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Prolonged exposure of pancreatic islets isolated from "pre-diabetic" non-obese diabetic mice to a high glucose concentration does not impair Beta-cell function

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Summary. In the early stages of Type 1 (insulin-dependent) diabetes mellitus patients present a deficient insulin response to glucose. The reasons for this defective response are unknown, but it has been suggested that it reflects a deleterious effect of excessive glucose stimulation on a reduced Beta-cell mass. Female non-obese diabetic (NOD) mice from our colony, at the age of 12–13 weeks, have a normal basal glycaemia but an impaired intravenous glucose tolerance test, insulitis and a defective insulin response to glucose. In order to characterize the potential effect of glucose on the Beta cells at that "pre-diabetic" stage, pancreatic islets were isolated from 12–13 week old female NOD mice. Immediately after isolation (day 0) the NOD islets displayed a defective insulin response to an acute stimulation with 16.7 mmol/l glucose. After seven days in culture at both 11 and 28 mmol/l glucose these islets showed an increased insulin release in response to an acute glucose stimulation. This increase was more pronounced in the islets cultured at 28 mmol/l glucose. Experiments performed in parallel, using islets obtained from a non-diabetes prone strain of mice (Naval Medical Research Institute, NMRI) showed that these islets had a similar insulin release in response to glucose both on day 0 and after seven days in culture at 11 mmol/l glucose. The insulin mRNA levels of NOD islets did not change over one week in culture at 11 or 28 mmol/l glucose, but culture at the high glucose concentration induced a decrease in the islet insulin content. The present data show that culture at high glucose concentrations does not impair the function of islets isolated from NOD mice. These observations make excessive glucose stimulation, as a single factor, an unlikely explanation for the defective insulin release observed in NOD islets in the "pre-diabetic" period.

Key words: Glucose, Type 1 (insulin-dependent) diabetes mellitus, insulin release, NOD mice, pancreatic islets, tissue culture.

There is much evidence to suggest that the clinical onset of Type 1 (insulin-dependent) diabetes mellitus is preceded by a long-lasting autoimmune assault to the pancreatic Beta cells [1]. In parallel with the decrease in the Beta-cell mass, the patients present with an increasingly defective insulin response to i. v. glucose, while they are still able to release insulin in response to non-nutrient secretagogues [2]. The reasons for this defective response to glucose remain unclear, but it has been suggested that this finding reflects a deleterious effect of excessive glucose stimulation on a reduced Beta-cell mass [3, 4]. The further clarification of these issues has been hampered by difficulties in dissociating the effects of toxic and/or immunologically mediated Beta-cell injury from that of the progressive hyperglycaemia, inherent to in vivo models of insulin-dependent diabetes mellitus.

The non-obese diabetic (NOD) mouse currently represents one of the best animal models of human Type 1 diabetes mellitus [5, 6]. The onset of hyperglycemia in these mice is preceded for several weeks by insulitis and ongoing Beta-cell destruction. During this "pre-diabetic" period the NOD mouse develops a progressively impaired glucose tolerance, and their Beta cells show a defective insulin response to glucose, coupled with higher response to arginine and theophylline [7, 8]. Thus, this "pre-diabetic" phase of the NOD mouse represents a good experimental model in which to study the putative deleterious effects of excessive glucose stimulation of the islets of Langerhans during the early phases of insulin-dependent diabetes mellitus.

In the present study, pancreatic islets were obtained from 12–13 week old female NOD mice, an age at which most female animals of our NOD mice colony already exhibit glucose intolerance, insulitis and an impaired islet insulin response to glucose in vitro [8]. The Beta-cell function was evaluated immediately after islet isolation or after one week in tissue culture at 11.1 or 28 mmol/l glucose, by the measurement of glucose-stimulated insulin

release, the number of islets retrieved after culture, and the islet DNA, insulin and insulin mRNA contents. As a control, designed to assess the impact of the culture condition alone on the islets, pancreatic islets from non-diabetic prone Naval Medical Research Institute (NMRI) mice were also studied.

