term, high dose treatment impairs islet function [70–72] and accelerates the development of T1D [73]. Low-dose TNF- α treatment has been shown to inhibit the autoimmune response in models of T1D [74–76], whereas TNF- α and IFN- γ also have dose- and duration-dependent inhibitory effects on islet function in vitro [21,77]. Differences among duration and dose of treatment [78,79], species being tested [28,80], and combination of cytokines [4,27,28] are all important factors that likely contributed to the variable results among previous studies. Further, synergistic activity among multiple cytokines can alter or amplify signaling pathways [81], adding an additional layer of complexity to cytokine action. In our hands, low-dose cytokine treatment resulted in elevated basal calcium and reduced response to stimulation, which we interpret as indications of islet dysfunction. An alternative interpretation is that these calcium effects are the result of a protective response in normal islets, akin to the ER stress response that limits ER activity until the stressor is removed. It should also be noted that low-dose cytokines may have markedly different effects on islets from normal healthy individuals as compared with diabetes-prone individuals in which underlying beta-cell defects may exist.

In summary, our findings indicate that much lower concentrations of pro-inflammatory cytokines are sufficient to produce marked effects on islet function than perhaps previously thought. Disruptions in ER calcium homeostasis, plasma membrane calcium flux, and endogenous oscillations may thus be among the early signs of islet dysfunction that lead to the targeted destruction of beta cells. Because defects in beta-cell development and/or function are crucial to the development of T2D, any stressor, including low-grade systemic inflammation, may have magnified effects on islets from T2D-prone individuals. We suggest that cytokines, produced from distal tissues and carried through the circulatory system, are sufficient in concentration to disrupt normal islet function and possibly contribute to beta-cell failure, particularly in diabetes-prone individuals.

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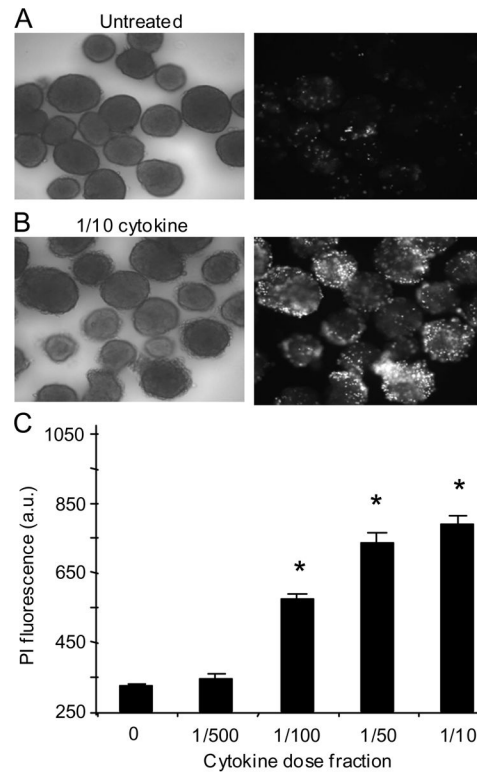
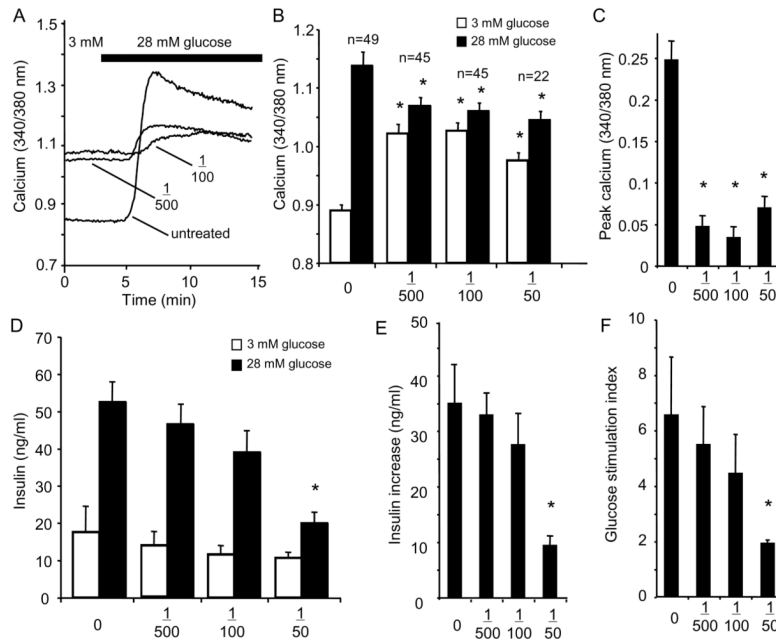


FIGURE 1.

