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Rescue of beta-cell exhaustion by diazoxide after the development of diabetes mellitus in rats with streptozotocin-induced diabetes

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Abstract

In this study, we attempted to demonstrate the possibility of rescuing beta-cell exhaustion by chronic intervention with an ATP-sensitive K^+ channel opener, diazoxide, which reduces the stress of insulin secretion, using rats with streptozotocin-induced diabetes. Three groups of male Wistar rats: (i) controls ($n=7$), (ii) streptozotocin (30 mg/kg i.v.)-induced diabetic rats ($n=10$), and (iii) streptozotocin-induced diabetic rats treated with diazoxide 30 mg/kg for 6 weeks ($n=10$), were studied. Intraperitoneal 2-g glucose tolerance testing was performed every 2 weeks, and pancreatic tissue was examined after 6 weeks of treatment with diazoxide. The insulin concentration in diabetic rats treated with diazoxide was significantly higher than in diabetic rats without diazoxide (6.6 ± 1.6 vs. 2.4 ± 1.0 ng/ml, $P<0.05$). The islet size and its cell number were reduced in diabetic rats compared to those in normal control rats. In normal control rats, 88% of pancreatic islet cells were insulin-positive, while 50% or less were positive in diabetic rats. However, islet size and its cell size appeared to be well preserved by diazoxide treatment. The average mass of islets in diazoxide-treated rats was significantly larger than that in untreated control animals. In addition, the degree of immunostaining for insulin was obviously higher in rats treated with diazoxide than in rats without diazoxide. Pancreatic proinsulin mRNA was restored in rats treated with diazoxide. The present study demonstrated that diazoxide protected from further damage the pancreatic beta-cells both functionally and morphologically in streptozotocin-induced diabetic rats by suppression of excessive insulin secretion. Our results strongly suggest the possibility that chronic intervention with an ATP-sensitive K^+ channel opener prevents the progress of deranged beta-cell function even after the development of diabetes mellitus.

Keywords: Diazoxide; Streptozotocin-induced diabetes; Beta-cell damage; Diabetes, rat

1. Introduction

A beta-cell defect essentially exists with hyperglycemia, which is common in patients with fully developed diabetes mellitus (DeFronzo, 1988; Rossetti et al., 1987). If diabetic subjects are not treated and remain hyperglycemic, the function of beta-cells deteriorates significantly, and this phenomenon is recognized as "glucose toxicity" (Rossetti et al., 1990). Therefore, it is important to take steps to protect beta-cells from further damage associated with the development of diabetes. One possible method is to reduce the level of glucose concentration by any procedure (Kosaka et al., 1980). Another possible intervention that has been suggested was the use of K^+-adenosine triphosphate (K_{ATP}) channel openers in diabetic subjects (Gomis et al., 1988; Greenwood et al., 1976).

Diazoxide acutely increases the plasma glucose concentration by inhibiting insulin secretion through opening K_{ATP} channels, and this effect is opposite to the action of sulfonylurea drugs (Trube et al., 1986). Since diazoxide can suppress insulin secretion from beta-cells, its use was originally advocated to interrupt the damage to beta-cells by resting their function if given in advance or simultaneously, when damage to beta-cells is in progress. Using streptozotocin-induced diabetic rats, it was shown that diazoxide has a protective effect if given in advance and simultaneously to rats (Culbert et al., 1974). More recently, this protective effect of K_{ATP} channel openers against the toxic effect of

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streptozotocin was confirmed morphologically and functionally for rat islets (Kullin et al., 2000).

The acute process of beta-cell damage is a typical interaction in the autoimmune-induced beta-cell damage that exists in type 1 diabetes mellitus. In type 1 diabetes mellitus that develops through an autoimmune mechanism, octreotide and diazoxide were used with the purpose of allowing beta-cell rest (Karlsson and Bjork, 1997; Bjork et al., 1998). This procedure was tried in adults with autoimmune diabetes (Bjork et al., 1996). There were attempts to show prophylactic effects of diazoxide in an animal model with impaired glucose tolerance and obesity such as Otsuka Long–Evans Tokushima fatty rats (Aizawa et al., 1995) and *db*/*db* mice (Lee, 1981). During the development of type 2 diabetes mellitus, insulin resistance causes significant stress in beta-cells. Diazoxide potently ameliorates hyperinsulinemia in insulin-resistant states (Alemzadeh et al., 1998; Surwit et al., 2000). Diazoxide reduced excess insulin secretion from residual beta-cells in 90% pancreatectomized rats resulting in the amelioration of hyperglycemia-induced exhaustion of beta-cells (Leahy et al., 1994). After transplantation of rat islets, diazoxide causes prolonged improvement of their function (Hiramatsu et al., 2000).

