Persistent impairment of the insulin response to glucose both in vivo and in vitro after streptozotocin exposure: Studies with grafted pancreatic islets and islets maintained in culture

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Abstract. The functional responses of the pancreatic B-cells after cytotoxic damage are still largely unknown. Using in vitro models to clarify this issue, we have recently observed a preferential reduction of glucose-stimulated insulin production and release in mouse pancreatic islets maintained in culture after in vitro exposure to streptozotocin. In order to evaluate the relevance of these findings in vivo, two sets of experiments were performed. First, mouse pancreatic islets were exposed in vitro to 2.2 mmol/l streptozotocin or vehicle alone, cultured for 6 days, and finally grafted under the kidney capsule of normoglycemic nude mice. Two weeks after transplantation there was no difference in the total DNA and insulin content between the two groups of islet grafts, but the insulin concentration, as expressed per μg DNA, was decreased by 40% in the streptozotocin-treated islets. The insulin release of the grafts, during perfusion of the graft-bearing kidney in situ with 16.7 mmol/l glucose was diminished in the streptozotocin group, whilst perfusion with 16.7 mmol/l glucose plus 5 mmol/l theophylline was able partially to counteract the reduction in insulin release. In the second set of experiments, NMRI mice were injected iv with 160 mg/kg streptozotocin or vehicle alone, and their islets isolated 15 min after the injections. After 6 days in culture, there was no decrease in DNA, glucagon and somatostatin contents, but the insulin content was decreased by 40% in the streptozotocin exposed islets. These islets also showed a 60% decrease in the insulin response to glucose, which was partly counteracted by incubation with 16.7 mmol/l glucose plus 5 mmol/l theophylline. These observations suggest that a defective response to glucose, in conjunction with a better response to non-nutrient secretagogues, may be a common property of pancreatic islets following toxin-induced disturbances.

Type I, or insulin-dependent diabetes mellitus (IDDM), is characterized by a severe insulin deficiency, secondary to impaired B-cell function and B-cell destruction. Despite the abrupt onset of symptoms, the autoimmune process leading to IDDM appears to evolve slowly over several years (1). A remarkable clinical feature in this 'prediabetic' period is a progressively developing inability to secrete insulin in response to intravenous glucose, while still maintaining a partial response to other secretagogues such as arginine and glucagon (2). Similar findings have been described in animal models of spontaneous (3,4) or drug-induced diabetes (5). The reasons for this selective loss of response to glucose are unknown, but it has been suggested to reflect a deleterious effect of an excessive glucose stimulation on a reduced B-cell mass (5). Another possibility could be that the damage inflicted to the B-cells could directly cause functional derangements, leading to such a response.

In an attempt to gain further information about this issue we have developed an in vitro approach, in which isolated islets are exposed to different assaults and subsequently maintained in culture for
one week. The data already obtained show that the surviving B-cells completely recover following exposure to interleukin 1 (6) or alloxan (7). However, after streptozotocin treatment there is a lasting damage to the B-cells, manifested essentially by a markedly diminished glucose-stimulated insulin release, which is partially reversible by compounds known to increase intracellular CAMP levels (8). The insulin production of these islets, as evaluated by insulin content, (pro)insulin biosynthesis and insulin mRNA content, is also decreased to approximately 50% of the controls, whereas other cellular functions remain normal. These findings suggest the intriguing possibility that, following streptozotocin-induced injury, the surviving B-cells are able to maintain most of their basal metabolic functions, but fail to maintain an adequate insulin biosynthesis and release.

In the present study we have carried out experiments which aimed to elucidate whether our previous in vitro findings with streptozotocin may also apply in vivo. For this purpose, mouse pancreatic islets were exposed in vitro to 2.2 mmol/l streptozotocin and, after one week in culture, grafted under the renal capsule of normoglycemic nude mice. After two weeks in vivo the graft-bearing kidneys were perfused (9) in order to evaluate their insulin secretory response. Furthermore, the grafts were recovered and their DNA and insulin contents evaluated. In a separate set of experiments, mice were injected intravenously with streptozotocin, and their islets isolated after 15 min. These islets were maintained in culture for 6 days, and then functionally characterized.

