Effect of High-Protein Feeding on Insulin Secretion in Mice Injected with Splenocytes from Diabetic Mice (43955)

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Abstract. **We report here our study of the *in vitro* glucose-induced insulin secretion from mice fed with a high-protein diet and injected with mononuclear splenocytes from streptozotocin-diabetic syngeneic donors. Results show that transfer of "diabetic" splenocytes caused significant diminutions in first phase of stimulated-insulin secretion. Nevertheless, insulin secretory levels attained in recipient mice fed with the high-protein diet were significantly higher than in mice fed with control diets.**

We also evaluated the effect of high-protein feeding on the capacity of "diabetic" splenocytes to transfer an impaired insulin secretion in recipient mice. For this purpose, splenocytes donor mice were fed with high-protein or control diets and injected with multiple low doses of streptozotocin. "Diabetic" splenocytes from high-protein, or control diets fed donors caused in recipient mice similar impairments in glucose-stimulated insulin secretion.

Taken together, these results seem to support the hypothesis of a protective effect of a high-protein diet on the β-cell function. [P.S.E.B.M. 1996, Vol 211]

Previous studies have shown that high-protein, low-carbohydrate (1, 2) or high-protein, carbohydrate-free diets (3, 4) may be beneficial both in reducing many of the signs associated with the diabetic state and in increasing pancreatic insulin contents in animals. Recently, we reported that administration of a high-protein, carbohydrate-free diet (HPD) produced a diminution in serum glucose levels and an improvement in both phases of stimulated-insulin secretion in mice given a single diabetogenic dose or multiple low doses of streptozotocin (SZ) (5). It was suggested that HPD can exert these beneficial effects by an initial protection against the diabetogenic action of SZ at the time of exposure (3, 6).

The diabetic syndrome induced by multiple low doses of SZ is not only due to a β-cytotoxic effect of the drug, but also to a subsequent destructive immune process (7, 8). Therefore, the question is whether the protective effect of HPD in the multiple low doses of SZ-diabetic mice is only on the toxicity of SZ or if the immune aggression process is also affected.

The present study explored the effect of a HPD on *in vitro* glucose-induced insulin secretion patterns from mice receiving mononuclear splenocytes (MS) from multiple low doses of SZ-injected donors. The ability of spleen cells from multiple low doses of SZ-diabetic mice fed HPD to induce an impaired insulin secretion in recipient mice was also investigated.

Materials and Methods

Male C57BL/6N inbred mice weighing 18–22 g were obtained from the Comisión Nacional de Energía Atómica (CNEA), Buenos Aires, Argentina. The mice were initially housed in a room with controlled temperature (23°C) and a fixed 12-hr artificial light:dark cycle. They had free access to water and a standard laboratory chow. Thereafter, mice were used in the assays as described below.

Diets. Three experimental diets were used: (i) a

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home-made high-protein, carbohydrate-free diet (HPD), (ii) a homemade balanced control diet (CD) or (iii) a commercial standard laboratory chow (SD). HPD (9) consisted of (w/w) 70% vitamin-free casein, 8% corn oil, and 15% cellulose; CD was composed of (also on a w/w basis) 17% vitamin-free casein, 53% corn starch, 8% corn oil, and 15% cellulose; SD contained the following percentage by weight: 18% protein, 57% starch (corn, sorghum, wheat, oats, barley), 5% fat, 13% fiber, 2% vitamin mixture, and 5% salt mixture as stated by the manufacturer (Romanelli SA, Buenos Aires, Argentina). The vitamin content and salt mixture added to both homemade diets followed the recommendations of the American Institute of Nutrition ad hoc Committee on Standards for Nutritional Studies (10, 11). HPD and CD were prepared twice a week and kept refrigerated at 4°C. CD was used to test the validity of SD as a control diet, due to the presence of putative diabetogens among standard laboratory chow ingredients (12, 13). All diets were approximately isocaloric and were available *ad libitum*.