Streptozotocin acutely and excessively damages pancreatic beta-cells by chemical toxicity (Saini et al., 1996). This effect itself does not last long. However, in the absence of insulin treatment, functional and morphological damage to beta-cells continues and hyperglycemia persists. Thus, streptozotocin-induced diabetic rats provide a model of beta-cell deterioration through glucose toxicity (Malaisse, 1991). In the present study, to demonstrate the protective effect of chronic pharmacological intervention in terms of rescue of beta-cell exhaustion due to sustained hyperglycemia, we examined the effect of a K_{ATP} channel opener, diazoxide, to prevent functional and morphological damage to beta-cells in streptozotocin-induced diabetic rats. This is the first report of the long-term effect of diazoxide on both functional and morphological recovery of deranged pancreatic beta-cell function in an animal model with chronic hyperglycemia.

2. Materials and methods

2.1. Animals

Male Wistar rats, 8 weeks of age with a body weight of ~ 270 g, were purchased from Japan Charles River (Yokohama, Japan). All rats were fed normal laboratory chow and kept under a standard light (6 a.m. to 6 p.m.) and dark (6 p.m. to 6 a.m.) cycle. One week after arrival, the rats were injected with 30 mg/kg of streptozotocin (Sigma, St. Louis, MO) in 0.05 M citrate buffer (pH 5.0) intravenously via the tail vein. A dose of 30 mg/kg of streptozotocin was used to obtain mildly diabetic rats (Junod et al., 1969). The same amount of vehicle was injected to control rats ($n = 7$). One week after streptozotocin treatment, the diabetic rats were randomized into two groups after confirming that prandial plasma glucose concentrations were more than 160 mg/dl: one with 30 mg/kg diazoxide (Wako, Osaka, Japan) injection per day ($n = 10$) and the other with the vehicle ($n = 10$). The protocol was reviewed and approved by the Committee for Institutional Laboratory Animal Care of Kawasaki Medical School.

2.2. Behavioural procedures

2.2.1. Study protocol

Diazoxide was dissolved in sterilized water to make a final concentration of 30 mg/ml, and its pH was adjusted to 11.0 with 0.5 N NaOH. Diazoxide, 30 mg/kg body weight, was given intraperitoneally around 5 p.m. once daily. Body weight and plasma glucose were measured after fasting for 12 h, once every week. Plasma glucose concentrations at noon, 6 p.m., and 6 a.m. the following morning, were measured every other week. Intraperitoneal glucose tolerance (2 g/kg body weight) was tested every other week. After 6 weeks of treatment with diazoxide or vehicle, the animals were killed under anesthesia with 50 mg/kg phenobarbital (Abbott Laboratories, Abbott Park, IL). Pancreases were rapidly excised into two parts: one piece was frozen in liquid nitrogen and the other was soaked in 34% formaldehyde solution (Wako). Frozen samples were stored at −80 °C until further assay. Soaked tissue was fixed in paraffin for histochemical analyses.

2.2.2. Intraperitoneal glucose tolerance test

After 12-h fasting, 2 g/kg body weight glucose was injected intraperitoneally. Blood samples were taken from the tail vein into heparinized capillary tubes at 0, 30, 60, and 120 min before and after glucose loading. Plasma samples were kept at −20 °C until glucose and insulin assays.

2.2.3. Chemical determinations

Plasma glucose was measured with the glucose oxidase and mutarotase method (Miwa et al., 1972) (Glucose CII test Wako, Wako). Serum and pancreatic insulin concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Bio-science, Yokohama, Japan). The coefficient of variation of intertest analysis and intratest analysis was less than 5%. All samples were tested in duplicate.

