Treatment With Diazoxide Causes Prolonged Improvement of β-Cell Function in Rat Islets Transplanted to a Diabetic Environment

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Prolonged hyperglycemia desensitizes β cells. A role for hyperglycemia-induced excessive stimulation can be tested by diazoxide, which inhibits glucose-induced insulin secretion. Using diazoxide, we have investigated in a rat transplantation model whether excessive stimulation can induce lasting effects on β cells. One batch with 150 islets and another with 20 islets isolated from Wistar-Furth rats were transplanted under the left-kidney capsule of syngeneic streptozotocin-diabetic recipients. In a first series, recipients were treated for 8 weeks with or without 0.2% diazoxide in the food. Graft-bearing kidneys were then perfused and excised. Diazoxide treatment increased by 5.5-fold the insulin response to 10 mmol/L arginine, by 4.1-fold the graft insulin content, and by 2.3-fold the preproinsulin mRNA versus nontreated diabetic controls. The persistence of these effects was assessed in a second series in which 8 weeks of diazoxide treatment was followed by 1 week of no treatment. Again, perfusion experiments showed a higher insulin response to arginine in diazoxide-treated rats (136.0 ± 25.7 *v* 62.3 ± 11.8 fmol/min, $P < .05$). Also, the response to 27.8 mmol/L glucose was increased (54.0 ± 17.1 *v* 13.6 ± 7.8 fmol/min, $P < .05$). The insulin content was increased (2.2 ± 0.6 *v* 1.0 ± 0.4 pmol/islet, $P < .05$), as well as the preproinsulin mRNA (0.60 ± 0.08 *v* 0.22 ± 0.02 pg/islet, $P < .05$). In a third series, we tested the impact of diazoxide treatment when given only during the first 2 weeks following transplantation. When tested 6 weeks later, insulin secretion was unaffected, whereas there was a strong tendency for a higher preproinsulin mRNA and insulin content in grafts of diazoxide-treated rats. In conclusion, this study demonstrates that β-cell function in transplanted islets is improved by diazoxide long after the end of treatment, an effect that is likely due to removal of hyperglycemia-induced excessive stimulation.

IT IS WELL RECOGNIZED that chronic hyperglycemia can impair β-cell function and perhaps survival.[1,2] Such negative effects are probably multifactorial. Based on prior studies,[3-5] it seems likely that overstimulation is one important factor. In a previous study[3] in which nondiabetic rats were made hyperglycemic for 48 hours by glucose infusion, we found that the hyperglycemia-induced attenuation of insulin secretion[6] could be avoided by coinfusion of diazoxide. Furthermore, Leahy et al[5] investigated the effects of removal of excessive stimulation by diazoxide on β-cell function in 90% pancreatectomized rats. In these experiments, rats were treated with diazoxide for 5 days and pancreas perfusion experiments were performed 2 days after the last administration of diazoxide. Also, these results showed an increase in insulin secretion and content in the treated rats.

Diazoxide is known to reversibly inhibit glucose-induced insulin secretion by opening adenosine triphosphate–sensitive K^+ channels in the cell membrane of β cells, thereby preventing β-cell depolarization.[7] No effects of the medication per se could be demonstrated to account for the protective effect of diazoxide.[4] Therefore, the effect of diazoxide was assigned to the prevention of excessive stimulation.

However, the relevance of these findings to the effects of more chronic hyperglycemia during diabetes is still in doubt. In particular, it is not clear to what extent any effects of long-term overstimulation are reversible. The effects of 48-hour glucose infusion in normal rats are known to be rapidly reversible.[6] However, more chronic hyperglycemia may possibly exert lasting and perhaps irreversible effects on β-cell function and could also induce structural damage.