Cytokine-induced islet cell death. A–B, Bright field image of control untreated islets (A, left panel) and corresponding PI fluorescence (A, right panel) compared with an image of islets treated overnight with 1/10 fractional dose of cytokines (B, left panel) and corresponding PI fluorescence (B, right panel). C, Islet PI fluorescence in response to a graded increase in cytokine concentrations. Each bar represents an average of 30–45 islets. Note that the pixel intensity of the background in this recording system was ~260 arbitrary units (a.u.). * indicates $P < 0.001$.

**FIGURE 2.**

Glucose-stimulated calcium (GSCa) and insulin (GSIS) responses following cytokine treatment. A, Representative traces of $[Ca^{2+}]_i$ changes in response to glucose stimulation for islets treated with 0, 1/500, or 1/100 cytokine dose. B, Mean $[Ca^{2+}]_i$ values at 3 and 28 mM glucose following different cytokine doses (n = number of islets for each treatment). C, Mean change in calcium following glucose stimulation (n = same as in B). D, Insulin secretion from 50 islets per condition during incubation in 3 mM glucose for 1 hour (white bars) followed by incubation in 28 mM glucose for 1 hour (black bars). E, Mean increase in insulin secretion following glucose stimulation. F, Mean increase in glucose stimulation index. * indicates $P < 0.05$ difference between insulin levels at 3 vs. 28 mM glucose for each cytokine dose. A total of 7 replicates for each condition were used for insulin measurements.

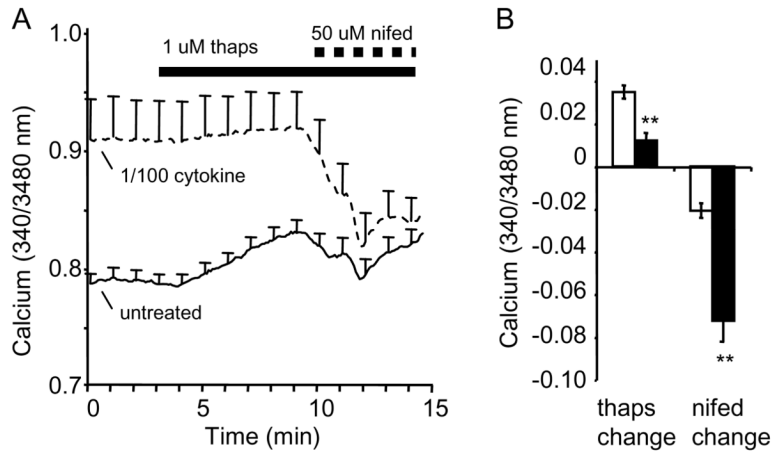
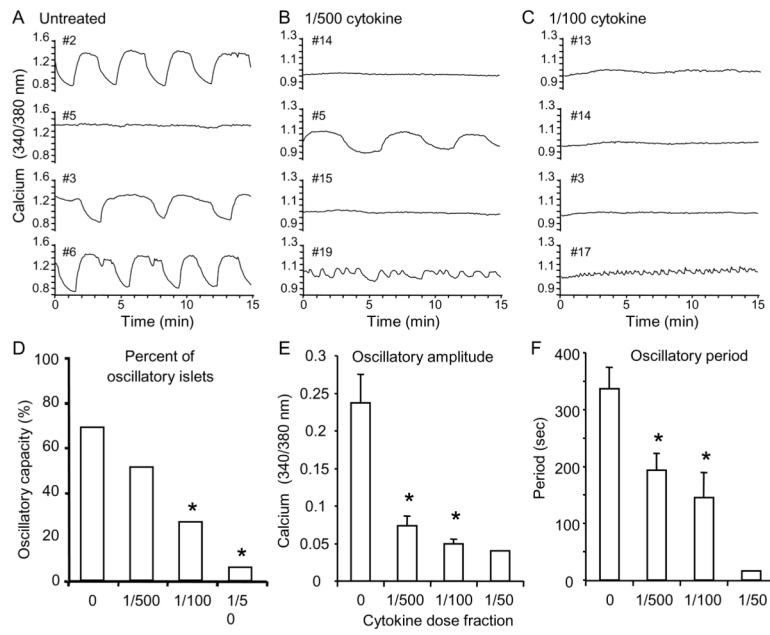
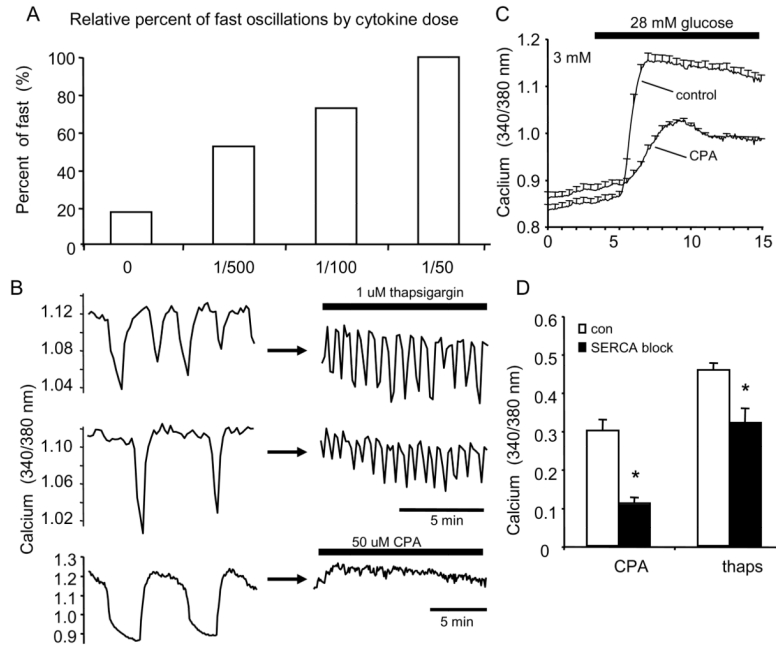


