Progression of diet-induced diabetes in C57Bl6J mice involves functional dissociation of Ca$^{2+}$ channels from secretory vesicles

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**Objective**—To elucidate the cellular mechanism underlying the suppression of glucose-induced insulin secretion in mice fed a high-fat diet (HFD) for 15 weeks

**Research Design And Methods**—C57Bl6J mice were fed a HFD or a normal diet (ND) for 3 or 15 weeks. Plasma insulin and glucose levels *in vivo* were assessed by intraperitoneal glucose tolerance test (IPGTT). Insulin secretion in vitro was studied using static incubations and a perfused pancreas preparation. Membrane currents, electrical activity and exocytosis were examined by patch-clamp technique measurements. [Ca\(^{2+}\)]\(_i\) was measured by microfluorimetry. TIRFM was used for optical imaging of exocytosis and submembrane depolarization-evoked [Ca\(^{2+}\)]\(_i\). The functional data were complemented by analyses of histology and gene transcription.

**Results**—After 15 weeks, but not 3 weeks, mice on HFD exhibited hyperglycemia and hypoinsulinemia. Pancreatic islet content and β-cell area increased 2- and 1.5-fold, respectively. These changes correlated with a 20-50% reduction of glucose-induced insulin secretion (normalized to insulin content). The latter effect was not associated with impaired electrical activity or [Ca\(^{2+}\)]\(_i\) signalling. Single-cell capacitance and TIRFM measurements of exocytosis revealed a selective suppression (>70%) of exocytosis elicited by short (50 ms) depolarization, whereas the responses to longer depolarizations were (500 ms) less affected. The loss of rapid exocytosis correlated with dispersion of Ca\(^{2+}\)-entry in HFD β-cells. No changes in gene transcription of key exocytotic protein were observed.

**Conclusions**—HFD results in reduced insulin secretion by causing the functional dissociation of voltage-gated Ca\(^{2+}\) entry from exocytosis. These observations suggest a novel explanation to the well-established link between obesity and diabetes.
Type-2 diabetes develops as a result of insulin resistance and β-cell failure (1). The link between obesity and insulin resistance is well-established (2). Diabetes results when genetically predisposed individuals are incapable of compensating the insulin resistance inherent to obesity with appropriate levels of insulin (3).

Longitudinal diet studies on islet function in vivo are best performed in experimental animals (4). When fed a high-fat diet (HFD), C57BL/6J mice develop glucose intolerance within 4 weeks (5-7). However, there is little information on the underlying mechanisms at the level of β-cell ion channel, electrical activity, Ca²⁺ homeostasis and exocytosis. We show here, in agreement with earlier in vivo (8; 9) and in vitro studies (10; 11), that whereas short periods on HFD enhance glucose-induced insulin secretion (GIIS), long periods results in its diminution. The inhibition of insulin secretion was not associated with any changes in β-cell electrical activity or [Ca²⁺]i, signalling that may explain the effect. We now demonstrate that the suppression of insulin secretion instead involves a selective inhibition of exocytosis in response to short (action potential-like) stimulation. These findings suggest that high-fat feeding inhibits insulin secretion by disrupting the tight association between voltage-dependent Ca²⁺-channels and the Ca²⁺-sensor of exocytosis.

**RESEARCH DESIGN AND METHODS**

Male C57BL/6J mice, without mutations in the NNT gene (12), were transferred to a 40% HFD (Special Diets Services, UK; composition given in Figure S5) at the age of 5 weeks as previously described (7). Age-matched control mice were maintained on a standard carbohydrate diet (ND; B&K Universal Ltd, Hull, UK). All mice were fed ad libitum and kept under a 12h light/dark cycle. All procedures were carried out according to national and institutional guidelines. Plasma insulin measured using an ELISA kit (Mercodia, Uppsala, Sweden).

IPGTTs were performed as previously described (7). For IPITT, mice were fasted overnight (4pm-9am). Fasting glucose levels were established using a one-touch Accuchek system (Roche). For the insulin tolerance test, a solution of 0.15U/kg insulin was administered ip. Blood glucose was determined at 15, 30 and 75 minutes afterwards.

