Effects of cyclosporin A and verapamil on mouse pancreatic islets

Chun I. Shi, Pál Rooth and Inge-Bert Täljedal

Department of Histology and Cell Biology, University of Umeå, Umeå, Sweden

The immunosuppressant drug cyclosporin A is used to prevent the rejection of organ transplants and the progression of autoimmune disease (1–3). One application is diabetes mellitus type I, with its immunological damage to the pancreatic islets, frequent need for kidney replacement and ongoing research on pancreas or islet transplantation.

The usefulness of cyclosporin A is hampered by the toxicity of the drug, notably against kidney and pancreatic islets. Cyclosporin A can induce morphological changes of the islet β-cells, depletion of insulin and abnormal insulin secretory responses (4–15). In both kidney (3, 16, 17) and pancreas (18), a decrease in blood flow has been reported. In islets transplanted under the kidney capsule, revascularization is hampered by cyclosporin A (19).

Studies on the microcirculation in mouse kidney cortex revealed that pretreatment with verapamil, a calcium antagonist, can prevent the inhibition of blood flow induced by cyclosporin A (16, 17); a similar effect has been confirmed in rat pancreas (18). Verapamil also improved the vascular ingrowth into islets grafted to kidney (19). In clinical studies on cadaver kidney recipients, verapamil has been found to improve blood flow, to ameliorate the acute cyclosporin A nephrotoxicity, to prevent the delay of renal function and to reduce the risk for rejection (3).

The results of cyclosporin A and verapamil treatments on the blood flow in kidney and on the revascularization of islets transplanted under the kidney capsule raise questions as to the direct effects of the drugs on the pancreatic β-cells. In the present study we have addressed this problem by in vivo and in vitro studies in normal untransplanted mice.

Materials and methods

Animals and chemicals

Adult male NMRI mice (38–44 g) from ALAB (Sollentuna, Sweden) were given free access to water throughout. Pelleted food was supplied until the night before each experiment.

Cyclosporin A from Sandoz (Basel, Switzerland; Sandimmun®, 50 g cyclosporin A/l cremophore) was diluted in saline to a concentration of 5 g/l cremophor from Sigma Chemical Co. (St Louis, MO, USA) was diluted in saline to a concentration of 100 g/l; and verapamil from Knoll AG (Ludwigshafen, Germany: Isoptin®, 2.5 g/l) was diluted in saline to a concentration of 0.125 g/l. Collagenses was from Boehringer Mannheim GmbH (Germany), tissue culture medium RPMI 1640 (containing 11.1 mmol/l d-glucose) from Flow Laboratories (Irvine, Scotland), fetal calf serum from Labkemi AB (Stockholm, Sweden), benzylpenicillin from Astra AB (Södertälje, Sweden), gentamicin (Garamycin®, 10 g/l) from Schering Corp (Kenilworth, NJ, USA), HEPES (N-2-hydroxyethyl-piperazine-N’-2-ethane sulphon acid) from Boehringer Mannheim GmbH (Germany) and bovine serum albumin (fraction 5) from Miles (Slough, UK). Reagents of analytical grade and twice-distilled water were used.

Isolation and culture of islets

In each experiment, six mice were fasted overnight. The pancreatic glands were excised and islets isolated by collagenase digestion and density centrifugation in dextran. The islets were incubated preliminarily at 37°C for 3 h in RPMI medium containing 10% heat-inacti-
**h**

Table 1. In vivo characteristics of mice treated with cyclosporin A and verapamil for 11 days. a

<table>
<thead>
<tr>
<th>Group</th>
<th>Insulin in pancreas (ng/mg wet wt)</th>
<th>Insulin in plasma (µg/l)</th>
<th>Fasting plasma glucose (mmol/l)</th>
<th>Fed plasma glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>185±16</td>
<td>2.4±0.5</td>
<td>4.6±0.4</td>
<td>10.6±2.1</td>
</tr>
<tr>
<td>CsA</td>
<td>136±13**</td>
<td>2.1±0.4</td>
<td>5.4±0.6</td>
<td>10.6±1.4</td>
</tr>
<tr>
<td>CsA + V</td>
<td>109±10**</td>
<td>2.3±0.4</td>
<td>4.9±0.2</td>
<td>10.1±0.9</td>
</tr>
<tr>
<td>V</td>
<td>203±15</td>
<td>2.2±0.4</td>
<td>5.1±0.9</td>
<td>11.5±1.2</td>
</tr>
</tbody>
</table>