Materials and methods

Animals

Female NOD mice, aged 12–13 weeks, were used in the experiments. The NOD mouse colony was established in Uppsala in March 1988, by brother and sister mating of the offsprings from three breeding pairs of inbred NOD mice obtained from the Clea Company (Aobadi, Japan). The cumulative incidence of diabetes in the colony at 28 weeks of age is 47% for the females and 7% for the males. On the day preceding the islet isolation (see below), a blood sample was obtained in non-fasting animals by retroorbital puncture and the serum glucose concentration was analysed using an automated glucose oxidase method (Glucose Analyser 2, Beckman Instruments, Fullerton, Calif., USA).

As an external control, designed to test the possibility that islet adaptation to a culture condition could by itself influence the results, islets from male NMRI mice (Anticimex AB, Sollentuna, Sweden) were also studied. NMRI mice are a non-diabetes prone strain of mice, not related to the NOD mice and without any known autoimmune disease. Islets from male NMRI mice has been extensively studied in our laboratory as regards islet metabolism and function. In some experiments (Table 3), islets obtained from female NMRI mice were also studied.

Morphology, islet isolation and culture

Non-fasted female NOD or male NMRI mice were killed by cervical dislocation and the pancreas was quickly removed. A piece of the pancreas (about 10–20% of the pancreatic mass) was fixed in Bouin's solution for light microscopical examination and the remaining material utilized for islet isolation (see below). The sections (at least 60 consecutive sections, 7 μm thick) were stained with haematoxylin and eosin and ranked in four classes for the presence of inflammatory cells, as previously described [9]. Class A denotes normal islet morphology; class B denotes a low degree of mononuclear cell infiltration especially in the periinsular area; class C denotes a heavy cell infiltration into a majority of islets and class D denotes that only a few residual islets could be identified, exhibiting an altered islet architecture and signs of cell degeneration.

Pancreatic islets were isolated by collagenase digestion [10] and hand-picked. By this method it was possible to obtain approximately 80 islets from each individual NOD mouse. A similar number of islets were picked from the NMRI mice. The islets were either immediately used for experiments (day 0) or cultured free-floating for seven days (day 7) in culture medium RPMI 1640 (Flow Laboratories, Irvine, UK) containing 11.1 or 28 mmol/l glucose and supplemented with 10% donor calf serum (Flow), benzylpenicillin (100 U/ml) and streptomycin (0.1 mg/ml) [11]. The culture medium was changed every second day, and samples of the medium frozen at −20°C for subsequent determination of insulin accumulation by radioimmunoassay [12]. On the last day of culture, the islets were recounted under a stereomicroscope, and the number of remaining islets expressed as a percentage of the islet number on day 0. Some of the cultured islets were fixed in Bouin's solution, paraffin embedded, sectioned and stained with haematoxylin-eosin.

Islet insulin release, DNA, insulin and insulin mRNA contents

For the insulin release experiments, the islets were divided into triplicate groups of seven islets each and placed in sealed glass vials containing 0.25 ml bicarbonate buffer [13] supplemented with 10 mmol/l HEPES and 2 mg/ml albumin (KRBH buffer). During the first hour of incubation at 37°C (O_2:CO_2; 95:5) the KRBH medium contained 1.7 mmol/l glucose. The medium was then gently removed and replaced by 0.25 ml KRBH supplemented with 16.7 mmol/l glucose, followed by incubation for a second hour. The insulin concentration in the incubation medium was determined by radioimmunoassay [12]. In each experimental group the insulin secretion was calculated as a mean of the values obtained from the three incubation vials. After the insulin release experiments, the islets were pooled and disrupted by sonication in 0.2 ml redistilled water. A 50 μl aliquot of the aqueous homogenate was mixed with 125 μl acid ethanol and the insulin extracted overnight at 4°C. DNA was measured fluorophotometrically in another fraction of the water homogenate [14, 15].

