Contribution of Different Mechanisms to Pancreatic Beta-cell Hyper-secretion in Non-obese Diabetic (NOD) Mice during Pre-diabetes

Background:

Pre-diabetic islet hyper-secretion is crucial to the development of the disease but the mechanisms remain unknown.

Results:

We reveal dynamic changes in beta-cell mass and function in non-obese diabetic (NOD) mice of different ages.

Conclusion:

Beta-cell mass increase and individual beta-cell secretory ability enhancement contribute to islet hyperactivity at different stages.

Significance:

This may provide insights into alteration of beta-cell function during the disease progression.

The development of insulin-dependent diabetes mellitus (IDDM) results from the selective destruction of pancreatic beta-cells. Both humans and spontaneous models of IDDM, such as NOD mice, have an extended pre-diabetic stage. Dynamic changes in beta-cell mass and function during pre-diabetes, such as insulin hyper-secretion, remain largely unknown. In this paper, we evaluated pre-diabetic female NOD mice at different ages (6, 10, and 14 weeks old) to illustrate alterations in beta-cell mass and function as disease progressed. We found an increase in beta-cell mass in 6-week-old NOD mice that may account for improved glucose tolerance in these mice. As NOD mice aged, beta-cell mass progressively reduced with increasing insulinitis. In parallel, secretory ability of individual beta-cells was enhanced due to an increase in the size of slowly releasable pool (SRP) of vesicles. Moreover, expression of both SERCA2 and SERCA3 genes were progressively down-regulated, which facilitated depolarization-evoked secretion by prolonging Ca2+ elevation upon glucose stimulation. In summary, we propose that different mechanisms contribute to the insulin hyper-secretion at different ages of pre-diabetic NOD mice, which may provide some new ideas concerning the progression and management of type I diabetes.

Type I diabetes is an autoimmune disease in which islets produce an auto-antigen that guides the infiltration of T lymphocytes (1). The subsequent destruction of beta-cells and insulin secretion deficiency lead to overt hyperglycemia. In addition to the reduction in beta-cell mass, numerous pieces of evidence have suggested that beta-cell function is altered during the progression of diabetes, which possibly contribute to its etiology (2).

Non-obese diabetic (NOD) mice spontaneously develop IDDM between weeks 12 and 30 after birth (2). As compared with normal non-diabetic mice, one of the early functional changes of beta-cells is enhanced insulin release, which is observed in mice as young as 4 weeks old and persists at least until the age of 8 weeks (3). At this point, insulinitis and a reduction in beta-cell mass are also observed (4). Islet hyperactivity is also reported at the pre-diabetic stage of biobreeding (BB) rat, another IDDM animal model (2). Therefore, islet hyper-secretion may be crucial in targeting lymphocytes to the islets and in the development of the disease. Two main mechanisms have been proposed to account for the transient islet hyperactivity: an increased beta-cell mass due to proliferation and neogenesis and/or enhanced beta-cell function due to environmental factors, e.g. cytokines, high glucose or altered regulatory neuronal or endocrine activities (2). However, beta-cell function has mainly been evaluated as the insulin secretory rates obtained from islets or whole pancreases (5) in which insulin secretion was profoundly affected by complex neuronal and endocirnal input signals (2). Therefore, whether or not the insulin secretion machinery in a single beta-cell is altered in the pre-diabetic stage has not been investigted. Moreover, glucose-stimulated insulin secretion has been reported to progressively decrease as NOD mice age, which leads to glucose intolerance and the onset of diabetes (6–7). The mechanism for the rise and fall in insulin secretion and its correlation with the autoimmune

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4 The abbreviations used are: NOD, non-obese diabetic; IDDM, insulin-dependent diabetes mellitus; SRP, slowly releasable pool; RRP, rapidly releasable pool of vesicles; GTT, glucose tolerance tests; SERCA, sarco/endoplasmic reticulum Ca2+-ATPase; TG, thapsigargin; BB, biobreeding.
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destruction of beta-cells are not known. To date, few studies have systematically compared blood glucose tolerance, beta-cell mass and cell function in NOD mice to normal mice at different ages. Whether multiple mechanisms contribute to the alterations in islet function during the pre-diabetic stages and how these mechanisms change during disease progression remain unanswered.