*All values refer to test samples taken on the 11th (glucose) or 12th (insulin) day after commencing treatment with cyclosporin A (CsA; 2.5 mg/kg per day) with or without verapamil (V; 0.4 mg/kg per day). Plasma glucose was measured after fasting overnight, as well as 1 h later when the mice had been given free access to food. Mean values ± SEM for four animals. Differences from control were tested by analysis of variance: **p < 0.01.

Table 2. Insulin in islets and medium during culture. a

<table>
<thead>
<tr>
<th>Group</th>
<th>Islets</th>
<th>Medium</th>
<th>Islets plus medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation for 1 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (4)</td>
<td>115±10</td>
<td>3.5±1.3</td>
<td>119±10</td>
</tr>
<tr>
<td>CsA (4)</td>
<td>103±14</td>
<td>4.0±1.3</td>
<td>107±14</td>
</tr>
<tr>
<td>CsA + V (4)</td>
<td>101±15</td>
<td>4.3±1.5</td>
<td>105±15</td>
</tr>
<tr>
<td>Culture for 24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (7)</td>
<td>135±15</td>
<td>86±16</td>
<td>221±28</td>
</tr>
<tr>
<td>CsA (7)</td>
<td>103±15</td>
<td>76±10</td>
<td>179±16*</td>
</tr>
<tr>
<td>CsA + V (7)</td>
<td>101±13</td>
<td>65±12</td>
<td>166±18*</td>
</tr>
<tr>
<td>Culture for 72 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (3)</td>
<td>71±6</td>
<td>21±0</td>
<td>92±6</td>
</tr>
<tr>
<td>CsA (3)</td>
<td>71±8</td>
<td>21±2</td>
<td>91±6</td>
</tr>
<tr>
<td>CsA + V (3)</td>
<td>77±6</td>
<td>19±1</td>
<td>96±7</td>
</tr>
</tbody>
</table>

* Mean values ± SEM are given for insulin in islets and medium (ng/isle) after the indicated periods of exposure to cremophor alone (control), 2 mg/l cyclosporin A (CsA) or cyclosporin A in combination with 37.5 µg/l verapamil (V). The numbers of separate culture experiments are shown within parentheses. Differences from control were tested by analysis of variance: *p < 0.05, **p < 0.01.

Experiments in vivo

To determine the normal concentration of insulin in NMRI mouse pancreas, seven untreated animals were used.

The in vivo effects of drugs were tested by injecting groups of four mice intraperitoneally for 11 days. Once daily the animals received cremophor alone (5 ml/kg body wt), cyclosporin A (25 mg in 5 ml cremophor/kg) or verapamil (0.4 mg/kg), followed 10 min later by cyclosporin A (25 mg in 5 ml cremophor/kg) or verapamil alone (0.4 mg/kg). Both before any injection had been given and on day 11, tail-tip blood samples (about 30 µl) were collected for determinations of the plasma glucose concentration. The blood samples were extracted immediately by ultrasonication in acid ethanol to determine their insulin content.

**Perfusion of islets**

The perfusion chamber was housed in an infant incubator at 37°C (20). Islets were perfused at a rate of 1.0 ml/min with Krebs–Ringer bicarbonate buffer continuously gassed with 95% O₂ and 5% CO₂ and containing 20 mmol/l HEPES, 1 g/l bovine serum albumin and D-glucose as required.