Immunohistochemistry is described in the Supplementary Material. Briefly, pancreases from 3 mice were dissected and 10 sections separated by 200 μm were analysed for β-cell positive area. Animals were killed by cervical dislocation after an overnight fast and islets were isolated as described previously (10). All experiments except Figs. 7, 8, and S6 were done on freshly isolated islets/tissues. Static and dynamic measurements of insulin release were performed using published methods (11; 13). All samples were measured by a commercially available RIA kit (IDS Ltd Euro-diagnostica distributor, Tyne & Wear, UK). [Ca²⁺]i in intact islets was assessed using a dual wavelength PTI system using the indicator fura-2AM (11). ATP measurements were carried out on batches of 10 islets pre-incubated for 1 h in 300 μl RPMI medium containing 2 mg/ml BSA and 1 mM glucose as described previously (11). ATP content was normalised to protein content (DC protein assay; BioRad, Hercules, California). Glucose-induced changes in whole-cell K<sub>ATP</sub>-conductance and membrane potential were monitored in intact islets using the perforated patch whole-cell technique (14). Ca²⁺-currents and exocytosis were measured in single β-cells, obtained by dissociation of islets in a
Ca\textsuperscript{2+}-free medium, using the standard whole-cell technique and media as reported elsewhere (15).

The TIRFM experiments were carried out on an inverted (16). The low-affinity Ca\textsuperscript{2+}-indicator Oregon Green 6F (10 µM) was used for the spatially resolved measurements of Ca\textsuperscript{2+}-influx (Fig 8). Intracellular Ca\textsuperscript{2+} diffusion was restricted by inclusion of 10 mM EGTA in the intracellular solution (see Fig. 8A). The data were quantified by calculating the standard deviation (σ) and mean fluorescence (µ) for the entire cell footprint and expressed as coefficient of variation (CV; i.e. σ/µ). Vesicles undergoing exocytosis were detected as the rapid brightening and dispersion punctuate spots of the fluorescent IAPP-mCherry peptide cargo (17). The rate of exocytosis was normalized to the number of docked vesicles (%N; number of granules released divided by number of visible granules).

Photorelease of “caged” Ca\textsuperscript{2+} was initiated using a JML-C2 flashlamp (Rapp Optoelektronik GmbH, Hamburg, Germany). The pipette solution consisted of (mM) 110 K-glutamate, 10 KCl, 20 NaCl, 1 MgCl\textsubscript{2}, 25 HEPES (pH 7.1 with KOH), 3 MgATP, 0.1 cAMP, 3 NP-EGTA (Synaptic Systems, Goettingen, Germany) and 2 CaCl\textsubscript{2}. The Ca\textsuperscript{2+}-indicator Fluo-4 was included in this medium at a concentration of 0.05 mM.

All experiments involving islets were done on the same day as isolation whereas experiments performed on dispersed cells were performed next day (12-24h) or after 48 hours when viral infection was needed (Fig. 7F).

Statistical analyses were performed using Origin 8 (OriginLab, Bucks, UK) and SPSS. Data are presented as mean ± SEM when data are normally distributed. Statistical significances were evaluated by two-tailed Student’s t-test.

RESULTS

High fat feeding results in increased body weight, glucose intolerance and insulin resistance. Mice fed the HFD for 3 weeks were hyperglycemic and exhibited impaired glucose tolerance following an intraperitoneal glucose load (Fig. 1A). However, insulin secretion in response to the glucose challenge was normal (Fig. 1B) Mice fed the HFD for 15 weeks exhibited marked basal hyperglycemia and impaired glucose tolerance 30 and 75 min after the glucose challenge (Fig. 1C). This correlated with increased basal plasma insulin, reduced 1\textsuperscript{st} phase insulin secretion (measured 15 min after the glucose challenge) whereas the 2\textsuperscript{nd} phase secretion (measured between 30 and 75 min; Fig. 1D) was improved. The HFD had a 20% increase in body weight after 15 weeks; from 35±1 g to 42±1 g (P<0.05) and were insulin resistant. Whereas insulin reduced plasma glucose to ~2.5 mM in ND mice, it remained >4 mM 75 min after insulin injection in HFD mice (Fig. 1E).