In the present study, we used intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), measurements and patch clamp experiments to evaluate functions of single beta-cell. This information was then correlated with the extent of beta-cell destruction and glucose intolerance in NOD mice at different ages as compared with age-matched, non-diabetic Balb/c mice. We revealed two mechanisms that contribute to islet hyperactivity at different stages in NOD mice. In 6-week-old NOD mice, islet hypersecretion mainly originated from an increase in the beta-cell mass as compared with Balb/c mice but not secretion from individual cells per se. In contrast, insulin hyper-secretion in 10- and 14-week-old NOD mice was mostly due to an enhanced secretory machinery of individual beta-cells, and down-regulation of the SERCA pump protein that led to sustained glucose-stimulated calcium elevations.

EXPERIMENTAL PROCEDURES

Islet and Single Beta-cell Preparations—Pre-diabetic NOD female mice, aged 6, 10, and 14 weeks, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., Chinese Academy of Sciences, Shanghai, China (SCXK 2007–0005). In this NOD colony, overt diabetes appeared in females at 15 weeks of age, and ~60–80% became diabetic by 8 months. NOD mice that developed overt diabetes (basal non-fasting glycemia >11 mm) were excluded from the experiment. Balb/c mice were non-diabetic, and their islets are often used as donor for allogenic islet transplantation to NOD mice (8). Therefore, age-matched Balb/c female mice were purchased from Vital River Experimental Animal Company (Beijing, China) to be used as the control. All animals were maintained under specific pathogen-free conditions. Mouse handling and experimental procedures were conducted in accordance with the Committee for the Use of Live Animals in Teaching and Research at the Capital Medical University. Animals were sacrificed by cervical dislocation, and primary islets and beta-cells were isolated from mice as previously described (9). Isolated cells were plated on coverslips that were precoated with poly-L-lysine and kept in a 37 °C, 5% CO$_2$ incubator for 24–48 h in modified RPMI 1640 medium. Islet and Single Beta-cell Preparations—Pancreases obtained from Balb/c and NOD mice of different ages were fixed in 10% neutral formalin, and embedded in paraffin (14). A series of 4.5-μm sections were obtained from different levels of the block. Deparaffinized sections (n = 5) were stained for beta-cells (insulin-positive) using an immunoperoxidase technique. The sections were first incubated with a polyclonal guinea pig anti-insulin antibody (Maixin Bio, China) and then with a biotinylated goat anti-guinea pig antibody (Maixin Bio, China). The sections were then incubated with a polymerized horseradish peroxidase-conjugated goat anti-mouse antibody (Maixin Bio, China) and developed with 3,3′-diaminobenzidine (DAB) (Maixin Bio, China), which stained insulin-positive cells a golden brown color. The sections were then counterstained with hematoxylin. The stained slides were observed under a light microscope to evaluate the severity of pancreatic islet inflammation (insulitis), which was graded on a 0–3 scale, according to the extent of intra-islet infiltration by leukocytes, with 0 = none, 1 = < 20% infiltration, 2 = 20–50%
infiltration, and $3 = >50\%$ infiltration (14). The mean insulitis score was calculated for each pancreas by dividing the sum of the insulitis scores for individual islets by the number of islets examined, and a minimum of 25 islets from 6 sections of each pancreas was independently evaluated blindly by two observers.

Measurement of Beta-cell Mass—Beta-cell mass was determined from insulin-positive stained sections using an image analysis system, which consisted of an Olympus light microscope (model BX50) attached to a video camera (Imaging micropublisher 5.0 RTV, Canada) and the Image-Pro Plus, Version 6.0 software (Media Cybernetics, Inc.). The relative cross-sectional area of beta-cells was determined by quantification of the cross-sectional area occupied by beta-cells and the cross-sectional area of all tissue in multiple fields per slide. Beta-cell mass per piece was estimated as the product of the cross-sectional area of beta-cells/total tissue and the weight of the pancreas piece before fixation (15), which was repeated by two individuals.

Western Blot Analysis and Quantitative RT-PCR—For the Western blotting experiments shown in Fig. 4, isolated islets (~400 for each batch) were cultured for 1 day before being labeled with a SERCA antibody (Santa Cruz, sc-30110, 1:800). A beta-actin antibody (Sigma, 1:2000 dilution) was used as a control. Incubation with the mouse primary antibody was followed by application of a rabbit anti-mouse IgG peroxidase conjugate (Sigma, 1:1000 dilution). The blots were then probed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce) (12).

Total RNA was extracted from pancreatic islets (~400 islets) from 6-, 10-, and 14-week-old NOD female mice and age-matched Balb/c female mice. Real-time PCR analysis was performed as previously described (16).