Materials and Methods

Experiments with islets exposed to streptozotocin in vitro and subsequently transplanted into nude mice

Male NMRI mice (Anticimex, Sollentuna, Sweden) which had been starved overnight were used. Islets were isolated with the aid of Ficoll gradients (10) from collagenase-digested pancreata and subsequently harvested from exocrine tissue by means of a braking pipette. The islets were maintained free-floating in tissue culture for 4–5 days at 37°C in air + 5% CO₂ (11) before exposure to streptozotocin (SZ; lot 2408A, a generous gift from Dr. A. Y. Chang, Upjohn Co, Kalamazoo, MI, USA). The culture medium was RPMI 1640 (Flow Laboratories, Irvine, UK) containing 10% calf serum, benzylpenicillin (10⁵ U/l), streptomycin (0.1 g/l), and 11.1 mmol/l glucose. The culture medium was changed every 48 h.

The islets were then exposed in vitro to 2.2 mmol/l SZ or pure citrate buffer (CB; 10 mmol/l, pH 4.5) (controls) for 30 min at 37°C, according to a previously described protocol (8). This SZ concentration (2.2 mmol/l) has been found to induce a long lasting inhibition of insulin biosynthesis and release (12). Furthermore, at that concentration it is still possible to recover most of the SZ-exposed islets (8,12). Immediately thereafter, the islets were transferred to culture medium RPMI 1640 and maintained in culture for 7 days. After this period, groups of 250 control or SZ-treated islets were suspended in Hank's salt solution (Statens Bakteriologiska Laboratorium, Stockholm, Sweden) and implanted, by means of a small glass pipette, beneath the left renal capsule (13) of ether-anesthetized normoglycemic male nude (nu/nu) Balb/c mice (Bomholtgaard, Ry, Denmark).

Two weeks after transplantation, the grafted animals were anesthetized with sodium thiobutabarbitral (Ryk Gulden, Konstanz, FRG; 130 mg/kg, ip). The kidneys were then removed and prepared for perfusion as previously described in detail (9). The organ preparation was perfused with a bicarbonate buffer (14) supplemented with 10 mmol/l HEPES, 20 g/l albumin, 20 mg Dextran T 70 (Pharmacia Fine Chemicals, Uppsala, Sweden) and either 2.8 mmol/l glucose, 16.7 mmol/l glucose or 16.7 mmol/l glucose plus 5 mmol/l theophylline. The perfusion medium was kept at 37°C and continuously gassed (O₂:CO₂; 95:5) while being administered at a flow rate of 1.5 ml/min, without recycling, for 85 min. The experiment started with a 15-min perfusion period with medium containing 2.8 mmol/l glucose; this was followed by 20 min perfusion with 16.7 mmol/l glucose; 15 min with 2.8 mmol/l glucose; 20 min with 16.7 mmol/l glucose + 5 mmol/l theophylline, and finally 15 min with 2.8 mmol/l glucose. A 1.5-ml sample of the effluent medium was collected every fifth min except for the first 5 min of perfusion with the high glucose or glucose + theophylline concentrations, when samples were taken every min. The insulin concentration of the effluents was measured by radioimmunoassay (15). After the perfusion, the grafts were carefully dissected in toto from the transplantation site under the kidney capsule and disrupted by manual homogenization (Potter-Elvehjem tubes) and subsequent sonication in redistilled water. An aliquot of the aqueous homogenate was mixed with acid-ethanol and the insulin extracted overnight at 4°C. DNA was measured fluorophotometrically in another fraction of the water homogenate (16,17).

Experiments with islets exposed to streptozotocin in vivo followed by islet isolation and subsequent culture for 6 days

Non-fasted male NMRI mice were injected with SZ or 0.2 ml vehicle (CB) only. SZ was dissolved immediately before use in CB and administered iv at a diabetogenic
dose of 160 mg/kg body weight (18). After 15 min the mice were killed by cervical dislocation, the pancreas quickly removed and the islets isolated from the collagenase-digested pancreas, without the aid of Ficoll gradients. By this means at least 150 islets per mouse were obtained within 45 min after SZ administration. The islets were subsequently maintained in culture, as described above, for 6 days.

The number of islets were recounted, using a stereomicroscope, on the last day of culture, and the islets utilized for morphological studies and determination of insulin release and insulin and DNA contents. Insulin release was studied in triplicate groups of 10 islets, as previously described (8). During the first hour of incubation the medium contained 1.7 mmol/l glucose. The medium was then gently removed and replaced by a solution containing either 16.7 mmol/l glucose or 16.7 mmol/l glucose plus 5 mmol/l theophylline, and the incubation was continued for a second hour. The insulin concentration in the incubation medium was determined by radioimmunoassay (15). In each experimental group the insulin secretion was calculated as a mean from the three incubation vials. After the release experiments, the islets were pooled and disrupted by sonication in redistilled water, and insulin and DNA content determined as described above. Furthermore, the glucagon and somatostatin contents of these islets were assayed according to the methods described by Faloona & Unger (19) and Peeters et al. (20), respectively, using assay kits provided by Milab AB, Malmö, Sweden.