2.2.4. Histochemistry

Samples embedded in paraffin were sliced 4-μm thick and set on silicon-coated glass slides. The samples were deparaffined, and then stained with hematoxylin–eosin and azan using standard methods. Antisera of guinea pig to rat insulin and C-peptide were purchased from Linco (St. Charles, MO). Anti-immunoglobulin G (H + L chain) of guinea pig was purchased from Sigma. Standard chemicals from Nichirei (Tokyo, Japan) were used for immunostaining. The chromogen substrate for peroxidase was 3,3′-diaminobenzidine. Cells were counted in three to five islets

Table 1
Changes in metabolic parameters

	Week 0	Week 2	Week 4	Week 6
ΔBody weight (g)				
Control	0	56 ± 7	85 ± 6	111 ± 11
STZ	0	66 ± 12	90 ± 13	116 ± 14
STZ + diazoxide	0	32 ± 5[a]	63 ± 18[a]	77 ± 22[a]
Mean AUC (glucose[b], mg/dl)				
Control	167 ± 24	188 ± 36	184 ± 36	178 ± 22
STZ	349 ± 45	344 ± 22	377 ± 52	411 ± 56
STZ + diazoxide	360 ± 28	312 ± 60	308 ± 38	240 ± 94[a]
Mean AUC (insulin[b], μg/ml)				
Control	0.9 ± 0.2	1.3 ± 0.5	1.1 ± 0.2	1.9 ± 0.4
STZ	2.3 ± 0.3	2.6 ± 0.6	1.8 ± 0.2	2.1 ± 0.8
STZ + diazoxide	2.6 ± 0.2	4.6 ± 0.8[a]	6.7 ± 1.2[a]	6.1 ± 1.3[a]

[a] $P < 0.05$ vs. streptozotocin-induced diabetic rats without treatment.
[b] Average of area under the curve (AUC) from 0 to 120 min.

in a pancreas to determine the ratio of beta-cells that were identified by insulin and C-peptide antibodies. Total islet area and total pancreatic area were measured in 10 consecutive slices in three different parts (head, center, and tail) of the pancreas from each rat. Islet cell mass was estimated by multiplying the average of the ratio of total islet area to total pancreatic area by the pancreatic wet weight.

2.2.5. mRNA determination

Pancreatic RNA was extracted from frozen samples. After weighing, ~ 100 mg of pancreatic tissue was homogenized at high speed with a Polytron homogenizer in 1 ml of TRI REAGENT (Molecular Research Center, Cincinnati, OH) (Chomczynski and Sacchi, 1987). After the homogenate was kept for 5 min at room temperature, 0.2 ml chloroform was added and shaken vigorously. After centrifugation, the lower phase was taken for insulin analysis and the upper aqueous phase was mixed with 0.5 ml of isopropanol, and centrifuged again. The RNA pellet was washed with 75% ethanol, then it was dissolved in water treated with diethyl pyrocarbonate. The final preparation with total RNA had a 260:280 ratio, 1.6–1.8.

Pancreatic RNA (20 μg) in 6 μl of water with 14 μl of northern sample buffer was denatured at 65 °C for 15 min, and applied to 1% agarose gel. RNA was transferred on a nylon membrane after electrophoresis, and was fixed under 120 mJ/cm^2 ultraviolet exposure. Proinsulin mRNA was detected by its hybridization to mouse proinsulin I single-stranded cRNA labeled with alpha-^{32}P-CTP (Amersham Pharmacia Biotech, Tokyo, Japan). Hybridized membranes were developed with an image plate (BAS 2000, Fuji, Tokyo, Japan), and densitometric analysis was done with an image processing system attached to the BAS 2000 system.

2.3. Statistical analysis

Two-way ANOVA (analysis of variance) was used on the three groups of rats for multiple comparisons. When ANOVA showed a significant difference, both Fisher's least-significant difference test and the unpaired two-tailed Student's *t*-test were used to evaluate differences between individual rat groups. A *P* value less than 0.05 was accepted as the criterion for statistical significance. All data are shown as the means ± S.E.M.