The experiments were started by 45 min of perfusion with 2.8 mmol/l D-glucose. The medium was then quickly changed to one of the same composition except that the glucose concentration was 16.7 mmol/l. After 15–25 min (as indicated in the Results section), the glucose concentration was switched back to 2.8 mmol/l for the final 15 min. During the last 10 min of the initial period at low glucose concentration, five samples of the effluent were collected to define the basal insulin secretory rate. After changing to the high glucose concentration, the effluent was sampled every minute for the first 5 min and then at intervals of 2, 3 and 5 min. Sampling at 5-min intervals was also used during the final period at low glucose concentration. Perifused islets were extracted by ultrasonication in acid ethanol and assayed for their insulin content.

vated fetal calf serum, 11.1 mmol/l D-glucose, 60 mg/l benzylpenicillin and 60 mg/l gentamicin. Groups of 50 islets were then distributed to sterile plastic Petri dishes (Heger Plastics AB, Stallarholmen, Sweden) containing 4 ml of the same culture medium supplemented with test substances. In one experimental series, each experiment comprised three parallel dishes containing cremophor alone (40 ml/l; control), cyclosporin A (2.0 mg/l with 40 ml/l cremophor), or cyclosporin A (2.0 mg/l with 40 ml/l cremophor) plus verapamil (37.5 µg/l). In a second series of experiments, the verapamil concentration was raised to 25 mg/l (51 µmol/l) and a fourth dish was included containing verapamil alone.

After 1, 24 or 72 h at 37°C in a water-saturated atmosphere of air and 5% CO₂, culture media were collected for insulin assay. In most of the experiments the cultured islets were transferred from the Petri dishes to a perfusion system for measuring insulin release. In two experiments, however, they were extracted immediately...
taken after an 18-h fast as well as 1 h after the animals again had been given free access to food. At the end of the experiment, the mice were killed by decapitation. The pancreas was dissected out, weighed and extracted by ultrasonication in 2 ml of acid ethanol. Blood samples for measuring the plasma insulin concentration were also taken from the neck wound of each animal.

**Glucose and insulin assays**

Plasma glucose was measured by a luciferin/luciferase system (21). Insulin was assayed radioimmunologically with crystalline mouse insulin as standard.

**Statistical analyses**

Mean values ±SEM are presented. The probability of zero difference between means was judged by student's t-test or by analysis of variance in conjunction with the Newman–Keuls test for multiple comparisons. Two-tailed tests were used throughout.
Results

Drug effects in vivo

The pancreas of untreated NMRI mice contained 203 ± 16 ng insulin/mg wet weight (mean ± SEM for seven animals). Mice treated for 11 days with cremophor alone (controls) or with verapamil showed a normal content of insulin in the pancreas (Table 1). Treatment with cyclosporin A, with or without verapamil, decreased the insulin content of the pancreas significantly. The plasma concentrations of insulin and glucose remained unchanged.

Drug effects in vitro

Islet content and release of insulin during culture. Exposure to cyclosporin A for 1 h had no effect on the islet insulin content or on the amount of insulin recovered from the culture medium (Table 2). However, after 24 h an inhibitory action of cyclosporin A on the islet content of insulin was evident. This effect was not modified by the presence of verapamil. Whether or not drugs were present, both the islet content of insulin and the rate of insulin accumulation in the medium were of the same magnitude after 24 h as after 1 h, but fell markedly after 72 h.

Dynamics of insulin release after culture. The presence of cyclosporin A during culture for 1 h did not influence the subsequent dynamics of insulin secretion in a microperfusion system (Fig. 1, Table 3). After 1 and 24 h of culture, the basal insulin release at 2.8 mmol/l glucose was unaffected by the islets having been exposed to cyclosporin A. However, the secretory response to a sudden challenge with 16.7 mmol/l glucose was decreased significantly in islets treated with cyclosporin A for 24 h. This effect of cyclosporin A seemed even more pronounced after 72 h of culture. The inhibitory action of cyclosporin A was not prevented by combining the drug with 37.5 µg/l verapamil in the culture medium.

In a second series of cultures for 24 h, cyclosporin A again was found to inhibit the subsequent insulin release during microperfusion (Fig. 2, Table 3). And, again, verapamil afforded no obvious protection, although the concentration of verapamil was increased to as much as 25 mg/l. The presence of this high concentration of verapamil during culture in itself caused a marked inhibition of the glucose-stimulated insulin secretion.