Calculations and statistical analysis
In the perfusion experiments, the insulin secretory rate per graft or per graft DNA content was calculated by multiplying the insulin concentration in the sample by the flow rate, and the values were thus expressed as ng insulin/min. The total insulin response to glucose or glucose plus theophylline of the grafts was obtained by planimetry of the area under the individual perfusion profiles, and expressed as ng insulin/20 min or ng insulin/µg DNA per 20 min.

All results were expressed as means ± SEM and statistical differences were calculated using Student’s t-test for unpaired data.

Results
Experiments with islets exposed to streptozotocin in vitro and subsequently transplanted into nude mice
After 2 weeks in vivo, the grafted islets could be macroscopically identified as a whitish spot under the kidney capsule. There was no difference in the DNA and insulin contents of grafted SZ-treated islets or CB-treated islets (Table 1). However, there was a 40% decrease in the insulin concentration of the grafted SZ-treated islets when the data were expressed per µg DNA.

The functional capacity of the grafts was assessed by perfusing each graft-bearing kidney. When the perfusion fluid was changed from a medium containing 2.8 mmol/l glucose to 16.7 mmol/l glucose, the secretion of insulin increased within a few minutes in both groups (Fig. 1). However, the total insulin release in response to 16.7 mmol/l glucose was decreased by 70% in the SZ group in comparison to the control group (Table 2). Perfusion with 16.7 mmol/l glucose plus 5 mmol/l theophylline induced a marked further increase in insulin release in the control grafts, compared with that observed after stimulation with 16.7 mmol/l glucose alone (p < 0.01). In the animals transplanted with SZ-treated islets there was also an increased stimulation with 16.7 mmol/l glucose plus 5 mmol/l theophylline compared with 16.7 mmol/l glucose alone (p < 0.01), but it still remained about 50% lower than in the control group. This difference did not, however, attain statistical significance.

Experiments with islets exposed to streptozotocin in vivo followed by islet isolation and subsequent culture for 6 days
In a second set of experiments, NMRI mice were injected with SZ or CB only, their islets isolated after 15 min and maintained in culture for 6 days. After this period it was possible to recover 92 ± 3%

Table 1.

<table>
<thead>
<tr>
<th>SZ (mmol/l)</th>
<th>Graft DNA content (µg)</th>
<th>Graft insulin content (ng/graft)</th>
<th>Graft insulin content (ng/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.58 ± 0.50</td>
<td>4335 ± 450</td>
<td>1307 ± 181</td>
</tr>
<tr>
<td>2.2</td>
<td>3.95 ± 0.27</td>
<td>3140 ± 442</td>
<td>784 ± 66*</td>
</tr>
</tbody>
</table>

Pancreatic islets were treated and grafted under the kidney capsule of nude mice as described in Materials and Methods. Data are given as means ± SEM of 7 (controls) or 5 (SZ) experiments. * denotes p < 0.05 when compared versus control islets.
of the control islets and 82 ± 4% of the SZ-treated islets (N = 6 for both groups; p < 0.05). The DNA contents of the remaining islets were similar irrespective of whether the donor mice had been treated with SZ or not (Table 3). Furthermore, there were no differences in the glucagon and somatostatin contents of islets isolated from SZ or CB injected mice. However, there was an almost 40% decrease in the insulin content of the SZ-exposed islets.

Table 2.
Insulin release of control or streptozotocin (SZ) treated islets grafted under the kidney capsule of nude mice.

<table>
<thead>
<tr>
<th>SZ (mmol/l)</th>
<th>Secretagogues (mmol/l)</th>
<th>Insulin release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ng insulin/graft per 20 min</td>
</tr>
<tr>
<td>0</td>
<td>Glucose (16.7)</td>
<td>22.4 ± 5.3</td>
</tr>
<tr>
<td>2.2</td>
<td>Glucose (16.7)</td>
<td>6.2 ± 0.8*</td>
</tr>
<tr>
<td>0</td>
<td>Theophylline (5)+glucose (16.7)</td>
<td>131.3 ± 30.1</td>
</tr>
<tr>
<td>2.2</td>
<td>Theophylline (5)+glucose (16.7)</td>
<td>64.1 ± 13.8</td>
</tr>
</tbody>
</table>

Pancreatic islets were treated and grafted under the kidney capsule of nude mice as described in Materials and Methods. The kidneys were perfused with KRBH medium containing 2.8 mmol/l glucose and subsequently stimulated for 20 min with 16.7 mmol/l glucose and 16.7 mmol/l glucose + 5 mmol/l theophylline, as shown in fig. 1. Total insulin output was estimated by planimetry of the individual perfusion profiles. Data are given as means ± SEM of 7 (controls) or 5 (SZ) experiments. * denotes p < 0.05 and ** p < 0.01 when compared versus control islets.
Table 3.
Islet DNA and hormonal contents in islets isolated and maintained in culture after in vivo exposure to streptozotocin (SZ; 160 mg/kg body weight).