3. Results

3.1. Body weight and daily profile of glucose concentrations

Body weights before treatment were not different among the three groups (Table 1). Body weights were similar in both control and streptozotocin-induced diabetic rats during study periods. Diabetic rats treated with diazoxide had a tendency to gain less, but the difference between the two diabetic groups did not reach statistical significance. Daily profiles of glucose concentration are depicted in Fig. 1. One week after the injection of streptozotocin, the glucose

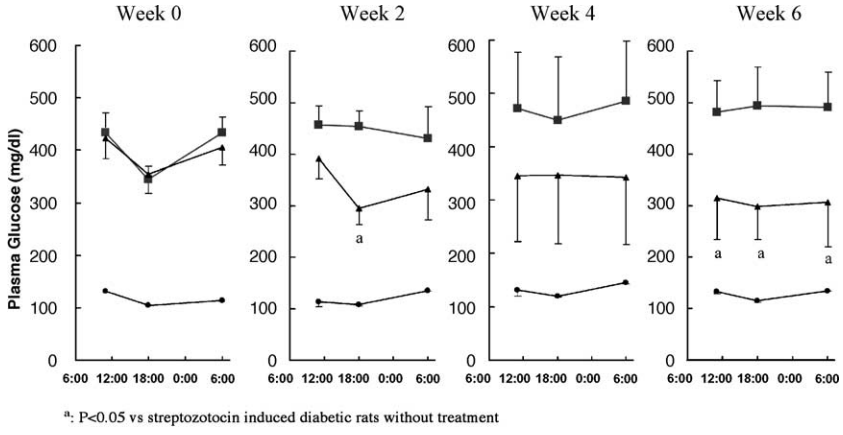

Fig. 1. Plasma glucose daily profiles. Plasma samples were obtained at noon, 6 p.m., and 6 a.m. the next morning before the treatment (Week 0), and 2 (Week 2), 4 (Week 4), and 6 (Week 6) weeks after the start of diazoxide treatment in control (closed circle) and streptozotocin-induced diabetic rats with (closed square) and without diazoxide (closed triangle). Data are shown as means ± S.E.M.

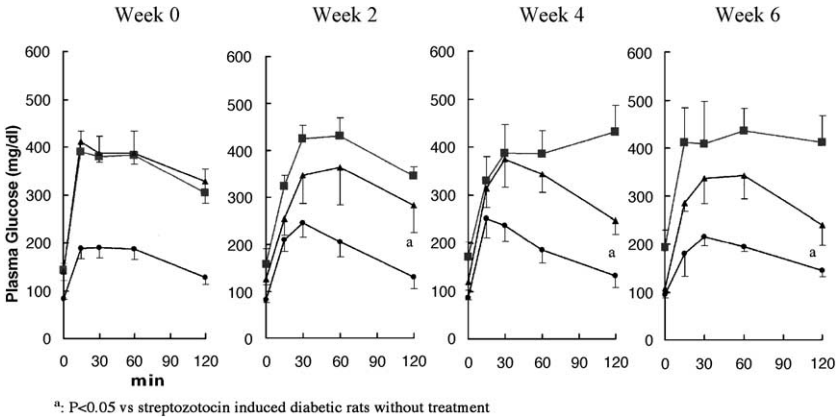

Fig. 2. Plasma glucose concentrations during intraperitoneal glucose tolerance test. Plasma samples were obtained before glucose loading, 15, 30, 60, and 120 min after the start of the injection before the treatment (Week 0), and 2 (Week 2), 4 (Week 4), and 6 (Week 6) weeks after the start of diazoxide treatment in control (closed circle) and streptozotocin-induced diabetic rats with (closed square) and without diazoxide (closed triangle). Data are shown as means ± S.E.M.

profiles were not different between the two diabetic groups as shown in the panel labeled Week 0. At 2 weeks of treatment with diazoxide, the plasma glucose concentration started to decline. This tendency got more prominent and the plasma glucose concentration at 6 p.m. was significantly lower in diabetic rats treated with diazoxide at 4- and 6-weeks treatment. Control rats had euglycemic profiles during the whole study period.

3.2. Intraperitoneal glucose tolerance test

Plasma glucose and insulin concentrations in the intraperitoneal glucose tolerance test are shown in Figs. 2 and 3, respectively. At Week 0, plasma glucose and insulin profiles were similar in both diabetic groups. Significantly higher insulin levels in diabetic animals compared with normal controls suggested that the beta-cell function of streptozotocin-induced diabetic rats was still preserved to a certain extent, but not sufficiently to lower plasma glucose concentrations. At Week 2, the mean glucose concentration at 2 h in the intraperitoneal glucose tolerance test appeared to be lower in diabetic rats treated with diazoxide than in rats without diazoxide, and the mean insulin concentration was higher in diazoxide-treated rats. At Week 6, the insulin concentration in diazoxide-treated rats reached significantly higher levels compared to rats without diazoxide. Mean glucose and insulin concentrations calculated from the area under the curve of response curves are summarized in Table 1. Obviously, insulin responses were much prominent in diazoxide-treated rats.