The aims of the present study were (1) to investigate the effect on β-cell function of reducing β-cell stimulation during a prolonged period of exposure to a diabetic environment with hyperglycemia, and (2) to investigate whether the effects are rapidly reversible. To this end, we used a transplantation model in which insulin secretion and the insulin and preproinsulin mRNA content of islet grafts transplanted to diabetic recipients were assessed after 8 weeks' treatment with diazoxide. Upon finding positive effects, reversibility was then tested by assessing the same parameters of β-cell function also after an ensuing 1-week period of no treatment. Lastly, we also tested for a lasting impact of treatment during the first 2-week period following transplantation.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Stockholm Ethics Committee for Animal Experiments. Male Wistar-Furth rats (B&K Universal, Sollentuna, Sweden) served as recipients. They were made diabetic by intravenous injection with 60 mg/kg streptozotocin (Sigma, St Louis, MO) at least 2 weeks before transplantation. Diabetes was confirmed by blood glucose greater than 20 mmol/L.

Isolation and Transplantation of Islets

Islets of Langerhans were isolated by collagenase treatment[8] from 12- to 15-week-old female Wistar-Furth rats. Islets from the nondiabetic rats were cultured overnight in RPMI 1640 (Life Technologies, Paisley, UK). Transplantation was then performed basically as previously described.[9] In our study, 2 batches of islets containing 150 to 200 and 20 islets, respectively, were transplanted to separate sites under the left-kidney capsule. To minimize interexperimental variation, the islets

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isolated from 1 donor were divided into transplants for both control and diazoxide-treated rats.

Experimental Protocols

Diazoxide was administered as a 0.2% admixture to the powdered control food (B&K Universal). In the first protocol (protocol 1), graft-bearing kidneys were isolated and perfused at the end of an 8-week treatment period. In the second protocol (protocol 2), an 8-week treatment period was followed by 1 week of no treatment before perfusions were performed. In protocol 2, we also restricted the food intake of the control diabetic (untreated) rats so that any difference in blood glucose levels between groups was minimized, such difference otherwise occurring because of a moderate reduction of food intake in the diazoxide-treated rats. In the third protocol (protocol 3), diazoxide was given for the first 2 weeks after transplantation, followed by 6 weeks without any treatment. In all experiments, blood glucose levels were examined weekly at 9 AM. In protocol 2, blood was also sampled during 1 day at several time points for insulin measurements.

Kidney Perfusion

Graft-bearing left kidneys were perfused with Krebs-Ringer bicarbonate buffer supplemented with 20 g/L bovine serum albumin (Sigma), dextran T-70 (Pharmacia, Uppsala, Sweden), and 3.3 mmol/L glucose. After an equilibration period of 10 minutes, sequential stimulations with 27.8 mmol/L glucose and 10 mmol/L arginine were performed.[10] The effluents were collected every minute and stored at −20°C pending insulin assay. Grafts were retrieved after perfusion experiments and stored for measurement of preproinsulin mRNA (main graft) and insulin content (smaller graft).

Point-Counting Morphometry

In protocol 2, isolated pancreases from the animals were excised, weighed, and fixed in phosphate-buffered 4% formaldehyde solution overnight and routinely embedded in paraffin. Immunohistochemical identification of β cells was made using a monoclonal antibody against insulin visualized by the avidin-biotin-peroxidase complex method (Vectastain; Vector Laboratories, Burlingame, CA). The number of β cells was estimated by conventional point-sampling, and the proportion of β cells was calculated by dividing the intercepts over β cells by the intercepts over islets.

Assays

Blood glucose was determined by a glucose oxidase method (Accutrend Sensor Glucose; Boehringer Mannheim, Mannheim, Germany). Insulin in the grafts was extracted as previously reported.[11] The insulin concentration was measured by radioimmunoassay.[12]