Assay A. Recipient syngeneic mice received HPD, CD, or SD during 15 days before being injected with mononuclear splenocytes. Mice fed with commercial chow (SD) were used as mononuclear splenocytes (MS) donors. The animals were injected ip with 40 mg/kg of body weight of either streptozotocin (dissolved in citrate buffer, pH = 4.5) or citrate buffer alone during five consecutive days. SZ or citrate buffer-injected mice were killed by cervical dislocation 15 days after the last injection, and MS were aseptically isolated by shredding the spleen with steel wire mesh followed by two washes in sterile saline solution (14, 15). Viability of MS was assayed by the Trypan blue exclusion test (16).

Mice adapted for 15 days to HPD, CD, or SD were injected ip with 5×10^7 viable MS (in 0.2 ml sterile saline solution) obtained from the SZ-injected donors ("diabetic" MS) or from the citrate buffer-injected donors (control MS). The recipient mice were maintained on the same type of diet they had consumed before, for another 15 days. After this period elapsed, mice were sacrificed and their pancreas quickly removed and processed for perifusion as described below.

Assay B. Male mice previously fed during 10 days with HPD, CD, or SD were injected ip with multiple low doses of SZ (40 mg/kg × 5) or citrate buffer alone as described above. Thereafter, mice were kept on the same type of food they had consumed previously and during SZ or citrate-buffer injections. The animals were sacrificed by cervical dislocation 15 days after the last injection, and "diabetic" or control MS were isolated from their spleens as described above. Isolated MS from SZ or citrate buffer-injected mice fed one of the three diets were injected ip (5×10^7 viable MS/0.2 ml saline solution) into syngeneic recipient mice fed a commercial chow. Recipient mice were sacrificed and their pancreas were perifused 15 days after having been transferred.

Blood samples from nonfasted recipient mice were obtained by retroorbital sinus puncture immediately before the transfer procedure and at the time of sacrifice.

Perifusion of Pancreatic Slices. The technique described by Burr *et al.* (17) with slight modifications (18) was used. Krebs-Ringer bicarbonate buffer was used as the perifusion buffer and was supplemented with 1 g% dextran 70 (Sigma-Chemical Co., St. Louis, MO), 3.3 m*M* glucose, and 100 KIU Trasylol/ml (Bayer, Buenos Aires, Argentina). The pH of the buffer kept under constant 95% O_2, 5% CO_2 gassing was 7.38–7.40. Thin slices (0.5–1 mm in thickness) from a whole pancreas of a single mouse were cut using ophthalmological surgery equipment under a stereoscope microscope and used in each perifusion. After an initial 15-min recuperation period (during which no fractions were sampled) 16.5 m*M* glucose stimulation started on the 3rd min and lasted until the end of perifusion. Samples were collected at 1-min intervals between Min 1 and 15, and at 5-min intervals between Min 15 and 40 of perifusion, in tubes at 4°C containing 0.2 ml of 0.25 *M* EDTA. Tubes were immediately frozen at −20°C. Samples from Min 1 and 2 were used for baseline determinations. Perifusion flux was 1.8–2.2 ml/min.

Analytical Methods. Nonfasted serum glucose levels were determined using Glicemia Enzimatica Kit (Wiener Lab, Buenos Aires, Argentina). Insulin was determined in samples from perifusion by the method of Herbert *et al.* (19). Pork monoiodine [^{125}I]insulin was obtained from CNEA, Buenos Aires, Argentina. Rat standard insulin was obtained from Novo Research Laboratories (Denmark). Guinea pig anti-porcine insulin antiserum was sufficiently "nonspecific" as to allow pork labeled insulin to be displaced by mouse insulin.

Insulin assay sensitivity was 2.5 μU/ml; intra-assay coefficient of variation (CV) was 8.7%, 6.2%, and 5.1% for 1–5, 5–10, and 10–50 μU insulin/ml determination ranges respectively; interassay CV was 6.6%, 5.0%, and 5.2% for the given ranges.