3.3. Histochemistry

Hematoxylin–eosin staining and immunostaining for insulin of typical islet glands in each group are shown in Figs. 4 and 5, respectively. The islet size and its cell number were reduced in diabetic rats regardless of treatment with diazoxide compared to those of normal control rats. On the

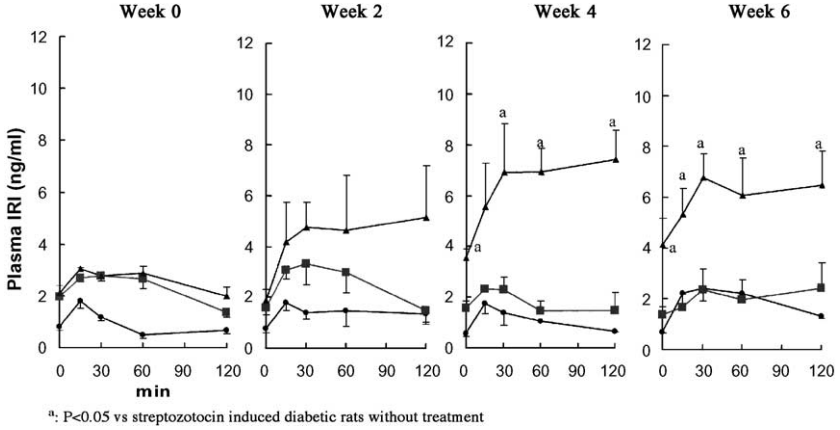

Fig. 3. Plasma insulin concentrations during intraperitoneal glucose tolerance test. Plasma samples were obtained before glucose loading, 15, 30, 60, and 120 min after the start of the injection before the treatment (Week 0), and 2 (Week 2), 4 (Week 4), and 6 (Week 6) weeks after the start of diazoxide treatment in control (closed circle) and streptozotocin-induced diabetic rats with (closed square) and without diazoxide (closed triangle). Data are shown as means ± S.E.M.