Insulin secretion in vitro is improved by short term HFD exposure and impaired in the longer term: Insulin content was identical in islets from mice fed ND and HFD for 3 weeks (Fig A.2). Basal insulin secretion (2-6 mM glucose) was 2-fold higher in HFD islets than in ND islets (Fig. 2B). In both ND and HFD islets, elevation of glucose to 12 and 20 mM stimulated insulin secretion ~3- and ~4.2-fold (relative 6 mM), respectively. After 15 weeks, islet insulin content was ~15% higher in HFD islets than in control islets (Fig. 2C). In ND (but not in HFD) islets, 6 mM glucose stimulated insulin secretion 1.7-fold above that seen at 2 mM glucose (from 0.07 % to 0.13 %; Fig. 2D, inset). Insulin secretion elicited by 12-20 mM glucose or 0.2 mM tolbutamide was reduced by 25-30% in islets from mice on the HFD (Fig. 2D-E). ; no difference between ND and HFD islets was detected when insulin secretion was evoked by high extracellular K+.
Effects of long-term HFD on 1st and 2nd phase insulin secretion: Perfused pancreases from ND mice displayed a ~100-fold increase in insulin secretion when stimulated with 20 mM glucose (Fig. 3A). Insulin secretion (normalised to insulin content) was strongly reduced in mice fed the HFD for 15 weeks with 1st and 2nd phase being equally suppressed (57% and 61%, respectively). Total pancreatic insulin content was twice as high in HFD mice as in ND mice (Fig. 3B).

High-fat feeding has little effects on \([\text{Ca}^{2+}]_i\): After 15 weeks on ND and HFD, glucose exerted a dual effect on \([\text{Ca}^{2+}]_i\) in mouse islets: after an initial lowering, glucose increased \([\text{Ca}^{2+}]_i\) (Fig. 4A-B). In the presence of 12 mM glucose, \([\text{Ca}^{2+}]_i\) oscillated with a period that averaged 14 s and 11 s in HFD and ND islets, respectively (Insets; \(P=0.08\)). As has been reported previously, these rapid oscillations were sometimes superimposed on a slower rhythm (18). Peak and average steady-state glucose-induced increases in \([\text{Ca}^{2+}]_i\) were similar in ND and HFD islets. \([\text{Ca}^{2+}]_i\) measured in the presence of 0.1 mM tolbutamide was also not different between ND and HFD islets (not shown). Finally, exposing islets to 70 mM extracellular K+, in presence of 2 mM glucose produced identical increases in \([\text{Ca}^{2+}]_i\) in ND and HFD islets (Fig. S1).

In mice fed the ND and HFD for 3 weeks, \([\text{Ca}^{2+}]_i\) changes elicited by glucose and tolbutamide were, apart from a higher initial peak in HFD islets after addition of glucose, almost identical (Fig. S2).

Islet β cell mass is increased by HFD exposure after 15 weeks but β-cells are structurally intact: Immunohistochemical analyses of pancreatic sections revealed no difference in β-cell area and average islet size between ND and HFD mice after 3 weeks of diet introduction (Fig. 5A). At 15 weeks, the average islet size and the total β-cell area was 1.2-fold and 1.5-fold higher in HFD than in ND mice (Fig. 5B) but the number islets were not affected and averaged 1.13±0.1 vs 1.02±0.1 islet/mm² in ND and HFD pancreases, respectively. Total islet protein content was 1.2-fold higher in HFD than in ND islets (p=0.04; not shown).

Electron microscopy revealed no alterations of β-cell ultrastructure in ND and HFD islets (Fig. S3A-D). The absence of degranulation is consistent with the finding that islet insulin content was not reduced by high-fat feeding (cf. Fig. 2D).

Effects of HFD on whole-cell \(K_{\text{ATP}}\)-conductance: Because islet function and insulin secretion were not impaired after 3 weeks on the HFD, the remainder of the study focus on the consequences of 15 weeks on HFD.

In both ND and HFD β-cells, the resting whole-cell conductance (\(G\)), principally reflecting \(K_{\text{ATP}}\)-channel activity, was maximally inhibited already in the presence of 12 mM glucose (Fig. S4A). No further reduction of \(G\) was observed at 20 mM glucose. The ability of glucose to block \(K_{\text{ATP}}\) channel activity was independent of diet type.