<table>
<thead>
<tr>
<th>SZ injection (mg/kg)</th>
<th>DNA content (ng/10 islets)</th>
<th>Islet hormonal contents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Insulin (ng/µg DNA)</td>
</tr>
<tr>
<td>0</td>
<td>143 ± 9</td>
<td>5840 ± 850</td>
</tr>
<tr>
<td>160</td>
<td>153 ± 14</td>
<td>3830 ± 310*</td>
</tr>
</tbody>
</table>

NMRI mice were injected with 160 mg/kg body weight SZ or citrate buffer only (control), the islets isolated after 15 min and maintained in culture for 6 days. Data are given as means ± SEM of 6 separate experiments. * denotes p < 0.05 when compared against control islets.

There was a slight decrease in the basal insulin release at 1.7 mmol/l glucose in SZ-treated islets (Table 4). When exposed to 16.7 mmol/l glucose, the control islets responded with an 8-fold increase in insulin release compared with the basal release at 1.7 mmol/l glucose. Incubation of such islets with 16.7 mmol/l glucose plus 5 mmol/l theophylline induced an enhanced insulin release. In the SZ-exposed islets the absolute values of insulin release were diminished after stimulation with both glucose and glucose plus theophylline, compared with control islets stimulated with the same secretagogues. This decrease was most pronounced when the SZ-exposed islets were stimulated with 16.7 mmol/l glucose alone, which resulted in only a 4-fold increase compared with the basal release at 1.7 mmol/l glucose (p < 0.02 versus control islets). Theophylline reversed the SZ-induced reduction of insulin release, if we compare the 17-fold stimulation above the basal release, with the 16-fold increase observed in the control islets. Expression of the insulin release data per islet (data not shown), instead of per µg/DNA (Table 4), did not alter the above described observations.

Table 4.
Insulin release of islets isolated and maintained in culture after in vivo exposure to streptozotocin (SZ, 160 mg/kg body weight).

<table>
<thead>
<tr>
<th>SZ injection (mg/kg)</th>
<th>Insulin release (ng insulin/µg DNA)</th>
<th>Glucose (16.7)</th>
<th>Theophylline (5) + glucose (16.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First hour 1.7 mmol/l glucose</td>
<td>Second hour different secretagogues (mmol/l) as indicated</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>52.9 ± 7.8</td>
<td>389.3 ± 50.8</td>
<td>158.3 ± 25.5**</td>
</tr>
<tr>
<td>160</td>
<td>38.1 ± 8.7</td>
<td>1030 ± 103</td>
<td>597 ± 81**</td>
</tr>
</tbody>
</table>

The islets were treated as described in Table 3. After 6 days in culture the islets, in triplicate groups of 10, were incubated in KRBH medium containing 1.7 mmol/l glucose. After 60 min the medium was removed and the islets incubated for a second hour with the secretagogues given in the second column. Data are given as means ± SEM of 6 separate experiments. * denote p < 0.05 and ** p < 0.01, respectively, when compared versus control islets stimulated with similar secretagogues.
Discussion

The present study aimed to elucidate if the lasting damage induced by SZ to islets in vitro can be reproduced when two key steps of the experimental procedure are transferred to the in vivo situation. These steps included the maintenance of the islets in vivo after in vitro exposure to SZ or the exposure of islets to the drug in vivo and subsequent culture in vitro.

The transfer of the in vitro SZ-treated islets to a normoglycemic in vivo environment was accomplished by grafting the islets beneath the kidney capsule of normoglycemic nude mice. Two weeks after transplantation, the insulin release after stimulation with glucose was diminished in the SZ-treated islets. Theophylline was able partially to counteract the SZ-induced reduction of insulin release. These data indicate that although functionally impaired, the islets exposed to SZ in vitro are viable and become engrafted in a proper way. To what extent their decreased functional activity after transplantation can be explained by a reduced insulin secretion per B-cell or rather reflects a reduced volume density of B-cells in the grafts remains unclear. The DNA content values do suggest that the cell number of the control and SZ-treated and grafted islets is similar. However, it must be kept in mind that these DNA values may include a contribution of cells constituting the fibrous capsule covering the islet graft. It should also be noted when interpreting these data that the total insulin content of the grafts, irrespective of the previous treatment with CB or SZ, was proportionally lower than the insulin content observed in non-grafted islets (Table 3). This finding suggests that either not all islet cells had survived in the new site, or that they tend to be less granulated than in the in vitro condition.