In order to obtain a sufficient number of islets for the insulin mRNA quantification on days 0 and 7, the pancreata of two NOD or two NMRI mice were pooled in each group, and the islets isolated as described above. Groups of 50 islets were sonicated and RNA extracted with phenol-chloroform. Insulin mRNA was quantified by dot blot analysis [16, 17], as previously described [18]. The probe for hybridization was pRI-7 [19]. After hybridization, the filters were washed and then exposed at −70°C to Hyperfilm-MP (Amersham International, Amersham, UK) with an intensifying screen. The intensities of the spots thus obtained were determined by densitometry.

Statistical analysis

Means ± SEM were calculated. The data were then analysed by analysis of variance, followed by multiple comparisons using the Student's paired t-test. The p-values were corrected for multiple comparisons using the Bonferroni method [20].

Results

The 12–13 week old NOD mice included in the study were not overtly diabetic. The observed serum glucose concentrations were 7.9 ± 0.9 mmol/l in the NOD mice ($n = 8$) and 10.0 ± 0.7 mmol/l in the NMRI mice ($n = 7$). Light microscopic examination of the pancreatic sections showed that all NMRI mice belonged to class A (normal islet morphology) ($n = 9$). In contrast, all NOD mice ($n = 10$) had some degree of mononuclear cell infiltration, with three animals ranked as class B and the remaining seven mice as class C.

The loss of islets after the seven-day culture period was less than 10% in all groups (Table 1). On day 0 the NOD islets had high DNA contents. This finding is probably explained by the contribution of infiltrating mononuclear cells to the total DNA content, as previously described [8]. After seven days in culture these mononuclear cells tend to disappear [8], and the NOD islets attain a similar DNA content as that of the NMRI islets. Exposure to a high glucose concentration in culture did not modify the islet retrieval or the DNA content of the islets obtained from both NOD or NMRI mice. Furthermore, light microscopical examination of NOD or NMRI

Table 1. Islet recovery, DNA content and insulin and insulin mRNA contents of islets isolated from NOD or NMRI mice and studied immediately or after seven days in culture at different glucose concentrations

Mouse strain	Medium glucose (mmol/l)	Islet retrieval (%)	DNA content (ng/10 islets)	Insulin content		Insulin mRNA (OD/50 islets)
				(ng/10 islets)	(ng insulin/ng DNA)	
NOD	– (Day 0)	–	855 ± 139	991 ± 61	1.46 ± 0.30	1.46 ± 0.52
	11 (Day 7)	91 ± 2	311 ± 45[a]	553 ± 48[c]	1.89 ± 0.27	2.01 ± 0.46
	28 (Day 7)	92 ± 3	291 ± 26[b]	271 ± 24[c]	0.96 ± 0.07	2.79 ± 0.69
NMRI	– (Day 0)	–	394 ± 49	1074 ± 87	2.40 ± 0.15	1.97 ± 0.45
	11 (Day 7)	93 ± 3	315 ± 33	802 ± 62[a]	2.54 ± 0.22	2.06 ± 0.38
	28 (Day 7)	93 ± 3	343 ± 26	273 ± 40[c]	0.75 ± 0.08[c]	2.75 ± 0.44

Pancreatic islets were isolated from NOD or NMRI mice and studied immediately (day 0) or after 7 days in culture (day 7) at different glucose concentrations (11 or 28 mmol/l). The recovery of islets on day 7 was expressed as the percentage of islets remaining in culture on day 7 as compared to day 0. The insulin mRNA was determined by dot blot analysis, quantified through densitometry and expressed as optical density (OD, arbitrary units). The results are means ± SEM of 8–11 observations. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ when compared to the respective islet group on day 0