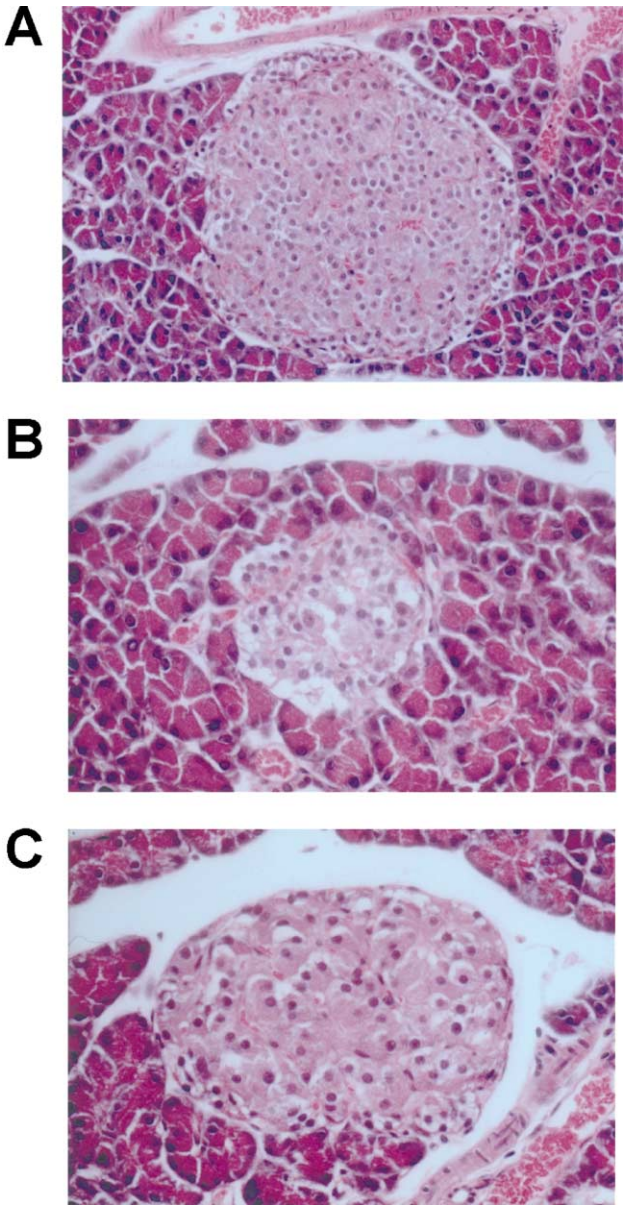

Fig. 4. Morphology of the pancreatic islet with hematoxylin–eosin stain. Control (A), streptozotocin-induced diabetic rat (B), and streptozotocin-induced diabetic rats treated with diazoxide (C) were compared after 6 weeks of induction of diabetes by streptozotocin. Magnification was × 100 in (A) and × 200 in (B) and (C).

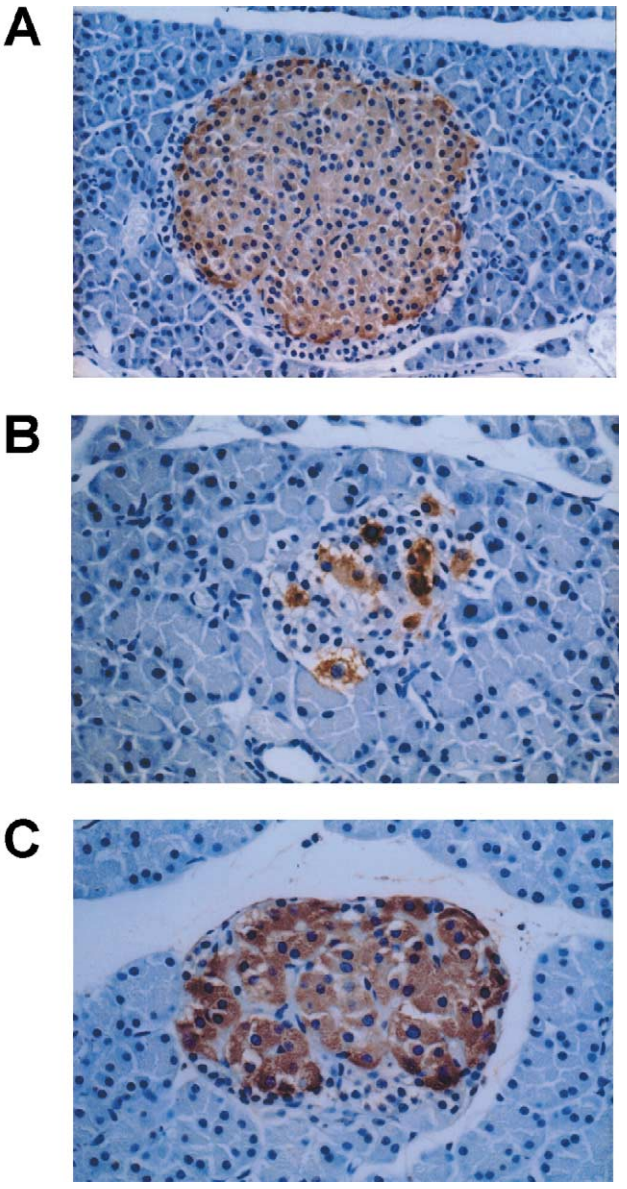

Fig. 5. Immunostaining of the pancreatic islet with rat insulin antibody. Control (A), streptozotocin-induced diabetic rat (B), and streptozotocin-induced diabetic rats treated with diazoxide (C) were compared after 6 weeks of induction of diabetes by streptozotocin. Magnification was × 100 in (A) and × 200 in (B) and (C).

other hand, the islet size and its cell size and number appeared to be well preserved in rats treated with diazoxide when compared to those in rats without diazoxide. The average mass of islets estimated from slices in three different parts of the pancreas in diabetic rats treated with diazoxide was significantly larger than that in untreated diabetic control animals (3.4 ± 0.3 vs. 1.6 ± 0.4 mg, $P < 0.05$). Azan staining revealed a significant increase of fibrous tissues in the islets of diabetic rats without diazoxide (not shown). The summary of immunostaining is shown in Table 2. Of pancreatic islet cells, 89% were insulin and C-peptide-positive in control rats, while ~ 50% were positive in tissues obtained from diabetic rats. However, the degree of immunostaining for insulin or C-peptide was obviously higher in diazoxide-treated diabetic rats than in rats without diazoxide.