Measurement of Preproinsulin mRNA

Total RNA of islet grafts was extracted by an acid phenol method.[13] Preproinsulin mRNA levels were determined by a nonsaturated solution hybridization assay using an RNA probe radiolabeled with ^{35}S-UTP.[14] An in vitro–synthesized 58-bp oligonucleotide corresponding to the last part of exon 3 of the rat preproinsulin II gene flanked by *Bam*HI and *Kpn*I restriction sites was inserted into pGEM-3Zf(+). The resulting vector was linearized by *Eco*RI and transcribed in vitro with SP6 RNA polymerase in the presence of 3 μmol/L ^{35}S-UTP for synthesis of the probe. Unlabeled sense RNA was obtained by transcription with T7 RNA polymerase after linearization with *Xba*I. The DNA template was removed by RQ1 deoxyribonuclease 1, and transcripts were separated from unincorporated nucleotides on nick columns. The concentration of unlabeled sense RNA was determined spectrophotometrically. Three serial dilutions of each RNA sample in 20 μL 0.2× SET (1× SET contains 1% sulfanilamide, 20 mmol/L Tris-HCl, pH 7.5, and 10 mmol/L EDTA) were mixed with 20 μL 2× hybridization solution (20,000 cpm probe, 1.2 mmol/L NaCl, 8 mmol/L EDTA, 1.5 mmol/L dithiothreitol, 50% formamide, and 40 mmol/L Tris-HCl, pH 7.5). After hybridization at 70°C for 18 hours, the samples were treated with 40 μg RNase A and 100 U RNase T1 in the presence of 100 μg herring-sperm DNA for 60 minutes at 37°C in a vol of 1 mL. Protected probe was precipitated with 100 μL 100% trichloroacetic acid. Precipitates were collected on glass-fiber filters and radioactivity was counted in a scintillation counter. Parallel hybridizations with increasing amounts of unlabeled sense RNA allowed the construction of a standard curve (Fig 1). The amount of preproinsulin mRNA was calculated by comparison to the standard curve. Background radioactivity, determined by hybridizations without graft extracts and sense RNA, was less than 1% of the input radioactivity. The assay was linear in the range of 1 to 50 times the background. All quantifications of preproinsulin mRNA in graft extract are based on at least 3 serial dilutions within the linear range of the assay.

Statistical Analysis

Data in the tables and figures are expressed as the mean ± SEM. The insulin secretory responses were calculated as the area under the concentration curve after subtracting values immediately preceding the introduction of the secretagogues. Tests of significance were performed using the Wilcoxon signed-rank test. Probability values less than .05 were considered significant.

RESULTS

Body Weight, Food and Diazoxide Intake, and Blood Glucose

The body weight of diazoxide-treated and control rats is shown in Table 1. No significant differences were found before or after the period of transplantation. The food intake in protocol 1 was lower in diazoxide-treated rats (73.2% ± 3.1% of control rats, $P < .05$). In the same protocol, blood glucose tended to be higher in control rats versus diazoxide-treated rats. In protocol 2, food intake in control rats was restricted to achieve similar blood glucose levels between the groups (Table 1 and Fig 2). The mean daily intake of diazoxide (pooled data from protocols 1 and 2) was 291 ± 13 mg/kg (mean ± SEM).

Plasma Insulin

Treatment with diazoxide was associated with a tendency, albeit nonsignificant, for lower levels of plasma insulin (Table 2).

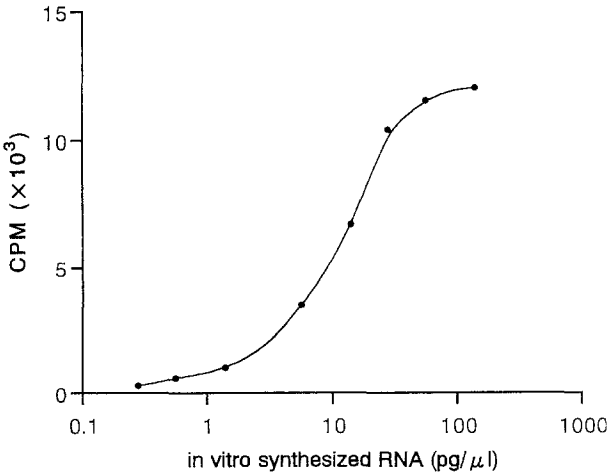

Fig 1. Standard curve relevant to the solution hybridization assay for rat preproinsulin mRNA.