islets exposed to 11.1 or 28 mmol/l glucose for seven days revealed well preserved islets, with no obvious signs of cell necrosis or remaining mononuclear cells (not shown). In both NOD and NMRI islets, culture at 11.1 mmol/l glucose induced a decrease in insulin content, as compared to day 0 (Table 1). On the other hand, when the islet insulin content was expressed per DNA this difference was not evident. In both strains, culture at 28 mmol/l glucose induced a further decrease in insulin content, both as compared to day 0 and compared to islets cultured at 11.1 mmol/l glucose ($p < 0.01$ or less). Correction of these results for DNA did not change the observed differences, except for a lack of significance when comparing NOD islets on day 0 and day seven. Immediately after isolation (day 0) islets obtained from NOD and NMRI mice had similar insulin mRNA content. After seven days in culture at 28 mmol/l glucose, both groups of islets tended to increase their insulin mRNA levels compared to the values observed in islets cultured at 11.1 mmol/l glucose, but these difference did not reach statistical significance (Table 1).

Culture at 28 mmol/l glucose, compared to 11.1 mmol/l, increased the insulin accumulation in the medium of NMRI islets during the whole period of observation (Table 2). In contrast, in the cultures containing islets obtained from NOD mice there was no increase in the insulin accumulation in response to the high glucose concentration (28 mmol/l) during the first four days of culture, whereas an increase was observed during days five to six.

Islets obtained from the NOD mice showed a decreased insulin response to an acute glucose stimulation on day 0 (Table 3). However, after seven days in culture at both 11.1 and 28 mmol/l glucose, there was a marked improvement in the insulin release in response to 16.7 mmol/l glucose. During the short-term incubation, islets previously cultured at 28 mmol/l glucose had a higher insulin release than islets cultured at 11.1 mmol/l glucose, both at 1.7 ($p < 0.001$) and 16.7 mmol/l glucose ($p < 0.01$). It is noteworthy that the basal insulin release at 1.7 mmol/l glucose was increased on day 0, as compared to the basal insulin release of islets cultured at 11.1 mmol/l glucose.

In contrast to the observations made in the NOD islets, the NMRI islets showed a similar insulin release in response to 16.7 mmol/l glucose both on day 0 and after one week in culture at 11.1 mmol/l glucose. Culture at 28 mmol/l glucose induced an even higher insulin release in response to both 1.7 and 16.7 mmol/l glucose, as compared to NMRI islets immediately after isolation (day 0)

Table 2. Insulin accumulation in the medium of islets isolated from NOD or NMRI mice over a six-day period in culture at different glucose concentrations

Mouse strain	Medium glucose (mmol/l)	Insulin accumulation in the medium ($ng \cdot 10\ islets^{-1} \cdot 48\ h^{-1}$)		
		Days 0–2	Days 3–4	Days 5–6
NOD	11	255 ± 38	169 ± 20	131 ± 12
	28	264 ± 35	184 ± 24	192 ± 24[a]
NMRI	11	270 ± 27	173 ± 22	126 ± 15
	28	476 ± 71[c]	391 ± 75[c]	415 ± 56[b]

The groups of islets were isolated and maintained in culture as described in Table 1. Medium was collected for insulin determination at 48-h intervals. The results are means ± SEM of 11–12 observations. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ when compared to the respective islet groups maintained at 11 mmol/l glucose

Table 3. Glucose-stimulated insulin release of islets isolated from NOD or NMRI mice and studied immediately or after seven days in culture at different glucose concentrations

Mouse strain	Medium glucose (mmol/l)	Insulin release ($ng \cdot 10\ islets^{-1} \cdot 60\ min^{-1}$)	
		1st h (1.7 mmol/l glucose)	2nd h (16.7 mmol/l glucose)
NOD	– (Day 0)	6.4 ± 1.1	14.0 ± 3.1
	11 (Day 7)	1.4 ± 0.2[b]	53.5 ± 8.2[c]
	28 (Day 7)	11.6 ± 1.2	75.4 ± 6.5[c]
NMRI (male)	– (Day 0)	5.5 ± 1.1	28.2 ± 3.8
	11 (Day 7)	1.4 ± 0.1[a]	34.2 ± 4.9
	28 (Day 7)	11.8 ± 1.0[b]	70.0 ± 6.6[c]
NMRI (female)	– (Day 0)	7.4 ± 2.3	25.2 ± 4.5
	11 (Day 7)	0.9 ± 0.3[a]	35.1 ± 7.9