By exposing the islets to SZ in vivo in the second set of experiments, we avoided a possible experimental bias derived from a potential non-homogeneous islet diffusion of SZ in vitro. The dosage of SZ injected (160 mg/kg) has been shown to be diabetogenic in NMRI mice (18). Thus, when the mice were killed 14 days after the SZ injection, there was a marked decrease in the number of islets, and the few remaining islets showed a severely altered architecture and pyknotic cell nuclei (18). Quite in contrast, a great majority of islets isolated 15 min after the SZ injection and subsequently kept in culture survived (Table 3) and, though functionally impaired in a way similar to that of islets exposed to SZ in vitro (8), did not show a severe derangement in architecture or the presence of pyknotic cell nuclei (data not shown). This discrepancy between in vivo and in vitro findings can not be explained just by the shorter time of B-cell exposure to SZ, owing to islet isolation 15 min after drug injection. The deleterious action of SZ in the B-cells in vivo seems to be established within 2 min of drug administration (21). Furthermore, islets isolated 10 min after SZ injection show a markedly decreased respiratory activity, insulin release and glucose oxidation (22).

One possible explanation for the discordant in vivo and in vitro observations on the islet survival after injection of SZ could be a contribution by the immune system for further in vivo islet destruction. During the first few days after SZ injection, there is a macrophage infiltration in the islets (23) and an activation of resident non-endocrine Ia-positive cells (24), which apparently ingest and eliminate the remnants of destroyed B-cells. Direct macrophage cytotoxicity against islet cells has also been demonstrated (25) and interleukin 1, a cytokine produced by activated macrophages, can be cytotoxic to B-cells (26). Therefore, it is conceivable that macrophages migrating to the islets for scavenging purposes after the SZ-induced damage, would also further damage the surviving B-cells.

Another possibility is that the chronic hyperglycemia induced by the SZ treatment could contribute to an accelerated death of already damaged B-cells. This notion is further supported by the recent findings that sustained hyperglycemia, superimposed on a reduced B-cell mass induced by pancreatectomy in dogs, caused a profound reduction in the number and size of the islets (27). However, the fact that culture of islets at 28 mmol/l glucose after in vitro SZ exposure (28) improved islet survival, in comparison with islets maintained in culture at 5.6 or 11.1 mmol/l glucose, makes it improbable that direct effects of hyperglycemia are the only explanation for this discordance between in vivo and in vitro observations.

After isolation of the islets exposed to SZ in vivo there was a slight but significant loss of islets during the 7-day culture period. In the remaining islets the number of cells per islet was preserved, as evidenced by their DNA content. Likewise, they had a normal content of both glucagon and soma-
tostatin. It is therefore conceivable that the 40% decrease in their insulin content does not reflect a shift in the volume density of B-cells in the islets. The findings of a defective glucose-stimulated insulin release and that theophylline, a phosphodies- terase inhibitor, could partially restore the impaired glucose-stimulated insulin release reinforce the possibility that, under these experimental conditions, we are studying mainly a population of B-cells with an impaired capacity to release insulin. Whether this impaired capacity is due to survival of partially damaged B-cells, or rather to the destruction of a subpopulation of more active B-cells by SZ, remains unresolved.

On the basis of these and previously reported data it can be suggested that a defective insulin response to glucose, in conjunction with a better response to non-nutrient secretagogues, may be a common property of pancreatic islets following toxic (e.g. streptozotocin) (8, and present data), immunologically (e.g. interleukin-1β) (6,29), or functionally (e.g. hyperglycemia) (30,31) induced disturbances. It has previously been proposed that the phase of a preferential loss of response to glucose during the course of IDDM may reflect a noxious effect of glucose on a reduced B-cell mass (5). However, this arises in a situation in which the presumptive functional impairment induced by hyperglycemia cannot be dissociated from functional effects of B-cell damage. The present experimental approach where B-cells are exposed to a tentative B-cell toxin in vitro, transplanted and subsequently examined functionally in vivo, may be a useful tool to study interactions between toxic events and in vivo factors which might cause B-cell impairment and destruction during the course of diabetes.

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