Statistical Analyses—All data were analyzed using the Igor Pro software (Wavemetrics, Lake Oswego, OR). Averaged results are presented as the mean value ± S.E. from the number of experiments indicated. Statistical significance was evaluated using either Student’s $t$ test for single Gaussian distributed datasets or Mann-Whitney rank sum test for non-single Gaussian distributed datasets. Asterisks ‘*’ and ‘**’ denote statistical significance with $p$ values less than 0.05 and 0.01, respectively.

RESULTS

Age-dependent Changes in Glucose Tolerance in NOD Mice—

In the present study, we examined beta-cell function in 6-, 10-, and 14-week-old NOD mice, which all exhibited near normal glycemia and no signs of diabetes. NOD mice and age-matched Balb/c mice, were intravenously injected with a 25% glucose solution (2 g/kg of body weight), and blood glucose concentrations at various times after injection were measured, as shown in Fig. 1. $A$–$C$. At age of 6 weeks, NOD mice exhibited better glucose tolerance on intraperitoneal GTT test than did control mice (Fig. 1A). The converse was true at 14 weeks age (Fig. 1C), and the GTT of NOD and control mice were superimposable at age of 10 weeks (Fig. 1B).

Age-dependent Destruction of Beta-cells in NOD Mice—NOD mice are characterized by an earlier onset of T lymphocyte infiltration and insulitis (1). We used histological staining to probe for these changes in our colony of 6-, 10-, and 14-week-old mice and compared them with age-matched, non-diabetic Balb/c mice. Histological examination of pancreatic sections revealed little lymphocytic infiltration of islets from control mice (Fig. 2A), minimal infiltration of 6-week-old NOD islets (Fig. 2B) (4), and progressively increasing infiltration at age 10 and 14 weeks (Fig. 2, C, D, and F). Islet inflammation scoring is described under “Experimental Procedures.” As islet inflammation increased, more beta-cells within the islets were replaced by infiltrated mononuclear cells. As shown in Fig. 2E, there was more beta-cell mass in 6-week-old NOD mice compared with the control Balb/c mice, which may explain the hypoglycemia seen in Fig. 1A. However, in contrast to the slight increases in beta-cell mass in older Balb/c mice, older NOD mice exhibited much reduced beta-cell mass. Ten-week-old NOD mice exhib-
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**FIGURE 2. Age-dependent destruction of beta-cells in NOD mice.** A–D, comparison of the degree of insulitis and beta-cell mass of Balb/c and NOD mice by immunohistochemical staining. Arrows indicate islet cells, and arrowheads indicate infiltrated lymphocytes. The scale bar in D represents 10 µm. A, 14-week-old Balb/c mice islets that showed normal structure without infiltration (for the example shown here, insulitis score = 0). B, 6-week-old NOD mice islets were surrounded by leukocytes, but most were not infiltrated (score = 1). C, 10-week-old NOD mice islets showed mild to moderate insulitis, and beta-cell mass was reduced (score = 2). D, 14-week-old NOD mice islets were heavily infiltrated by leukocytes, and beta-cell mass was reduced significantly (score = 2). E, quantification of average beta-cell mass from age-paired Balb/c and NOD mice. 6-week-old NOD mice showed slight increases in beta-cell mass (Balb/c 1.01 ± 0.07 mg, NOD 1.19 ± 0.05 mg, mean ± S.E., p < 0.05). In contrast, 14-week-old NOD mice displayed significant decreases in beta-cell mass in comparison with normal Balb/c mice (Balb/c 1.27 ± 0.05 mg, NOD 0.83 ± 0.1 mg, p < 0.01). F, average insulitis scores for NOD mice. There was a progressive loss of beta-cells in NOD mice as a function of age.

A small but not significant reduction in beta-cell mass, while 14-week-old mice had significantly less beta-cell mass compared with the Balb/c controls (Fig. 2E). Overall, these data indicate that the progressive loss of beta-cells in NOD mice occurs as a function of age.