Table 1. Body Weight and Blood Glucose Before and After Treatment With Diazoxide

Protocol/Group	No. of Rats	Body Weight (g)		Blood Glucose (mmol/L)	
		Before	After	Before	After
Protocol 1					
Control diabetic	7	221 ± 8	230 ± 6	27.5 ± 1.4	39.2 ± 1.2
Diazoxide-treated diabetic	7	218 ± 7	239 ± 6	26.2 ± 1.6	31.6 ± 1.4
Protocol 2					
Control diabetic	6	249 ± 11	249 ± 8	23.7 ± 1.0	22.5 ± 1.6
Diazoxide-treated diabetic	6	249 ± 8	240 ± 6	24.5 ± 1.2	23.9 ± 1.2
Protocol 3					
Control diabetic	6	231 ± 3	243 ± 5	22.8 ± 1.4	25.2 ± 0.6
Diazoxide-treated diabetic	6	231 ± 3	231 ± 4	22.3 ± 3.5	22.6 ± 4.0

Effects of 8-Week Treatment With Diazoxide on Insulin Secretion From Islet Grafts

The insulin response to 27.8 mmol/L glucose of grafts from untreated control rats was abolished, whereas subsequent stimulation with 10 mmol/L arginine induced significant secretion (Table 3). These results are in agreement with previous data from the same transplantation model.[10] In kidney-graft perfusions performed sequentially to 8 weeks of diazoxide treatment (protocol 1), the insulin response to arginine was increased relative to grafts from control animals; however, glucose-stimulated insulin secretion was still absent (Fig 2 and Table 3). In the second series of experiments (protocol 2), perfusions were performed 1 week after the last administration of diazoxide. Under these conditions, the previous treatment was still associated with a marked enhancement of arginine-induced insulin secretion. Additionally, and in contrast to protocol 1, a moderate but significant restoration of glucose-induced insulin secretion could be demonstrated (Fig 3 and Table 3).

Effects of 8-Week Treatment With Diazoxide on Insulin and Preproinsulin mRNA Content

Diazoxide treatment, when immediately followed by perfusion (protocol 1), was associated with a 4.1-fold increase in insulin and a 2.3-fold increase in preproinsulin mRNA content (Table 4). Such effects persisted in grafts from recipients in which treatment was withheld for 1 week (protocol 2), showing a 2.2-fold increase in insulin content and a 2.7-fold increase in preproinsulin mRNA (Table 4).

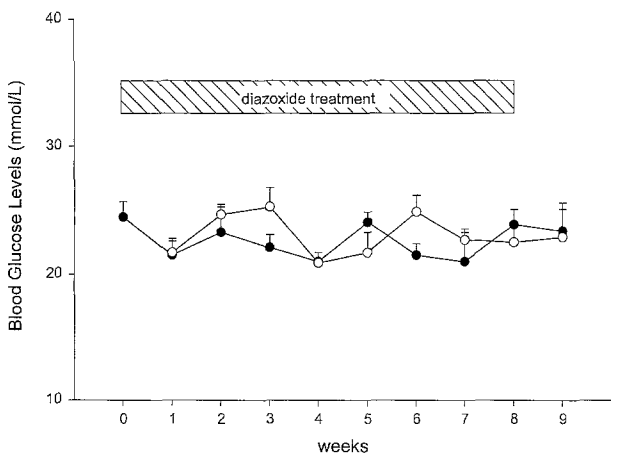

Fig 2. Blood glucose levels of control (○) and diazoxide-treated (●) rats in protocol 2.

Table 2. Plasma Insulin in the Rats

Group	No. of Rats	Plasma Immunoreactive Insulin (μU/mL)		
		1 wk	3 wk	7 wk
Control diabetic	6	24.0 ± 3.5	32.3 ± 4.6	29.2 ± 7.1
Diazoxide-treated diabetic	6	15.9 ± 0.7	17.8 ± 2.3	12.9 ± 4.2

Point-Counting Morphometry

The proportion of β cells in islets did not differ between control and diazoxide-treated pancreases (13.9% ± 4.1% *v* 13.3% ± 2.4% in control rats).

Effects of 2-Week Treatment With Diazoxide on β-Cell Function of Islet Grafts

In protocol 3, diazoxide treatment for 2 weeks was followed by 6 weeks without treatment. The 2-week period of treatment failed to affect insulin secretion in the presence of arginine or elevated glucose (Table 3). However, there was a strong tendency ($P < .1$) for increased insulin and preproinsulin mRNA content in islet grafts from treated rats (Table 4).