FIGURE 3.

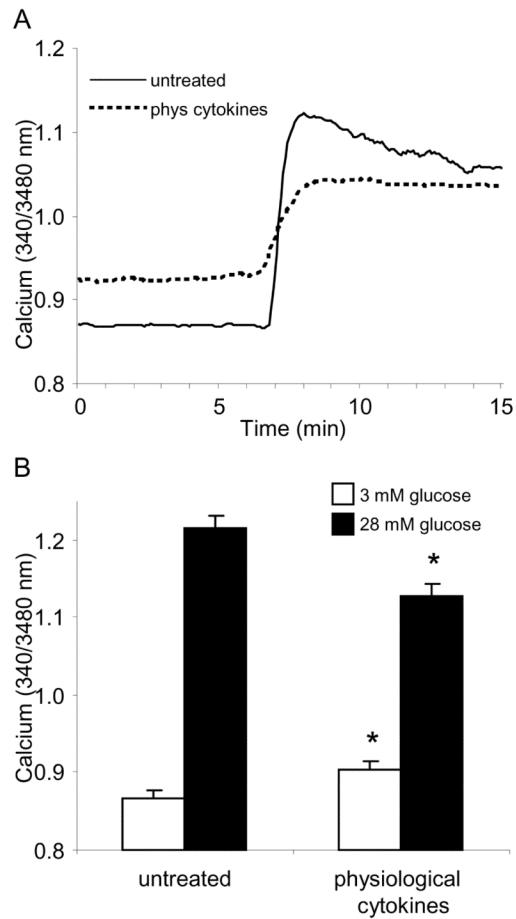
Contributions of the endoplasmic reticulum and ion-channel activity to cytokine-induced changes in basal $[Ca^{2+}]_i$. A, Average of several traces from untreated (solid, $n=9$) and overnight cytokine-treated islets (dashed, $n=8$). Islets began in 3 mM glucose and were then treated with thapsigargin and then thapsigargin + nifedipine as shown. B, Mean change in $[Ca^{2+}]_i$ during thapsigargin treatment and thapsigargin + nifedipine treatment. * indicates $p<0.05$.

**FIGURE 4.**

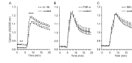
Cytokine-induced changes in islet oscillations. A–C, $[Ca^{2+}]_i$ patterns during a 15-min record in steady-state 11 mM glucose for untreated islets (A) and for islets treated with a 1/500 cytokine dose (B) or 1/100 dose (C). D–F, The percentage of islets displaying oscillations (D), mean peak-to-peak amplitude of oscillations (E), and mean period of oscillations (F) declined with increased cytokine concentration. The number of oscillatory islets among total islets recorded was $n=34/49$ for untreated, $n=22/45$ for the 1/500 dose, $n=11/45$ for the 1/100 dose, and 1/22 for the 1/50 cytokine dose. * indicates $P < 0.01$. Note the different scales in (A–C).