Statistical Analysis. Statistical analysis of the data was performed with analysis of variance (ANOVA) and Scheffe's test. The results are expressed in all cases as mean ± SEM.

Results

Assay A. Before being sacrificed, MS donor mice showed the following values of nonfasted plasma glucose: citrate buffer–injected mice—157 ± 4 mg/dl, n = 22; SZ-injected mice—453 ± 19 mg/dl, n = 21, $P <$ 0.01.

Table I. Nonfasted Serum Glucose Values (mg/dl) from Mice Fed a High-Protein Diet (HPD), a Commercial Standard Chow (SD) or a Homemade Balanced Control Diet (CD), and Transferred with Control or "Diabetic" MS

	Serum glucose values (mg/dl)		
	HPD	SD	CD
Before transfer	160 ± 6 *n* = 14	159 ± 5 *n* = 13	164 ± 4 *n* = 11
15 days after control MS transfer	166 ± 13 *n* = 5	162 ± 7 *n* = 6	168 ± 2 *n* = 6
15 days after "diabetic" MS transfer	163 ± 8 *n* = 9	164 ± 9 *n* = 7	169 ± 3 *n* = 5

Note. Values before transfer correspond to animals that had been already kept on one of the three different diets for 15 days and were bled 1 day before the injection of MS. Results are expressed as mean ± SEM.

MS-recipient mice, fed during 15 days with HPD, SD, or CD presented before the transfer procedure, similar weight gains (X ± SEM: HPD—0.28 ± 0.03 g/day, $n = 14$; SD—0.27 ± 0.03 g/day, $n = 13$; CD—0.26 ± 0.04 g/day, $n = 11$) and similar values of nonfasted serum glucose whichever the diet administered (Table I). Transfer of either control or "diabetic" MS to HPD, SD or CD fed recipient mice did not affect their serum glucose levels determined 15 days after MS injections (Table I).

Basal insulin secretion from perifused pancreatic slices was similar in mice fed HPD, SD, or CD, transferred with control or "diabetic" MS (Table II).

In our experimental conditions, the first peak of glucose-induced insulin secretion reaches its highest level at perifusion time 4 min, while the second phase rises to its highest value at perifusion time 40 min. Table II shows that the type of diet administered had no effect on glucose-induced insulin secretion values from mice injected with control MS.

Injection of "diabetic" MS caused significant diminutions in both phases of glucose stimulated insulin secretion from recipient mice fed SD, CD, or HPD; however, the first insulin secretory peak from HPD fed mice transferred with "diabetic" MS was higher than in mice fed CD or SD (Table II). Second phases of glucose-induced insulin secretion from "diabetic" MS-recipient mice were significantly diminished no matter which diet was administered (Table II).

Figure 1 shows the wave forms of insulin secretion patterns from mice injected with control or "diabetic" MS; in this figure, we included one control group (mice fed CD and injected with control MS) and two groups of mice (fed CD or HPD) injected with "diabetic" MS.

Assay B. MS-donor mice fed during 10 days with HPD, SD, or CD presented, before injections of SZ or citrate-buffer, similar weight gains (Table III). Donor mice injected with citrate-buffer showed, 15 days after the last injection and before being sacrificed, similar values of nonfasted plasma glucose (Table III).

MS-donor mice that had been injected with SZ showed, compared with control groups ($P < 0.01$), significantly increased plasma glucose levels; however, SZ-injected mice fed HPD presented lower serum glucose levels when compared with mice fed SD or CD (Table III).

MS-recipient mice transferred with control or "diabetic" MS from HPD-, SD-, or CD-fed donors presented no significant differences in their serum glucose values (Table IV).

Mice transferred with control or "diabetic" MS from HPD-, SD-, or CD-fed donors presented similar levels in basal insulin secreted by perifused pancreatic slices (Table V).