DISCUSSION

The present study demonstrates major positive effects of diazoxide treatment on β-cell function in islet grafts in diabetic recipients. All parameters, ie, insulin secretion and insulin and preproinsulin mRNA content, were markedly increased after 8 weeks of treatment. The effect on preproinsulin mRNA is important, since it shows that the present beneficial effects of diazoxide extend beyond protection against exhaustion of insulin stores, an effect previously proposed to be of sole significance.[5]

Our results furthermore show for the first time that the effects of diazoxide are not rapidly reversible and may even be permanent. Hence, 1 week without treatment following diazoxide only partly diminished the difference in insulin content between control and treated rats vis-à-vis results obtained without such a procedure. Furthermore, 1 week without treatment failed to diminish the beneficial treatment effects on insulin secretion.

The effects of diazoxide treatment also appear considerable with reference to β-cell function in transplants to nondiabetic rats. Hence, an 8-week transplantation period reduced the insulin mRNA of transplants to diabetic rats by 72% compared with transplants to nondiabetic rats, and reduced arginine-induced insulin secretion by 67%.[10]

A slowly reversible or permanent effect could be secondary, at least in part, to the preservation of β-cell numbers in relation to control grafts. It follows that the effects on insulin mRNA that we observe could be to some extent secondary to effects on β-cell death.

Removal of excessive stimulation failed to affect β-cell numbers in the pancreases. It may relate to the effect of

Table 3. Insulin Secretion From Islet Grafts

Protocol/Group	No. of Rats	Insulin Secretion (fmol/min) 3.3 mmol/L Glucose	27.8 mmol/L Glucose	10 mmol/L Arginine
Protocol 1				
Control diabetic	7	0.2 ± 0.1	0.7 ± 0.5	26.9 ± 13.5
Diazoxide-treated diabetic	7	0.1 ± 0.1	8.1 ± 6.5	148.8 ± 38.7*
Protocol 2				
Control diabetic	6	10.6 ± 5.0	13.6 ± 7.8	62.3 ± 11.8
Diazoxide-treated diabetic	6	3.3 ± 2.1	54.0 ± 17.1*	136.0 ± 25.7*
Protocol 3				
Control diabetic	6	0.0 ± 0.0	0.0 ± 0.0	42.0 ± 14.1
Diazoxide-treated diabetic	6	0.0 ± 0.0	1.9 ± 1.5	31.5 ± 19.7

*$P < .05$ *v* control.

Table 4. Insulin and Preproinsulin mRNA Content in Islet Grafts

Protocol/Group	No. of Rats	Insulin Content (pmol/islet)	Preproinsulin mRNA Content (pg/islet)
Protocol 1			
Control diabetic	7	1.0 ± 0.1	0.42 ± 0.04
Diazoxide-treated diabetic	7	4.1 ± 0.6*	0.97 ± 0.25
Protocol 2			
Control diabetic	6	1.0 ± 0.4	0.22 ± 0.02
Diazoxide-treated diabetic	6	2.2 ± 0.6*	0.60 ± 0.08*
Protocol 3			
Control diabetic	6	1.1 ± 0.2	0.45 ± 0.09
Diazoxide-treated diabetic	6	1.6 ± 0.2	0.67 ± 0.03

*$P < .05$ *v* control.

streptozotocin treatment, which, when given neonatally, severely diminishes the replicative potency of surviving β cells.[15,16] When streptozotocin was given at an adult age (present experiments), the replicative potential may have been additionally exhausted by the marked hyperglycemia.