β-cells electrical activity is marginally increased but ATP levels are not affected by HFD: Insulin secretion is tightly linked to β-cell electrical activity (19). The membrane potential of β-cells exposed to 2 mM glucose was identical in ND and HFD β-cells within freshly isolated islets and averaged -66±2 mV and -67±2 mV, respectively. Upon stimulation with 12 mM glucose, the β-cells depolarized to -40±3 mV in ND islets and to -39±2 mV in HFD islets. In both ND and HFD islets, membrane depolarization was associated with the initiation of oscillatory electrical activity consisting of bursts of action potentials superimposed on depolarized plateaux separated by repolarised silent intervals (Fig.
The fraction active phase at 12 mM glucose averaged 65-70% in both ND and HFD islets. Both ND and HFD islets responded with increased electrical activity when exposed to 20 mM glucose. Under the latter conditions, the inclusion of tolbutamide in the perfusion medium exerted no additional stimulatory effect.

Consistent with the lack of obvious changes in [Ca\(^{2+}\)]\(_i\) handling and electrical activity, ATP production (normalized to islet protein) was unaltered in HFD islets (Fig. S4B).

**Abnormal exocytotic pattern after 15 weeks on HFD:** We compared the exocytotic capacity of β-cells from mice fed the ND and the HFD for 15 weeks. Exocytosis (monitored as increases in membrane capacitance) was evoked by a train of twenty 50-ms (Fig. 7A) or ten 500-ms (Fig. 7C) depolarizations from -70 mV to 0 mV. In Fig. 7B the cumulative change in C_m (\(\Delta C_m\)) is displayed against the cumulative charge of the Ca\(^{2+}\)-currents (\(\Delta Q\)) during the train of the 50-ms pulses. In β-cells from ND and HFD mice, the slopes of the relationship were 3.4±0.8 fF/pC and 0.9±0.4 fF/pC (P<0.006), respectively. Fig. 7D compares the exocytotic responses in ND and HFD β-cells evoked by the individual 500-ms depolarizations during the pulse train. Exocytosis evoked by the first 500-ms depolarization tended to be slightly inhibited (by ∼35%; P<0.06) in HFD β-cells but the responses during the rest of the trains were not affected. The total responses evoked by the trains of the 50-ms and 500-ms in ND and HFD β-cells are summarized in Fig. 7E. It is evident that HFD selectively inhibits exocytosis elicited by the short pulses.

We also monitored exocytosis optically in β-cells from ND and HFD mice by TIRF imaging. To visualize the granules, cells were infected with IAPP-mCherry. The granule density in the footprint of the cells averaged 0.67±0.06 µm\(^{-2}\) (n=5) and 0.72±0.05 µm\(^{-2}\) (n=7) in ND and HFD β-cells, respectively. The surface area of the β-cell (estimated from the cell capacitance and using a specific membrane capacitance of 9 fF·µm\(^{-2}\)) used in these experiments averaged 682±63 µm\(^2\) (ND) and 785±055 µm\(^2\) (HFD). From these values we estimated that the number of near-membrane granules (N) was ∼500 for both ND and HFD β-cells, similar to the number of docked granules determined by electron microscopy (20). Exocytosis was elicited by a 1-s depolarization to zero mV (Fig. 7D). The depolarization triggered the release of some of the docked granules (no newcomers (21) were released during this period). We found that cargo release was significantly reduced during the first 600 ms of the 1s-long depolarization in the HFD cells.

**Dispersion of local [Ca\(^{2+}\)]\(_i\) transient reduces the chances of vesicle fusion:** Exocytosis in β-cell is dependent on a tight association of Ca\(^{2+}\) channels to secretory granules (22). We performed spatially resolved measurements of the submembrane [Ca\(^{2+}\)]\(_i\) by TIRFM (Fig. 7E-F). With low intracellular buffering (0.5 mM EGTA), even depolarizations as short as 50 ms evoke widespread increases in [Ca\(^{2+}\)]\(_i\) across the entire footprint of the β-cell and the CV was ∼0.70 (Fig. 8Ai). It can be noted that the increase in [Ca\(^{2+}\)]\(_i\) is restricted to the depolarization and that it promptly return towards the baseline upon repolarization (vertical dotted lines in Fig. 8A). As the depolarization was extended to 500 ms, the increase in [Ca\(^{2+}\)]\(_i\) became more uniform and CV was accordingly reduced. When intracellular Ca\(^{2+}\)-diffusion was restricted by inclusion of 10 mM EGTA (Fig. 8Aii) or 0.5 mM BAPTA (Fig. 8Aiii), Ca\(^{2+}\)-entry was confined to discrete areas and the CVs increased 1.6- to 1.9-fold. The [Ca\(^{2+}\)]\(_i\) increases evoked by 50-ms depolarizations from -70 to 0 mV were less concentrated in HFD than in ND β-cells and the CV was
reduced by ~14% (Fig. 8B). The CV of the prestimulatory fluorescence was also analyzed (to assess differences in factors like dye loading and cell adherence) but no differences were observed (0.18±0.01 vs 0.16±0.01 in ND and HFD β-cells, respectively). The magnitude of the \([Ca^{2+}]_i\) transient was unaffected (109±4% in HFD vs. ND β-cells; \(P>0.05\)) and the the integrated \(Ca^{2+}\)-currents were -9.1±1.4 pC and -9.9±1.8 pC in ND and HFD β-cells, respectively. In Fig. 7F we noted that hardly any granules underwent exocytosis during the first 100-200 ms. This is at variance with our previous observations made in β-cells isolated from NMRI mice (16). We measured exocytosis in C57Bl6J β-cells in response to an instant and uniform elevation of \([Ca^{2+}]_i\) produced by photoliberation of Ca\(^2+\) (Fig. 8C). The estimated delay (derived by linear back-extrapolation of the capacitance increase to the baseline) averaged 209±70ms (n=7).