Mice injected with control MS from donors fed HPD, SD, or CD presented similar values of glucose induced insulin secretion (Table V). On the other hand, injection of "diabetic" MS from HPD, SD, or CD donor mice caused, in MS-recipient mice, similar diminutions in their first and second phases of glucose-induced insulin secretion (Table V).

Table II. Basal and Glucose-Induced Insulin Secretion (at Min 4 and 40 of Perifusion), in Pancreatic Slices from Mice Fed SD, CD, or HPD, and Injected with Control or "Diabetic" MS

	Perifusion time					
	1 min (basal)		4 min		40 min	
	Control MS	"Diabetic" MS	Control MS	"Diabetic" MS	Contol MS	"Diabetic" MS
SD	52.5 ± 4.1 *n* = 6	50.7 ± 2.8 *n* = 7	354.7 ± 9.5 *n* = 6	225.3 ± 8.9[a,c] *n* = 7	437.7 ± 7.5 *n* = 6	382.3 ± 16.1[b] *n* = 7
CD	50.3 ± 3.2 *n* = 6	52.8 ± 1.3 *n* = 5	367.7 ± 10.0 *n* = 6	216.2 ± 5.4[a,c] *n* = 6	435.2 ± 4.7 *n* = 6	388.6 ± 5.8[a] *n* = 6
HPD	57.4 ± 3.8 *n* = 5	56.0 ± 3.8 *n* = 9	372.6 ± 12.5 *n* = 5	316.9 ± 6.2[a] *n* = 9	445.0 ± 9.5 *n* = 5	395.9 ± 10.3[a] *n* = 9

Note. Insulin was expressed as μU/min/100 mg wet tissue. Values are shown as mean ± SEM.
[a] $P < 0.01$ and [b] $P < 0.05$ versus mice fed the same type of diet and injected with control MS.
[c] $P < 0.01$ versus mice fed HPD and injected with "diabetic" MS.

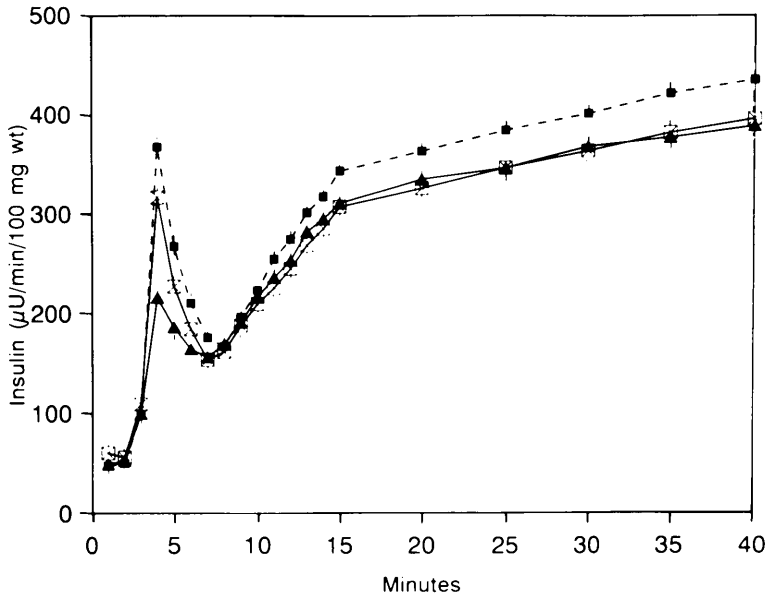

Figure 1. Insulin secretion stimulated by 16.5 m*M* glucose in perifused pancreatic slices from recipient mice fed CD and transferred with control MS (■---■), and from recipient mice fed CD (▲—▲) or HPD (□—□), both transferred with "diabetic" MS. Results are expressed as mean ± SEM.