We find only nonsignificant inhibition by diazoxide of plasma insulin levels in the recipient rats. These levels reflect pancreatic rather than graft secretion, since the pancreatic β cells, although reduced by about 80%, would still contribute a vast majority. Of relevance to the present findings also are our previous observations that β cells from neonatally streptozotocin-diabetic rats are partly unresponsive to an inhibitory effect of diazoxide on secretion.[17]

It is interesting to note that diazoxide treatment followed by 1 week without treatment was associated not only with an improved response to arginine but also with a definite improvement of glucose-induced insulin secretion. Previous studies have shown that the insulin response to arginine are more robust during diabetic conditions than the response to glucose, and the latter response may indeed be abolished in the face of a normal or even exaggerated response to arginine.[18] In previous experiments using the model of 48-hour glucose-infused rats, glucose-induced insulin secretion was specifically decreased by the glycemia and preserved by diazoxide.[3] It seems possible that glucose-induced insulin secretion, for reasons presently unclear, is more sensitive to overstimulation than the responses to other secretagogues. It is intriguing, though, that the diazoxide effect on glucose-induced insulin secretion was apparent only after 1 week without treatment. A possible explanation is a remaining inhibitory effect of diazoxide in protocol 1 in the kidneys with grafts at the time of perfusion. However, such lasting inhibition was not previously found in perfused pancreases[3] or under other conditions.[4]

It was previously shown that hyperglycemia is a negative factor during the early period following islet transplantation.[19] Our data from a 2-week period of diazoxide treatment are suggestive of an important effect of diazoxide during this period of islet implantation. Hence, an adverse effect of hyperglycemia during transplantation could include excessive stimulation. Because diazoxide has a vasodilating effect, as well as an insulin secretion–blocking effect, it is possible that the treatment with diazoxide increased renal blood flow and that such action could affect engraftment.

The present study adds to a number of reports demonstrating the positive effects of long-term diazoxide treatment, albeit by mechanisms that may be partly different from the present findings.[20,21] The present study provides further evidence that diazoxide may be potentially useful as a therapeutic agent in

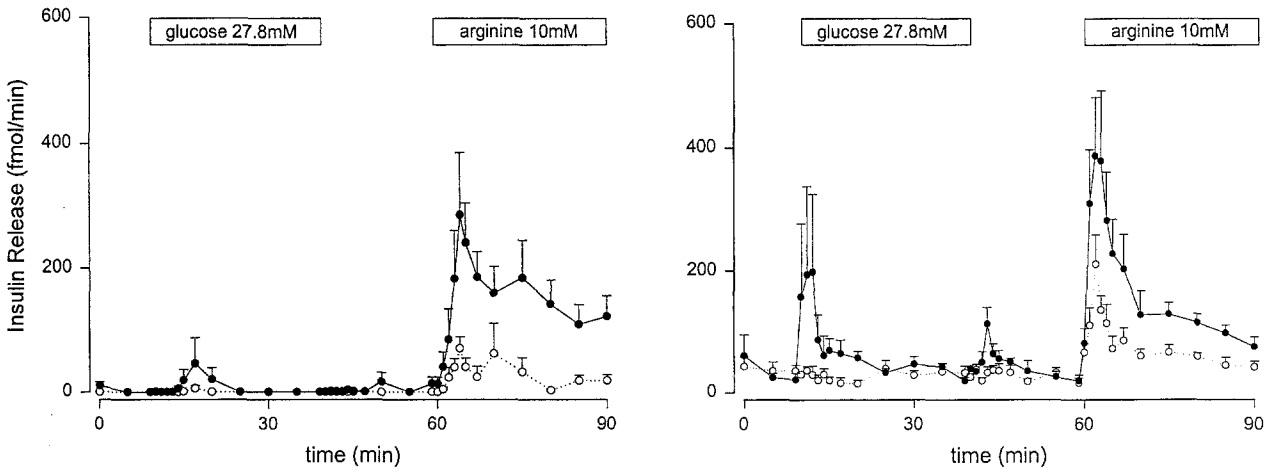

Fig 3. Insulin secretion from perfused transplants in control (○) and diazoxide-treated (●) rats after 8 weeks of treatment in protocol 1 (8-week treatment without washout, left) and protocol 2 (8-week treatment followed by a 1-week washout period, right).

different forms of diabetes. In conclusion, the results of the present study indicate that long-term treatment with diazoxide causes a prolonged improvement of the insulin secretory response and—whether by protecting β-cell survival and/or function—also the capacity for insulin biosynthesis. These effects are of importance when considering optimal procedures for the transplantation of islets or β cells and could also be of relevance to the treatment of type 2 diabetic patients.[22]

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