**FIGURE 5.**

Cytokines or thapsigargin treatment similarly increase the frequency of islet oscillations. A, Relative percent of fast oscillations among all oscillatory islets at each cytokine dose. B, Two representative examples of islets treated with thapsigargin showing a shift from slow to fast oscillations. C, Example of 48-hour treatment with the SERCA inhibitor CPA showing impaired GSCa compared to untreated control islets. D, Summary of effects of thapsigargin (n=22 untreated, n=16 thapsigargin-treated, $p < 0.001$) or CPA (n=17 untreated, n=11 CPA-treated, $p < 0.01$) on peak change in $[Ca^{2+}]_i$ during glucose stimulation.

**FIGURE 6.**

Cytokines can reproduce key inhibitory effects on islet function at the low physiological concentrations found in circulating blood. (A) Examples of GSCa traces for islets exposed overnight to “physiological” concentrations (10 pg/ml TNF- α + 15 pg/ml IL-1 β + 1 pg/ml IFN- γ) or untreated. (B) Mean values for basal and peak calcium values during glucose stimulation among cytokine-treated (n=40) and untreated (n=38) islets.

**FIGURE 7.**

Effects of individual cytokines on islet calcium handling. (A-C) GSCa traces for islets exposed overnight to treatments of (A) 15 pg/mL IL-1B (B) 10 pg/mL TNF-a (C) 1 pg/mL IFN-g, compared to untreated islets. N=22–35 islets were used for each treatment condition. **, $p<0.01$; ***, $p<0.001$.

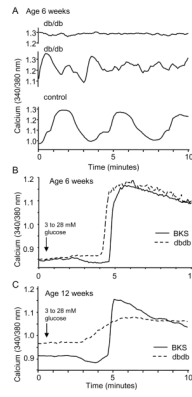


FIGURE 8.

Db/db islets show decreased oscillatory capacity and a decreased response to glucose stimulation. A, Representative examples of db/db islets (top and middle) and a control (bottom) in 11 mM glucose. B–C, Representative example of a db/db and control islet responding to acute glucose stimulation from 3 to 28 mM glucose at 6 weeks (B) and 12 weeks of age (C).

TABLE 1

Progressive islet dysfunction in db/db mice.



measurement	6 weeks		12 weeks	
	control	db/db	control	db/db
body weight (g)	21.1 ± 0.8 N = 12 mice	22.9 ± 1.1 N = 19 mice	27.7 ± 0.4 N = 6 mice	*40.7 ± 1.2 N = 14 mice
blood glucose (mg/dl)	112.3 ± 5.4 N = 12 mice	123.5 ± 10.9 N = 19 mice	96.7 ± 10.5 N = 6 mice	*344 ± 27.3 N = 14 mice
serum IL-1B (pg/ml)	14.2 ± 5.0 N = 6 mice	*42.6 ± 15.1 N = 7 mice	2.8 ± 0.1 N = 4 mice	52.0 ± 17.1 N = 3 mice
basal calcium (3G)	0.815 ± 0.011 N = 25 islets	0.817 ± 0.012 N = 23 islets	0.776 ± 0.015 N = 14 islets	*0.922 ± 0.022 N = 21 islets
peak calcium (28G)	1.122 ± 0.013 N = 25 islets	1.101 ± 0.010 N = 23 islets	1.339 ± 0.026 N = 14 islets	*1.126 ± 0.023 N = 21 islets
GSCa	0.307 ± 0.019 N = 25 islets	0.284 ± 0.014 N = 23 islets	0.563 ± 0.038 N = 14 islets	*0.204 ± 0.041 N = 21 islets
percent oscillating	92% (13 of 14)	*42% (14 of 33)	92% (45 of 49)	*17% (8 of 48)

* p < 0.05.

3G = 3 mM glucose, 28G = 28 mM glucose. Units for calcium measurements = fura-2 ratio (340/380 nm).

TABLE 2

Comparing islets from the db/db mouse with low-dose cytokine-treated islets.

Oscillation parameter	cytokines	6-week db/db	12-week db/db
Basal Ca	↑		↑
GSCa peak	↓		↓
Percent Oscillation	↓	↓	↓