Table III. Weight Gains (g/day) and Nonfasted Serum Glucose Levels (mg/dl) from MS-Donor Mice Fed HPD, SD, or CD

	HPD	SD	CD
Weight gains before injections (g/day)	0.26 ± 0.04 *n* = 20	0.26 ± 0.02 *n* = 21	0.25 ± 0.03 *n* = 20
Serum glucose values 15 days after citrate buffer injections (mg/dl)	157 ± 5 *n* = 10	160 ± 4 *n* = 10	157 ± 4 *n* = 10
Serum glucose values 15 days after SZ injections (mg/dl)	288 ± 14[a,b] *n* = 10	443 ± 15[a] *n* = 11	403 ± 22[a] *n* = 10

Note. Values before injections of SZ or citrate buffer correspond to those animals that had been already kept on one of the three different diets for 10 days.
[a] $P < 0.01$ versus serum glucose values from mice injected with citrate buffer and fed the same type of diet.
[b] $P < 0.01$ versus SZ-injected mice fed SD or CD.

Discussion

It became increasingly clear that insulin-dependent diabetes is a disease that reflects a variety of genetic, environmental, and immunological factors (20). Among these environmental factors, diet plays a critical role (21–23).

Table IV. Serum Glucose Values from Recipient Mice Transferred with Control or "Diabetic" MS

	Serum glucose values (mg/dl)		
	HPD	SD	CD
Before transfer	166 ± 3 *n* = 12	159 ± 5 *n* = 13	166 ± 3 *n* = 10
15 days after control MS transfer	166 ± 4 *n* = 6	162 ± 7 *n* = 6	163 ± 4 *n* = 5
15 days after "diabetic" MS transfer	155 ± 7 *n* = 6	164 ± 9 *n* = 7	167 ± 2 *n* = 5

Note. Donor mice were fed with HPD, SD, or CD. Results are expressed as mean ± SEM.

Previous studies from several laboratories, performed with rats and mice fed HPD or a balanced diet supplemented with branched-chain amino acids, showed a protective and restorative effect against the diabetogenic action of SZ (2, 3, 24). This effect also entails an increased insulin pancreatic content and serum levels (3).

Recently, we reported a beneficial effect of HPD on serum glucose levels and on both phases of glucose-stimulated insulin secretion in perifused pancreatic slices from rats and mice given a diabetogenic dose of SZ (5). A direct interference with the chemical action of the SZ molecule on the β cell was postulated as the mechanism by which HPD protects against the noxious effect of the drug (3).

We also reported a restorative effect of HPD on glycaemia and on *in vitro* patterns of stimulated-insulin secretion in mice made diabetic by multiple low doses of SZ (5). There is now overwhelming evidence that multiple low doses of SZ-induced diabetes has immune

Table V. Insulin Secretion (as μU/min/100 mg wet tissue) at Min 1, 4, and 40, in Perifused Pancreatic Slices from Mice Injected with Control or "Diabetic" MS Obtained from Mice Fed SD, CD, or HPD

	Perifusion time		
	1 min (basal)	4 min	40 min
Control MS from SD-fed mice	52.5 ± 4.1 $n = 6$	354.7 ± 9.5 $n = 6$	437.7 ± 7.5 $n = 6$
Control MS from CD-fed mice	46.6 ± 3.8 $n = 5$	358.2 ± 9.2 $n = 5$	442.8 ± 12.0 $n = 5$
Control MS from HPD-fed mice	48.2 ± 2.1 $n = 6$	343.3 ± 16.7 $n = 6$	432.5 ± 14.1 $n = 6$
"Diabetic" MS from SD-fed mice	50.7 ± 2.8 $n = 7$	225.3 ± 8.9[a] $n = 7$	382.3 ± 16.1[b] $n = 7$
"Diabetic" MS from CD-fed mice	49.2 ± 3.7 $n = 5$	229.0 ± 9.5[a] $n = 5$	382.4 ± 5.5[a] $n = 5$
"Diabetic" MS from HPD-fed mice	50.8 ± 3.4 $n = 6$	238.7 ± 7.4[a] $n = 6$	391.0 ± 5.0[b] $n = 6$