Discussion

When mouse pancreatic islets were transplanted under the kidney capsule, prolonged treatment with cyclosporin A was found to inhibit the establishment of a functioning blood flow in the grafts (19). In acute infusion experiments, cyclosporin A also inhibited the subcapsular microcirculation of blood in the kidney cortex (3, 16, 17). In view of those results, cyclosporin A possibly could damage the function of the transplanted islets in two ways: directly by adverse effects on the islets per se, and indirectly by nephrotoxic damage to the surrounding recipient tissue. To elucidate these mechanisms, it seemed necessary to perform the present corollary study of untransplanted normal mouse islets.

In vitro perfusion of the rat pancreas has revealed no immediate effect of cyclosporin A on insulin secretion (11). After 2–3 h of exposure to the drug (0.1–100 mg/l), isolated rat islets displayed a dose-dependent inhibition of the insulin secretory response to glucose (6). When culturing human (5), rat (7) or mouse (4) islets with cyclosporin A (0.1–10 mg/l) for several days, various signs of β-cell dysfunction have been observed, including inhibited synthesis of nucleic acids and proinsulin, diminished insulin content and inhibited insulin secretion.

In our experiments, mouse islets were cultured with 2 mg/l cyclosporin A for up to 3 days. During that time period we saw only relatively small effects of the drug on the insulin contents in islets or culturing medium, although a significant decrease occurred in 24 h. However, when the cultured islets were subsequently challenged with 16.7 mmol/l glucose in a microperfusion system, it became evident that cyclosporin A had induced a time-dependent secretory defect in the β-cells. It was not detectable as early as after 1 h of exposure to cyclosporin A but became obvious after 24 h. There are many reports of disturbed β-cell morphology and function, as well as of glucose intolerance and hyperglycaemia, in rats treated with cyclosporin A for several days (e.g. 9, 10 and 13–15). The present in vivo results in mice are in keeping with this pattern. Thus, the
insulin content in the pancreas was decreased after 11 daily injections of 25 mg cyclosporin A kg body wt, although we observed no effects on insulin or glucose in plasma. The absence of overt disturbances of glucose homeostasis most probably reflects the vast reserve capacity of the mouse endocrine pancreas. It suggests that experimentation on kidney–islet interactions in transplanted mice should be feasible, without hindrance from the direct cytotoxicity of cyclosporin A against the β-cells.

The previously described adverse effects of cyclosporin A on capillary blood flow in mouse kidney cortex and transplanted islets could be prevented by injecting verapamil together with the immunosuppressant (3, 16, 17, 19). In non-transplanted rats fed cyclosporin A for 7 days, diltiazem, another calcium antagonist, was reported to afford a certain protection of the first phase of glucose-stimulated insulin release (12). Interestingly, calcium transport in the β-cells has been considered a target for cyclosporin A (8).

In one series of our culture experiments, the concentration of verapamil was lower than normally used for blocking calcium transport and insulin release in short-time in vitro studies (22). This low concentration was chosen to mirror the verapamil/cyclosporin A dosage ratio in their interactions on the microcirculation (16, 17, 19). Neither in vivo nor in vitro did this low concentration of verapamil counteract the effects of cyclosporin A in non-transplanted mouse islets. Cyclosporin A decreased the content of insulin in pancreas, decreased the content of insulin in cultured islets and inhibited insulin release from cultured islets as much in the presence as in the absence of verapamil. Moreover, when verapamil was tested at a high concentration known to inhibit strongly the insulin release in short-time experiments (22), again there was no obvious protection against the release-inhibiting action of cyclosporin A. Instead, the post-culture secretory response to glucose was suppressed, although not blunted, by the prior exposure to this concentration of verapamil alone.

The present observations are in contrast to the quite striking beneficial effects of verapamil on the renal cortical blood flow (16, 17) and the vascularization of islets transplanted to the kidney cortex (19). Therefore, we suggest that the action of verapamil on blood flow in transplanted islets is due to events in the recipient renal tissue rather than to a primary interaction between verapamil and cyclosporin A in the grafts.

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References

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