**Age-dependent Slowing of [Ca^{2+}], Clearance in NOD Mice—** The infiltration of T lymphocytes leads to an increased concentration of cytokines within islets (17), which profoundly affects multiple aspects of beta-cell function, e.g. intracellular calcium signaling pathways (18). Pulsatile insulin release from individual beta-cells depends on glucose-induced calcium oscillations. The duration and amplitude of these oscillations are mainly determined both by calcium influx and calcium clearance mechanisms (9, 19). These factors can be evaluated by measuring [Ca^{2+}], before and after depolarization in isolated beta-cells, as previously demonstrated (9). As shown in Fig. 3A, transiently perfusing cells with 70 mM KCl for 6 s triggered a pronounced [Ca^{2+}], elevation, which was followed by the slow return of [Ca^{2+}], to the basal level in normal extracellular solutions. Both the amplitudes of the depolarization-triggered [Ca^{2+}], transient and the time constants for clearance were almost identical in beta-cells from 6-week-old NOD and Balb/c mice, which suggested that these beta-cells had similar Ca^{2+} homeostasis mechanism. In contrast, calcium clearance was ∼18% slower in 10-week-old NOD beta-cells compared with the Balb/c cells (Fig. 3F, p < 0.05). Thapsigargin (TG) was used to irreversibly inhibit the cellular activity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), the major clearance mechanism in primary beta-cells (9, 20). Inhibition of the SERCA pump led to a considerably slowed clearance in beta-cells from both NOD and Balb/c mice (Fig. 3, D–F). Moreover, the difference in the rate of [Ca^{2+}], clearance between beta-cells from NOD and Balb/c mice was abolished by TG pretreatment, which indicated that SERCA function may be impaired in the NOD mice. Similarly, the [Ca^{2+}], decay rate was ∼25% slower in 14-week-old NOD beta-cells compared with the Balb/c cells (Fig. 3, H and I, p < 0.01), which was abolished by SERCA inhibition. Taken together, these data suggest that SERCA activity is progressively down-regulated in 10-week-old and older NOD beta-cells.

**Age-dependent Down-regulation of the SERCA Pump in NOD Islets—** To directly measure the possible changes in SERCA protein level, we performed Western blotting experiments to compare its expression in islets isolated from 10-week-old NOD and Balb/c mice. As shown in Fig. 4, A and B, the total SERCA protein level, which was detected by an antibody that recognized all three SERCA subtypes, was significantly reduced in NOD islets compared with Balb/c islets.

Because the pancreatic islets expressed the SERCA2 and SERCA3 subtypes (21), we further used real-time quantitative reverse transcription PCR (RT-PCR) to compare relative mRNA expression levels of SERCA2 and SERCA3 in NOD and Balb/c islets from mice of different ages. No differences in SERCA2 or SERCA3 expression were found between 6-week-old NOD islets and age-matched Balb/c islets, which agreed with their similar rates of [Ca^{2+}], decay after depolarization (Fig. 3A). In contrast, there was ∼51–75% less SERCA2 and SERCA3 mRNA in 10-week-old NOD islets compared with the control islets (Fig. 4, C and D). This result agreed with the statistically significant reduction in SERCA protein levels (Fig. 4B) and slowed [Ca^{2+}], clearance in NOD beta-cells (Fig. 3, F and I). Moreover, the SERCA down-regulation became more severe as the mice aged. The SERCA2 and SERCA3 mRNA levels from 14-week-old NOD islets were ∼22–26% of the levels in Balb/c islets (Fig. 4, C and D). Therefore, expression of both SERCA subtypes in pancreatic islets decreases as a function of age in NOD mice.

**Changes of Secretory Ability in 10-Week-old NOD Beta-cells—** Using patch clamp experiments, we further compared the secretory machinery of individual beta-cells isolated from 10-week-old NOD and Balb/c mice. To probe sizes of different vesicle pools within individual beta-cells, exocytosis was elicited by homogenous [Ca^{2+}], elevation generated by flash photolysis of caged Ca^{2+} (22). Exocytosis was quantified as an increase in the whole-cell membrane capacitance (Cm), which was mainly due to fusion of insulin granules to the plasma
membrane (22). As the averages shown in Fig. 5A, flash-photolysis induced a much more pronounced capacitance increase in 10-week-old NOD beta-cells as compared with age-matched controls. The earlier phase of the rise (within 4 s after flash) in capacitance can be fitted with a two-component exponential function; the fast component mainly represented release of rapidly releasable pool of vesicles (RRP), and the slow component represented release of slowly releasable pool of vesicles (SRP) (22). The later phase of the rise in capacitance (within 6–9 s after flash) can be described by a linear fit, which corresponded