Table 2
β-cell content in the rat pancreatic islets

	Insulin-positive cells (%)	C-peptide-positive cells (%)
Control	89.3 ± 2.1	88.3 ± 5.7
STZ	44.0 ± 12.4[a]	42.3 ± 15.1[a]
STZ + diazoxide	43.8 ± 13.4[a]	44.4 ± 11.5[a]

[a] $P < 0.05$ vs. control.

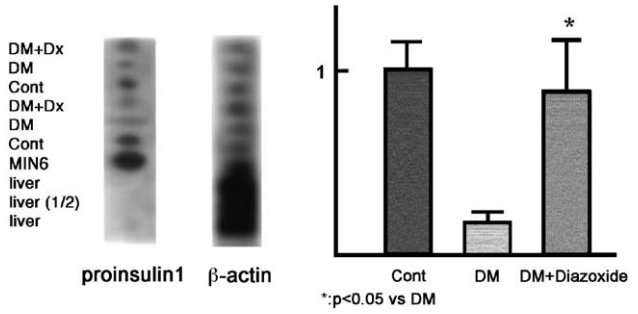

Fig. 6. Expression of proinsulin mRNA in the pancreas. MIN6 cells and liver samples were compared for positive standard for proinsulin 1 and beta-actin. Typical expression is shown in the left panel. Relative expression of proinsulin mRNA normalized by beta-actin is depicted in the right panel.

3.4. Insulin content and proinsulin mRNA

The insulin content in diazoxide-treated diabetic rats was about 10 times higher than that in rats without diazoxide (5.3 vs. 0.4 μg/g protein). The expression of pancreatic proinsulin mRNA is compared among three groups in Fig. 6. Proinsulin mRNA was restored in diazoxide-treated rats. This result was consistent with the results for insulin response observed in the intraperitoneal glucose tolerance test and for insulin measurement in the pancreas.

4. Discussion

The present study showed clearly that the chronic treatment of streptozotocin-induced diabetic rats with a K_{ATP} channel opener, diazoxide, induced a reduction of plasma glucose concentrations—an improvement of glucose tolerance with enhanced insulin secretion in the intraperitoneal glucose tolerance test, and preserved insulin content and proinsulin mRNA in beta-cells. In addition to this functional improvement of the diabetic state, our study demonstrated that diazoxide treatment protected islet cells from morphological damage in streptozotocin-induced diabetic rats. Among K_{ATP} channel openers, diazoxide has been extensively studied and its safety for human use was well accepted. The diazoxide dose, 30 mg/kg body weight per day, used in this study has been validated in previous publications. Once-daily injection was performed based on the half-life of diazoxide in human, 28 h (Anonymous, 1971), although the pharmacological data for rats were not always available. Under these study conditions, diazoxide administration did not induce any inappropriate event during the study.

The mechanism by which diazoxide preserves pancreatic beta-cells from damage by streptozotocin is not yet clear. Kullin et al. (2000) had reported that islets treated with streptozotocin in the presence of K_{ATP} channel openers including diazoxide in vitro showed preservation of morphology, although islets exposed to streptozotocin showed extensive morphological damage. This in vitro incubation study suggested that diazoxide could protect pancreatic beta-cells against acute toxic damage, indicating the protective effect on ongoing insulitis. Streptozotocin is known to damage acutely pancreatic beta-cells through methylation of DNA, which leads to strand breaks and activates poly (ADP-ribose) polymerase for DNA repair (Smulson et al., 1977).