The groups of islets were isolated and maintained in culture as described in Table 1. Islet insulin release experiments were performed by initially incubating the islets, in triplicate groups of seven, in KRBH containing 1.7 mmol/l glucose. After 60 min at 37 °C (O_2/CO_2, 95:5) the medium was removed and the islets incubated for another 60 min in medium containing 16.7 mmol/l glucose. Values are given as means ± SEM for 6–8 observations. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ when compared to the respective islet group on day 0

or after one week in culture at 11.1 mmol/l glucose ($p < 0.01$).

In a separate series of experiments, the effects of culture in islets obtained from female NMRI mice were also studied. As observed in the male NMRI islets, these islets had a similar DNA content immediately after isolation and after seven days in culture at 11.1 mmol/l glucose (day 0, 294 ± 33; day 7, 294 ± 44 ng DNA/10 islets [$n = 6$]). Furthermore, there was a similar insulin release in response to an acute stimulation with 16.7 mmol/l glucose both on days 0 and 7 (Table 3).

Discussion

Female NOD mice exhibit a long period of insulitis preceding the overt hyperglycaemia [5, 6]. The female NOD mice in our colony have a severe insulitis at 12–13 weeks of age, and with increasing age there is a progressive glucose intolerance and deterioration of insulin release in response to glucose, but the mice are still able to maintain a normal basal glycaemia ([8], present data). Interestingly, when islets are removed from 12–13 week old NOD mice and maintained in culture for one week, there is a clear improvement in the insulin response to an acute glucose challenge ([8], present data). However, when islets are isolated from the non-diabetes prone NMRI mice, islet culture for seven days does not change the insulin response to glucose. These findings suggest that some factor(s) of the in vivo NOD mouse environment affects the insulin secretion of the islet Beta cells, and that this inhibition is reversible in vitro. Two possible explanations for the inhibition include a direct suppressive action by the immune system on the surviving islet cells [21, 22] or a potentially deleterious effect of increasing glycaemic levels [3, 4].

In order to test this second hypothesis, islets obtained from 12–13 week old female NOD mice were exposed to 11.1 or 28 mmol/l glucose over a period of seven days in culture. It can be argued that 11.1 mmol/l glucose is already a high glucose concentration. However, repeated studies have shown that in order to preserve an adequate insulin response to glucose in vitro it is necessary to maintain rodent islets at a slightly higher glucose concentration than that prevailing in vivo [11, 23, 24]. If increasing glucose levels in vivo cause a Beta-cell defective response to glucose [3], a deleterious functional effect of 11.1 and especially 28 mmol/l glucose in the NOD islets in vitro could be anticipated. Nevertheless, a period of seven days in culture at 28 mmol/l glucose neither increased the islet loss compared to 11.1 mmol/l glucose, nor decreased the islet DNA content, islet insulin mRNA content or the glucose-induced insulin release. On the contrary, islets cultured at 28 mmol/l glucose released more insulin in response to a 16.7 mmol/l glucose stimulation than both freshly isolated NOD islets or NOD islets cultured at 11 mmol/l glucose. Interestingly, the acute insulin release in the presence of a low glucose concentration (1.7 mmol/l) was also increased in islets cultured at 28 mmol/l glucose. This finding has been reported previously [23, 25–27], and probably reflects the high glycogen contents in the islet cells [28], high levels of insulin mRNA and activated rates of insulin biosynthesis and release [29]. It is also noteworthy that the insulin content of NOD and NMRI islets decreased in culture, mainly at 28 mmol/l glucose. This decrease is probably related to a high functional activity in vitro, and reflects a depletion of insulin in the Beta cells rather than damage to these cells [30].