FIGURE 3. Age-dependent slowing of [Ca^{2+}] clearance and changes in cellular function in NOD mice. A, D, G, averaged calcium traces after high K⁺ (70 mM) stimulation in beta-cells from Balb/c (open symbol) and NOD (filled symbol) mice at different ages with (Δ) or without (○) TG pretreatment (1 mM, 3 min). (Six weeks Balb/c n = 40, NOD n = 41; 10 weeks control, Balb/c n = 39, NOD n = 43; TG pretreated, Balb/c n = 16, NOD n = 10; 14 weeks control, Balb/c n = 36, NOD n = 30, TG pretreated, Balb/c n = 20, NOD n = 22). B, E, H, depolarization-triggered Δ[Ca^{2+}] in different beta-cells. C, F, I, recovery time constants (τ) in different situations are summarized. Individual calcium recovery traces were fitted with a single-exponential function.

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FIGURE 4. Age-dependent down-regulation of the SERCA pump in NOD islets. A, B, RT-PCR analysis of the mRNA levels of SERCA2b and SERCA3 expression in islets from 10-week-old mice was detected by Western blot. Beta-actin was blotted to verify that there was equal loading of total protein, and the figure is representative of three similar experiments. C, relative intensity of SERCA protein versus actin in Balb/c islets was normalized as 1, and the relative intensity of SERCA protein versus actin in NOD islets was normalized accordingly. Significant less SERCA protein was expressed in 10-week-old NOD mice (n = 3, p < 0.05).

The slowly priming process of released vesicles pool gaining fusion competence was determined. The size of the RRP and the rate of released granules fusion were about the same in the health and disease beta-cells (Fig. 5B and C). In contrast, the size of the SRP of vesicles in NOD beta-cells was ~3.5-fold to that of the control (Fig. 5C), indicating a mechanistic acceleration of vesicle fusion process in 10-week-old NOD mice.

Next, we tested whether depolarization-evoked insulin secretion coupling was also altered in 10-week-old NOD mice. The voltage-gated calcium channel currents generated by depolarization from −70 to 0 mV was slightly but not statistically smaller in control cells (Fig. 5, D and E). The membrane capacitance triggered by the first depolarization, which represents fusion of immediately releasable pool (IRP) of vesicles close to the voltage-gated calcium channel on the plasma membrane (23), was not significantly different in the health and disease beta-cells (Fig. 5G). In contrast, trains of ten depolarizations evoked a significantly larger increase in the membrane capacitances in diabetic beta-cells compared with the control (Fig. 5, F and H), possibly due to the larger SRP of vesicles. Added together, these data indicate that the beta-cells from 10-week-old NOD mice exhibit enhanced secretory capability.

Age-dependent Changes in Glucose-stimulated Calcium Transient in NOD Beta-cells—Under physiological conditions, elevated blood glucose levels repeatedly depolarize pancreatic beta-cells, which stimulate calcium oscillations and pulsatile insulin release. Therefore, glucose-stimulated calcium transient is often used as an indicator of cell function, and defects in SERCA function are often associated with an altered response (24–25). We tested changes in the pattern of glucose-stimulated calcium transient in beta-cells from NOD mice at various ages. The application of 25 mM glucose induced a robust [Ca^{2+}], oscillation in beta-cells from 6-week-old NOD and Balb/c mice (Fig. 6A). First, we determined the maximal amplitude of [Ca^{2+}]_i ([Ca^{2+}]_i,max) that was reached during glucose stimulation. We calculated the first time point at which the increase in the [Ca^{2+}]_i, trace over the basal level was greater than 50% of [Ca^{2+}]_i,max as the time to rise, which defined the relative speed of the [Ca^{2+}]_i, response to glucose. Moreover, we defined the percentage of total time over 50% of [Ca^{2+}]_i,max elevation in [Ca^{2+}]_i, as the plateau time to describe how long [Ca^{2+}]_i, stayed elevated.

There was no difference in either the time to rise or the plateau time for 6-week-old NOD beta-cells (Fig. 6, C and D), which suggests that their cellular functions are similar. In both 10- and 14-week-old NOD mice, [Ca^{2+}]_i, of beta-cells stimulated with high glucose stayed elevated for longer period of time as compared with control cells (Fig. 6D), which will prolong glucose-stimulated insulin secretion in addition to the enhanced secretory ability shown in Fig. 5. On the other hand, the response of [Ca^{2+}]_i, to glucose only from 14-week-old beta-cells was delayed (Fig. 6). Therefore, glucose-stimulated calcium transient exhibited age-dependent different changes in NOD mice, which may relate to the adaptation of beta-cell secretion and function to the requirement of glucose regulation.