Note. Values are expressed as mean ± SEM.
[a] $P < 0.01$ and [b] $P < 0.05$ versus mice injected with control MS from donor mice under the same type of diet.

components such as an inflammatory process leading to macrophages and subsequent lymphocytes infiltration with concomitant β cell lysis (7). In spite of this, direct toxicity of SZ on β cells is an initial and important phenomenon in this model. Insulin secretion is seriously impaired before insulitis and hyperglycemia are evident (8). Furthermore, a single low dose of SZ can result in a slow and gradual development of diabetes over a period of 10–60 days without insulitis (25). Finally, *in vivo* injections of low doses of SZ in baboons also induced a dysfunction of β cells, even in the absence of hyperglycemia (26). The above cited studies do not clarify if the previously observed beneficial effects of HPD on multiple low doses of SZ-induced diabetes was (i) on the direct toxic action of the drug, (ii) on the immune aggression, or (iii) on both components. In order to gain information on these points, we looked for a model in which immune aggression was acting while no direct toxic effect of SZ was present.

All these arguments lead us to use the transfer of MS from multiple low doses of SZ-diabetic mice to syngeneic normal mice (14), an experimental model with impaired tolerance to an oral glucose load and altered glucose-stimulated insulin secretion pattern (27). We also observed that mitomycin C treatment of "diabetic" MS, prior to transfer, completely abolished the effects on recipient mice, suggesting that this effect implicates transferred splenocytes proliferation in recipients (27).

When using athymic and euthymic mice, enough evidence was obtained to support that both donor and recipient T lymphocytes play an important role in the progression to the diabetic state. Furthermore, MS from diabetic donors showed a specific homing towards pancreas which might indicate an early stage in the cellular immune aggression (27).

The present study shows that HPD exerts a protective and restorative effect on the insulin secretion patterns from an experimental model in which cellular immune aggression, but not a direct toxic action of SZ was present, since recipient mice fed HPD and injected with "diabetic" MS showed a partial restoration in the first phase of stimulated insulin secretion. The reason why there is only a partial restoration on the first but not on the second phase of the insulin response is rather confusing. We are tempted to speculate that since the first phase is an early and sensitive parameter of insulin secretion impairment, its restoration should also be achieved before that of the second phase. However, in a previous study we observed that HPD partially restored both phases of insulin secretion in the multiple low doses of SZ diabetic model, in which the β-cell aggression is stronger than in the "diabetic" MS transfer (5).

Scott *et al.* (21) and Rao *et al.* (12) raised the possibility that diabetogens were frequent constituents of commercial rat chow, and also, that chow constituents vary with market conditions (10). In addition, Coleman *et al.* (13) showed that unknown substances in natural-ingredient chow can enhance the development of diabetes in NOD mice. Bearing in mind these observations, we included in our study, groups of mice fed with a homemade balanced control diet (CD).

As it has been suggested that the source of dietary protein is a major component in the modulating effect of diet upon autoimmune diabetes in the BB rat and NOD mouse (21, 22), we used CD with casein as protein source, since HPD is also a casein-based diet.

The mechanism(s) by which HPD partially restores insulin secretion patterns remains to be clarified. Several hypothesis have been proposed: (i) the insulin secretion mechanism, (ii) the β-cell regeneration, (iii) the mechanisms of cellular repair, (iv) the

extrapancreatic metabolic effects, and (v) the immune system. In the last years a growing body of evidence suggests that dietary constituents influence the immune system reactivity. Dietary modulation of diabetes in BB rat and NOD mouse has been reported (13, 21, 22, 28). However, in our experimental conditions, the data show that HPD feeding has no effect on the capacity of "diabetic" MS to impair insulin secretion in recipient mice.

More information is needed concerning cellular and molecular mechanisms by which HPD exerts its effects, especially in specific cells and systems. The present study suggests that environmental factors such as HPD may be related to changes in metabolism of the β cell and possible interrelationship of immune system and β-cell function as a target.

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