To further characterize the impact of islet isolation and culture on Beta-cell function, islets from NMRI mice were also studied. The use of islets from NMRI mice, a strain non-related to the NOD mice, was promoted by the difficulty in obtaining proper non-diabetic controls for the NOD mice. Indeed, the non-obese non-diabetic (NON) mice, a genetically related control for the NOD mice, present mild glucose intolerance and hypoinsulinaemia [31], precluding their use in the current series of experiments. Thus, it cannot be excluded that some of the apparent differences observed between the NOD and NMRI islets can be explained not only by the "pre-diabetic" condition of the NOD mice, but also by strain differences. In any case, the data obtained on the male and female NMRI islets suggest that culture of pancreatic islets in medium RPMI 1640 at 11.1 mmol/l glucose is, indeed, able to preserve a normal response to glucose stimulation. Thus, there was an identical insulin response to 16.7 mmol/l glucose on both days 0 and 7. Similar findings have been recently reported for islets isolated from Sprague-Dawley rats and maintained under similar culture conditions [32]. However, it is conceivable that the collagenase treatment slightly damaged some Beta cells, as suggested by the increased insulin release at 1.7 mmol/l glucose on day 0, and the increased insulin accumulation in the medium during the first two days of culture. It is also possible that leakage of insulin from non-viable Beta cells, in the first few hours following the islet isolation, can contribute to this finding.

Culture of NMRI islets in the presence of 28 mmol/l glucose potentiated the glucose-induced insulin release, as compared with both freshly isolated islets and islets cultured in the presence of 11 mmol/l glucose. These findings confirm recent observations showing that culture for a 21-day period at 28 mmol/l glucose neither impairs the function of normal NMRI islets, nor potentiates the long-lasting damage induced by streptozotocin [27]. As a whole, the observations in islets obtained from NMRI and NOD mice tend to exclude the possibility that culture at high glucose concentrations is cytotoxic to both normal and immunologically assaulted mouse Beta cells. Furthermore, the results make hyperglycaemia, as a single factor, an unlikely explanation for the impaired insulin release observed in NOD islets in the "pre-diabetic" period, and reinforce the possibility of a direct suppressive effect of infiltrating mononuclear cells on the Beta cells. It remains to be determined which are the mediators of this suppression. The observation that the islet suppression can be improved in culture suggest a situation similar to that observed in rat [33] or mouse islets (Eizirik, unpublished data) following exposure to human interleukin-1β, i.e., there is an impairment in islet function that can be reverted if the islets are cultured in a cytokine-free environment for seven days.

It has been previously shown that intensive insulin treatment in the early stages of Type 1 diabetes mellitus can improve Beta-cell function [34–36], suggesting that the decrease in glucose levels could somehow protect the Beta cells. If high glucose is not directly deleterious to the islets, as suggested by the current in vitro data, how can these findings be explained? Recently it has been reported that culture of rat pancreatic islets at high glucose concentrations stimulates the expression of islet cell autoantigens, namely the 64,000-M_r protein [37]. Conversely, metabolic Beta-cell suppression in vivo reduces the expression of pancreatic monosialogangliosides, also considered a potential target for the autoimmune assault to the Beta cells [38]. If decreased metabolical demands decrease islet antigenic expression, the beneficial effects of intensive insulin therapy could be explained by a decrease in the immunological aggression to the islets. Indeed, exposure of rat islets to interleukin-1, one of the possible mediators of the autoimmune reaction in Type 1 diabetes, is able to induce a deficient insulin release in response to glucose, coupled to a better response to non-glucose secretagogues [22, 33], similar to the response observed during the early stages of human Type 1 diabetes [2].

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