DISCUSSION

In the present study, we evaluated beta-cell mass and its functions in mice of different ages. Blood glucose levels 20–60 min after intravenous glucose infusions were significantly lower in 6-week-old NOD mice compared with the control (Fig. 1A), which suggested an excessive insulin release, as has been previously proposed (3, 26). The Ca^{2+} clearance and glucose-stimulated calcium oscillations of individual NOD beta-cells were not significantly different from those of Balb/c cells (Figs. 3A and 6A), which indicated that there were no changes in beta-cell function at this stage. In contrast, despite the appearance of mononuclear cells with islets, there were significantly more beta-cells in NOD mice compared with young control Balb/c mice (Fig. 2E). Therefore, increase in the beta-cell mass represents the major mechanism for the up-regulation of insulin secretion and hypoglycemia in young NOD mice. The cause of this increase in beta-cell mass is unknown but may be reminiscent of the beta-cell replication and neogenesis observed after partial pancreatectomy (27). Because primary periinsulitis lesions appeared in NOD mice as young as 3 weeks old, islet replication may be an adaptive response to inflammation (2).

Recently, it was also suggested that islet cell proliferation is reinitiated in response to an autoimmune attack in type I diabetic human patients (28). This indicates that inflammation-triggered beta-cell replication may be a widely conserved mechanism for insulin hyper-secretion among rodent and human species.

Because of progressive lymphocyte infiltration, beta-cell mass in 10-week-old NOD mice decreased significantly from previously elevated levels. However, compared with the control age-matched mice, the beta-cell mass was similar. Significant down-regulation of the SERCA2 and SERCA3 genes in NOD mice was apparent, which led to the sustained oscillations of [Ca^{2+}], triggered by glucose, similar to what has been observed.
in SERCA3 KO mice (24). SERCA inhibition may also facilitate insulin secretion via augmenting the amplitude of depolarization-triggered \([\text{Ca}^{2+}]_{i}\) transients (9, 20). On the other hand, secretory ability of individual beta-cell at this stage was also enhanced via increasing the size of SRP of vesicles (Fig. 5B), possibly due to an up-regulation of SNARE proteins. This is in contrast to the decreased expression of SNARE proteins in islets of type II diabetic rats and human patients reported previously (29–30), which could be due to different types of diabetes studied. Alternatively, our data may suggest that the expression levels of SNARE proteins are subjected to dynamic regulation during the progression of the diseases, corresponding to different insulin secretory ability observed at different stages. Added together, both increased size of SRP of vesicles and enhanced \([\text{Ca}^{2+}]_{i}\) transient contribute to the enhanced insulin secretion evoked by train depolarization in individual beta-cells (Fig. 5F). Therefore, NOD islets hyper-responsiveness at this stage mainly originated from altered \([\text{Ca}^{2+}]_{i}\) handling and enhanced secretory ability of single beta-cells.