On the other hand, there is no report of a chronic effect of diazoxide after the development of mild diabetes mellitus on administration of low-dose one-shot streptozotocin in adult rats. The aim of the present study was to prove the long-term protective effect of diazoxide on beta-cell exhaustion, which is commonly shown in human type 2 diabetes, after the development of diabetes induced by streptozotocin treatment. Thus, the concept of our study was entirely different from that of the in vitro study previously reported (Kullin et al., 2000). Although we used this animal model that represents a stress on beta-cells, a chronic effect of streptozotocin still cannot be ruled out. It is important that a clear distinction be made between these features of beta-cell glucose toxicity and other etiopathogenic factors of beta-cell dysfunction such as a long-term deleterious effect of streptozotocin on the activity of key mitochondrial dehydrogenases (Malaisse, 1991). Since diazoxide acts on K_{ATP} channels in the mitochondria and affects rat islet mitochondrial membrane potential (Szewczyk and Marban, 1999; Grimmsmann and Rustenbeck, 1998), it may be argued that the effect of diazoxide now shown was mediated through its action on mitochondria membrane. However, the concentration of diazoxide necessary to yield such an effect is much higher (Kullin et al., 2000). Thus, it is not probable that one can attribute the effect of diazoxide observed in the present study to its action on mitochondria.

It is possible that diazoxide affected extrapancreatic tissues and modified insulin sensitivity. Although we did not test any specific measure of insulin sensitivity, the product of fasting plasma glucose and insulin concentrations that represents a degree of whole body insulin sensitivity was not decreased at Week 6 in the diazoxide-treated group. Diazoxide suppresses hyperinsulinemia resulting in an improved ability to stimulate lipolysis, and a loss of adipose tissue mass (Alemzadeh et al., 1996, 1998). Diazoxide restores beta3-adrenergic receptor function (Surwit et al., 2000). In our study, the mean increase in body weight was lowest in diazoxide-treated diabetic rats. One possible mechanism to explain the lower rate of weight gain is a reduction of food intake due to the effect of diazoxide on brain capillary insulin receptor binding (Alemzadeh and Holshouser, 1999). Thus, the possibility is not excluded that the metabolic effects of diazoxide now shown may be a partial result of food restriction.

Diazoxide can accelerate proliferation of certain cells (Xiong and Harmon, 1995) by increasing intracellular

Ca^{2+} concentration. On the other hand, beta-cells have opposite characteristics in terms of intracellular Ca^{2+} handling with the use of diazoxide. At least diazoxide was shown to reduce cell proliferation in RINm5F insulinoma cells (Sjoholm, 1995). Therefore, it is not to be expected that proliferation of residual beta-cells is induced after treatment with diazoxide in streptozotocin-induced diabetic rats. A slight increase in the number of islet cells in diazoxide-treated animals as shown in Figs. 4 and 5, might instead have been caused by a secondary preventive effect from beta-cell stress through apoptosis that could be a consequence after small dose administration of streptozotocin or glucose toxicity (Malaisse, 1991; Saini et al., 1996). Since in a steady state such as in our study conditions, it is very difficult to demonstrate the existence of apoptosis. It is possible that a counter effect on apoptosis may cause the protective effect of diazoxide. The ratio of beta-cell content in the pancreatic islets was not changed by diazoxide treatment as shown in Table 2.

An obvious protective effect of diazoxide, a K_{ATP} channel opener, on pancreatic beta-cells in the diabetic state was shown in this study using full-blown diabetic rats. This suggests that glucose toxicity in the pancreas may be mediated through beta-cell stress rather than the existence of chronic hyperglycemia. Use of insulin sensitizers, the thiazolidinediones, has been shown to have a protective effect on beta-cell function (Sunaga et al., 1999; Shimaya et al., 2000). Somatostatin has been also tried for suppressing insulin secretion from beta-cells (Basabe et al., 1983). To compensate for insulin resistance, one of the major features of type 2 diabetes, beta-cells are under stress especially in the pre-diabetic state to secrete insulin excessively (Sato et al., 1997). Although it seems to be paradoxical to use K_{ATP} channel openers for the treatment of diabetes mellitus, this kind of pharmacological intervention should be a new approach for prevention and treatment of type 2 diabetes mellitus. Although the original idea of using a K_{ATP} channel opener for the diabetes was to prevent acutely progressing beta-cell damage (Culbert et al., 1974), it is still useful to rescue beta-cell exhaustion in chronic hyperglycemia and insulin resistant state.

In summary, we have clearly demonstrated that a pharmacological intervention using diazoxide prevents the progress of pancreatic beta-cell dysfunction in the diabetic state by reducing stresses that produce an excessive insulin secretion from beta-cells.

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