**Gene transcription not affected in HFD islets:** We compared the mRNA levels of a panel of 22 selected genes in HFD and ND islets (Supplementary Table 1 which can be found in an online appendix at http://diabetes.diabetesjournals.org). No differences in gene expression were observed.

**DISCUSSION**

**Early effects of HFD: partial but insufficient β-cell compensation:** After 3 weeks, both basal plasma glucose and glycemic values during the IPGTT were elevated in HFD mice. These observation are in agreement with the report that 2 weeks on a HFD results in improved GIIS in Sprague-Dawley rats (23).

The stimulation of insulin secretion observed after 3 weeks on the HFD was not secondary to enhanced elevation of \([Ca^{2+}]_i\), and the responses in ND and HFD islets were virtually superimposable. Previous studies in vitro have indicated that the acute stimulatory effect of FFAs on insulin secretion involves increased whole-cell peak Ca\(^{2+}\)-currents (10) and/or inhibition of the \(K_v\)-current that underlies action potential repolarization (24); both effects would be expected to elevate \([Ca^{2+}]_i\). The lack of any major increase in the glucose-induced elevation of \([Ca^{2+}]_i\), argues that such effects contribute little to the stimulation of insulin secretion seen in vivo. The acute stimulatory effects of the FFA palmitate in vitro are principally attributable to enhanced exocytosis (10). The observation that insulin secretion was enhanced in the face of an unchanged \([Ca^{2+}]_i\), indicates that short-term HFD likewise leads to enhanced exocytosis.

**Long term effects of HFD: impaired β-cell function:** Our data confirm the adverse effects of long-term HFD on insulin secretion previously reported by others (25-27). In agreement with earlier observations (27), we observed a 1.5-fold and 2-fold increase in pancreatic β-cell area and insulin content, respectively. Mice on the HFD developed impaired glucose tolerance as a result of insulin resistance. Insulin secretion was apparently insufficient to meet the biological demand; again in agreement with previous reports (28; 29).

What is the cellular mechanism leading to reduced insulin secretion? The similar reduction in glucose- and tolbutamide-induced insulin secretion in the HFD group (Fig. 2D-E) suggests that the suppression of insulin secretion does not result from impaired glucose sensing. Indeed, both glucose-induced \([Ca^{2+}]_i\) increases and electrical activity were normal in HFD islets and glucose-induced ATP production was likewise unperturbed (Fig. S4B). Collectively, these observations indicate that insulin secretion is impaired at the level of exocytosis itself. We now demonstrate that HFD β-cells have a selective suppression of exocytosis evoked by action potential-like stimulation (trains of 50-ms depolarizations). Exocytosis monitored by capacitance measurements was
reduced by 70% in response to such stimulation. Interestingly, much weaker effects were obtained in response to trains of 500-ms depolarizations. We also monitored exocytosis of granules by TIRF imaging during a 1-s depolarization. Again, there was inhibition of granule exocytosis in HFD β-cells compared to control cells during the first 400 ms of depolarization. However, at later times, the rate of exocytosis was actually enhanced in the HFD β-cells compared to what was seen in the ND cells. Thus, it appears that it is the kinetics of exocytosis rather than the release competence of the granules which is affected by high-fat feeding.