**FIGURE 5. Enhanced secretory ability of beta-cells from NOD mice at 10 weeks old.** A, average capacitance traces evoked by homogenously \([\text{Ca}^{2+}]_{i}\) elevation generated by flash photolysis of NP-EGTA in beta-cells isolated from 10-week-old Balb/c and NOD mice. The initial increase in capacitance (within 4 s after flash) can be fitted with two-component exponential functions. The amplitude and the time constant of the fast and the slow component represent the size and the fusion kinetics of RRP and SRP, respectively. B, as compared with Balb/c mice, NOD beta-cells exhibited a comparable size of RRP of vesicles, but an enlarged SRP of vesicles (\(p < 0.01\)). C, release rates of the sustained component were obtained by a linear fit to the range of 6–9 s after flash in each Cm trace, which were not significantly different in NOD and control cells. D, average calcium current trace in Balb/c and NOD beta-cells stimulated with 100 ms depolarization from −70 to 0 mV (\(n = 8\)), the amplitudes of which were no statistically different as summarized in E. F, beta-cells were depolarized by a series of ten 200-ms pulses from −70 to 0 mV applied at 1 Hz. Average capacitance (Cm) traces recorded from primary pancreatic beta-cells isolated from 10-week-old Balb/c and NOD mice (\(n = 8\)) are shown here. G, sizes of IRP vesicle pool from Balb/c and NOD beta-cells were not significantly different (in fF: Balb/c = 34.4 ± 2.8, NOD = 62.9 ± 14.2). H, trains of depolarization evoked more increase in membrane capacitance in diabetic beta-cells as compared with the control (in fF: Balb/c = 179.3 ± 47.3, NOD = 436.9 ± 99.2, \(p < 0.05\)).
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In 14-week-old NOD mice, beta-cell mass was ~68% of the values of 6-week-old mice and ~66% of the values for Balb/c mice of the same age. The responsiveness of beta-cells to glucose elevations was also significantly delayed, similar to islet cells deficient of insulin receptor substrate (31), or diabetic islet cells with impaired glucose metabolism (32). Smaller $[\text{Ca}^{2+}]_{i}$ transient evoked by KCl depolarization was also observed only in 14-week-old NOD beta-cells (Fig. 3F). It has been shown previously that depolarization triggered calcium release from endoplasmic reticulum (ER) calcium store as well as $\text{Ca}^{2+}$ influx in primary beta-cells (33–34). Severe SERCA inhibition leads to partial depletion of ER calcium stores, and possible changes of ER release pathway during diabetes has also been proposed (35). Therefore, we believe that neither the delayed responses nor reduced $[\text{Ca}^{2+}]_{i}$ transient evoked by KCl in 14-week-old NOD islet is not due to the further reduction in SERCA expression, but rather a age-dependent decline in metabolism and cellular signaling pathways. Nevertheless, sustained $\text{Ca}^{2+}$ transient and enhanced secretory ability of individual beta-cells are able to compensate for the defects in beta-cell mass and responsiveness to glucose. As the result, only transient glucose-intolerance was observed and non-fasting blood glucose was still close to the normal range in these mice. However, depletion of ER calcium store, excessive insulin synthesis and occasional hyperglycemia will cause ER stress that impairs beta-cell function and survival, and finally lead to glucose intolerance.

Transient hyperactivity of beta-cells has been found in other type I diabetic models, e.g., BB rats (36). Earlier transient hyperinsulinemia has also been found to predate insulin resistance in type II diabetic rodents and animals (2). Numerous studies have shown that hyperinsulinemia in fasting and/or glucose-stimulated conditions reliably predicts progression to diabetes in individuals that are susceptible to type II diabetes and in first-degree relatives of type II diabetic patients (2). Therefore, the age-dependent mechanisms of earlier insulin hyperactivity in NOD mice revealed here may have implications in other diabetic models and provide insight into the role of beta-cell dysfunction in all types of diabetes. In fact, the down-regulation of the SERCA protein in NOD mice is shared in type II pre-diabetic and diabetic mice, rats and patients (21, 37–38). We also find that beta-cells from db/db mice also exhibit significant reductions in both SERCA2 and SERCA3 expression (data not shown). Moreover, SERCA function is also impaired in diabetic cardiomyocytes (39), skeletal muscle (40), smooth muscle cells (41), platelets (42), and neurons (43). Therefore, we propose that down-regulation of the SERCA pump is a hallmark of pre-diabetic stage. It not only enhances secretion from pancreatic beta-cells to meet the increasing demand for insulin during the progression of diabetes but also plays a unifying key role in adaptively adjusting the functions of downstream target cells to cope with the various complications from pre-diabetes to diabetes. In type II diabetes, a high glucose (44) or high saturated fatty acid (45) level has been proposed to impair SERCA function. However, these may not contribute to the down-regulation observed here because blood glucose levels in NOD mice were properly regulated. In contrast, cytokine production by lymphocytes may lead to the deterioration of SERCA function in nearby beta-cells (18, 46). The insulin receptor signaling pathway has been associated with SERCA function in beta-cells (47). Therefore, increased insulin release leads to enhanced exposure of beta-cells to high concentrations of insulin, which possibly inhibits the SERCA pump and thereby further enhances secretion in an autocrine manner. Determining the amounts of cytokines or excess insulin that contribute to SERCA inhibition awaits further investigation.

In conclusion, we have shown that different mechanisms contribute to insulin hyper-secretion in NOD mice of various ages, which could provide insight into the general etiology of diabetes. Our study also identifies a central role for SERCA pump down-

![Image](https://via.placeholder.com/150)
regulation in the hyperactivity of individual beta-cells in type 1 diabetes, which contributes to the understanding of calcium signaling pathway alterations in diabetic complications.

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