Ion channels are modulated by FFA application (30-33). Whole-cell K<sub>ATP</sub>-voltage-gated Ca<sup>2+</sup>-currents were not affected by the HFD. The delayed-rectifier K<sup>+</sup>-current (K<sub>DR</sub>), which is responsible for the repolarisation of the Ca<sup>2+</sup>-induced action potential in mouse β-cells, has been shown to be modulated upon application of FFA (32; 33). A reduced K<sub>DR</sub>-current would be expected to prolong the action potential. It has been reported that the K<sub>DR</sub>-current reduction is a GPR40 dependent process (33). Overexpression of GPR40 protects mice against the diabetogenic action of HFD (34). It is possible that the latter effect is mediated by the marked prolongation of the action potential that protects the β-cell against the inhibitory effects on insulin secretion produced by Ca<sup>2+</sup>-channel dispersion. However, we observed no differences in the duration and upstroke or downstroke velocities of the action potentials recorded from ND and HFD β-cells (not shown).

In β-cells, exocytosis in response to brief depolarizations depends on a close association of Ca<sup>2+</sup>-channels and secretory granules (35). It is therefore of interest that whereas Ca<sup>2+</sup>-entry in ND β-cells concentrates to discrete areas, it becomes more diffuse in HFD β-cells. The effect is fairly small but this may reflect partial recovery from the in vivo effect during tissue culture. The [Ca<sup>2+</sup>]<sub>i</sub> transients echoed the membrane depolarizations, no transients were observed when the depolarization to +60 mV (reversal of the Ca<sup>2+</sup>-current) and their amplitude was unaffected by pretreatment of the cells with thapsigargin (Fig. S6). These findings suggest that they reflect localized increase in [Ca<sup>2+</sup>]<sub>i</sub> due to Ca<sup>2+</sup>-influx through individual Ca<sup>2+</sup>-channels with little contribution by release from intracellular stores.

We have previously demonstrated that the β-cell Ca<sup>2+</sup>-channels aggregate into triplets and argued that this arrangement is a prerequisite for rapid exocytosis (22). The fact that the Ca<sup>2+</sup>-entry becomes dispersed in HFD β-cells is suggestive of less efficient clustering. In the HFD β-cells, Ca<sup>2+</sup> still enters the β-cell during electrical activity but the increases in [Ca<sup>2+</sup>]<sub>i</sub> occur too far away from the release-competent granules to trigger their release. This accounts for the apparent reduction of the Ca<sup>2+</sup>-sensitivity of exocytosis (Fig. 7B). During longer depolarizations, however, the Ca<sup>2+</sup>-channels stay open long enough to allow the [Ca<sup>2+</sup>]<sub>i</sub> increase to spread through the cytosol (compare 50 and 500-ms depolarizations in Fig. 8Ai). Under these conditions, granules situated too far away from the Ca<sup>2+</sup>-channels to be released during brief stimulation would be exposed to exocytotic levels of [Ca<sup>2+</sup>]<sub>i</sub>. In this context it is of interest that insulin secretion evoked by high-K<sup>+</sup> stimulation was the same in HFD and ND islets. This stimulation protocol also activates the Ca<sup>2+</sup>-channels long enough to allow [Ca<sup>2+</sup>]<sub>i</sub> equilibration throughout the β-cell and release any release-competent granule regardless of its proximity to the Ca<sup>2+</sup>-channels. The fact that high-K<sup>+</sup> was tested in the presence of 20 mM glucose also allows us to exclude an effect of HFD on the amplifying action of glucose. In this context it may be worth pointing out that tolbutamide
fails to restore insulin secretion from HFD islets to that seen in ND islets because the action potentials triggered by the sulphonylurea are of the same (brief) type as those elicited in response to glucose (36). The fact that 1st phase and 2nd phase insulin secretion are similarly affected (Fig. 3) is perfectly consistent with these observations. Both 1st and 2nd phase insulin secretion is triggered by action potential firing (37). First phase secretion is larger because it coincides with a brief period of continuous action potential firing whereas action potentials are grouped in bursts at steady state (Fig. 6). Another factor that contributes to the prominence of 1st phase insulin secretion is the number of RRP granules. It is likely that RRP is larger to begin with and that the rate of secretion will decline simply because there are fewer granules for Ca$^{2+}$ entering the cell to act on. The concept that short-lived localized increases in [Ca$^{2+}$], triggers exocytosis may seem difficult to reconcile with the finding that few granules undergo exocytosis during the initial 100-200 ms. In β-cells from NMRI mice we have observed a delay between photorelease of caged Ca$^{2+}$ (to produce a uniform step increase in [Ca$^{2+}$], and the initiation of exocytosis of only 10 ms (22). In this study, based on β-cells from C57BL6J mice, the latency was >200 ms. These observations suggest that (for reasons that remain to be clarified) there is a significant delay between the triggering and initiation of exocytosis.

Why is there a selective inhibition of exocytosis evoked by rapid depolarizations in HFD islets? The effects of high-fat feeding on exocytosis are reminiscent of those recently found to occur following reduced expression of Tcf7l2 (38). However, no changes in Tcf7l2 expression could be detected (Supplementary Table 1). We also failed to detect any major changes in the expression of 16 genes encoding key exocytotic proteins. In this context it is of great interest that the microRNA miR34a becomes upregulated in rodent β-cells exposed to palmitate (39). However, the fact that insulin secretion evoked by high-K$^+$ and exocytosis in response to long depolarizations were similar in HFD and ND β-cells militates against any major defects in β-cell exocytosis. Rather, the dispersion of Ca$^{2+}$-entry is likely to be key to the understanding of the mechanisms leading to impaired insulin secretion from HFD islets. Results obtained in isolated islets exposed to the free fatty acid palmitate for >72 h are very similar to those described here (16). The present data extend these observations by providing a direct link between increased dietary lipids and impaired β-cell function. In mice fed the HFD there is a 20-fold increase in pancreatic triglycerides (16). Increased adipocyte content of the pancreas will result in increased flux of FFAs in the extracellular space, some of which will be adjacent to the pancreatic islets (16), and thereby suppress insulin secretion by the mechanism that we have previously documented in vitro (16). If these data can be extended to man, they may have both dietary and therapeutic implications.

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FIGURE LEGENDS

Figure 1: Long-term HFD induces glucose intolerance and insulin resistance. A-D: plasma (p)-

glucose and insulin levels measured in mice fed ND (black traces and symbols) or HFD (grey traces and symbols) for 3 (A,B) and 15 (C,D) weeks during an IPGTT. Data are mean values ±

S.E.M. of n=49 (A), n=24 (B), n=33 (C) and n=14 (D). *P<0.05; **P<5.10⁻³; ***P<5.10⁻⁴. E) IPGTT performed in 12 weeks old mice (n=23 in each group). The traces show means ± S.E.M. *P<0.001 for comparisons between ND and HFD values.

Figure 2: Effects of HFD on GIIS in vitro. (A-E): Insulin content (A,C) and insulin secretion (B,D,E) in ND (black) and HFD (grey) islets. Data are from mice fed ND or HFD for 3 (A,B) and 15 (C-E) weeks, respectively. In B, D the islets were challenged with glucose at the indicated concentrations. In E(i), islets were stimulated by 0.2 mM tolbutamide in the presence of 12 mM glucose. In E(ii), islets were stimulated by 70 mM KCl in the presence of 20 mM glucose. The data highlighted within the box (panel D) compares insulin secretion at 1 and 6 mM in ND and HFD islets. The number below each point represents the minimum number of cases in that group. At least 4 different animals were used in each group. Data are mean values ± S.E.M. *P<0.05; **P<0.005; ***P<0.0005 for differences between ND and HFD. †P<0.05 indicates difference between 6 mM and 1 mM glucose. Numbers in parentheses in B, D and E are number of experiments performed.

Figure 3: HFD reduces 1ˢᵗ and 2ⁿᵈ phase release equally. GIIS in perfused pancreases from control (black) and HFD (grey) mice (A) at 15 weeks: The rates of insulin secretion are given as mean values ± S.E.M. and are normalised to pancreatic insulin content. (B): Total pancreatic insulin content in ND (black) and HFD (grey) mice. *P<0.05 vs. control (n>15 mice in each group).

Figure 4: Effects of HFD on [Ca²⁺]. Glucose- and tolbutamide-evoked changes in [Ca²⁺] in islets from mice fed the ND (A) and HFD (B) for 15 weeks. Representative traces are shown for each group. Values given above the traces are means ± S.E.M (n>13 islets from seven mice). *P<0.05 for comparisons of [Ca²⁺] under the respective conditions in ND and HFD islets.

Figure 5: Effects of HFD on β-cell area. β-cell surface area ratio to pancreatic surface area (left) and mean islet size (right) after 3 (A) or 15 weeks (B) on ND and HFD. Up to 24 sections taken from 3 mice were analysed for each group. Data are mean values ± S.E.M. In the case of average islet size. Significance levels (indicated above histograms) were calculated on transformed data (logarithm).

Figure 6: Effects of 15 weeks HFD on β-cell electrical activity. A-B: Representative membrane potential (V_m) recordings from β-cells in intact ND (A) and HFD (B) islets. (n=7 islets in both groups, from 4 different animals). Segments of the records obtained at 12 mM glucose (taken as indicated) are shown on an expanded time base below for both ND and HFD islets.
Figure 7: 15 weeks HFD treatment inhibits depolarization-evoked exocytosis. (A): Representative recordings of exocytosis during a train of 50-ms (10 Hz) depolarizations from -70 mV to zero mV in ND (black) and HFD (grey) β-cells. (B): Relationship between integrated current charge (ΣQ) and changes in membrane capacitance (ΣΔC_m) during the train of twenty 50-ms depolarizations (10 Hz). The responses during the four first depolarizations (not associated with exocytosis) are not shown. The lines superimposed on the data points indicate the slopes of the relationships. (C): Representative increases in cell capacitance evoked by a train of ten 500-ms (1 Hz) depolarizations from -70 mV to zero mV in ND (black) and HFD (grey) β-cells. (D): Increase in cell capacitance for each pulse during the train of 500-ms depolarizations in ND (black) and HFD (grey) β-cells. Mean values ± S.E.M. for 75 (ND) and 61 (HFD) cells. *P<0.06. (E): Total capacitance increments (in fF) following the train of 50-ms (black) and 500-ms (grey) pulses. Data are means ± S.E.M. for at least 61-75 β-cells from 6 animals from each group in the case of the long pulses experiment and for at least 23 β-cells from 3 animals from each group in the case of the short pulses experiment. **P<0.005. (F): Average rates of optically measured release of vesicles by discharge of fluorescent IAPP-mCherry cargo during a 1000 ms membrane depolarization to 0 mV. Data are means ± S.E.M. for 5 and 6 cells from 2 animals in each group for both ND (black) and HFD (grey) β-cells. * P<0.05.

Figure 8: HFD causes dispersion of Ca^2+ entry. (A): Evanescent-field illumination of voltage-clamped cells infused with 0.5 mM EGTA (i), 10 mM EGTA (ii) or 0.5 mM BAPTA (iii) and Ca^{2+} Green 6F (10 μM). The images in Ai show the prestimulatory fluorescence and the increase produced by a 50- and a 500-ms depolarizations, respectively. Numbers below images are CV values for the Ca^{2+} signal (mean values ± S.E.M.) for 6 (i), 11 (ii) and 11 (iii) different cells. ***P<5.10^{-5} (unpaired t-test) or ††P<0.005 (paired t-test) for comparisons with CV measured following a 50-ms depolarization with 0.5 mM EGTA. Scale bars: 2 µm. The vertical lines superimposed on the [Ca^{2+}]i traces indicate the end of the depolarization. (B): As in A(ii) but using ND or HFD β-cells (as indicated). CV values for the Ca^{2+} signal during a 50-ms depolarization to 0 mV for control (n=12 from three mice) and HFD (n=15 from three mice) β-cells are shown to the right of each image alongside. **P<0.001 for comparison between HFD and ND β-cells. (C) Immediate increases in [Ca^{2+}], and delayed increase in membrane capacitance (ΔC_m) in β-cells from C57Bl6J mice in response to photoliberation of caged Ca^{2+} (Ca^{2+}-np-EGTA preloaded into the cell). The trace is the average response recorded in 7 cells from 3 different animals. The continuous red line represents the back-extrapolation towards the baseline (dashed horizontal line). The intersection of the two lines was taken as the delay.
Figure 5

A

![Graph showing β cell area (%) and islet size (μm²) for 3 weeks.](image)

B

![Graph showing β cell area (%) and islet size (μm²) for 15 weeks.](image)

Figure 6

A

![Graph showing Vm (mV) response to 0.1 mM tolbutamide and 20 mM glucose for ND.](image)

B

![Graph showing Vm (mV) response to 0.1 mM tolbutamide and 20 mM glucose for HFD.](image)
