EXPERIMENTAL STUDY

Influence of a high-fat diet during chronic hyperglycemia on β -cell function in pancreatic islet transplants to streptozotocin-diabetic rats

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Abstract

Chronically elevated non-esterified fatty acids (NEFAs) can exert negative effects on β -cell function both *in vitro* and *in vivo*. Negative effects of fatty acids have been difficult to evaluate in overt diabetes because of the attendant hyperglycemia that gives rise to the confounding influence of 'glucotoxicity'. In this work, we tested for the effects of NEFAs in diabetes by (i) taking into account potential effects of prevailing levels of hyperglycemia, and (ii) focusing on lingering (and therefore possibly more serious) effects. A diabetic transplantation model was used in which two islet grafts with 200 and 20 rat islets respectively were transplanted under the kidney capsule of syngeneic recipients previously made diabetic by streptozotocin injection. Rats were then fed either a high-fat or a low-fat diet for 7 weeks, followed by 1 week of normal laboratory chow. During dietary intervention, food was consumed *ad libitum* in one protocol, but was restricted in the low-fat group in a second protocol (in order to match blood-glucose levels). A high-fat diet did not affect body weight. At the end of the protocols, graft-bearing kidneys were isolated and perfused. Insulin responses to 27.8 mM glucose in perfusion were uniformly absent, in keeping with previously documented effects of chronic hyperglycemia. In contrast, 10 mM arginine induced a marked increase in insulin secretion after a low-fat diet, an effect that was significantly reduced after a high-fat diet (109 ± 39 vs 13 ± 15 fmol/min ($P < 0.05$) and 95 ± 18 vs 32 ± 5 fmol/min ($P < 0.05$) in the 2 protocols respectively). Regardless of protocol, no effect of diet could be detected on graft contents of insulin or preproinsulin mRNA. Thus, under conditions in which influences of chronic hyperglycemia could be accounted for, a previous high-fat diet with elevated NEFAs inhibited arginine-induced insulin secretion; however, the results indicate that insulin biosynthesis and/or β -cell mass were not affected.

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Introduction

Insulin secretion declines over time in type 2 diabetes. The reasons for the decline are not clear. Chronic hyperglycemia and elevated non-esterified fatty acids (NEFAs) have been implicated as causal factors. This is because these metabolic abnormalities may be negative for β -cells. It has been established that chronic hyperglycemia inhibits insulin secretion and other β -cell functions (1–3). Although less thoroughly documented, NEFAs, with ensuing increased fatty acid oxidation, may exert similar effects (4–10).

Hyperglycemia and elevated NEFAs usually occur together in type 2 diabetes. An important question is whether chronic hyperglycemia and elevated levels of NEFAs interact on β -cells. The two metabolic abnormalities have been shown to exert additive negative

effects on human pancreatic islets *in vitro* (10). However, interactions *in vivo* in overt diabetes have not been explored. This is due to the inability to keep one factor constant while varying the other.

Against this background, we have tested for *in vivo* effects of elevated NEFAs on chronic hyperglycemia. To this end, we have used a diabetic transplantation model. In this model, rats were made diabetic by streptozotocin and then transplanted with normal islets from syngeneic donors. Notwithstanding the special conditions associated with transplantation (with regard to microcirculation and innervation), this model offers important advantages in comparison to other *in vivo* models. This is because the effects of the metabolic perturbations can be tested on β -cells that were normal at onset (unlike the β -cells of the streptozotocin-diabetic recipients or the β -cells of spontaneous diabetic

models). Furthermore, the size of the transplant can be kept small enough to prevent its insulin secretion from affecting the level of hyperglycemia.

Non-reversible, or only slowly reversible, effects of hyperglycemia and NEFAs would be the ones relevant to β -cell deterioration in type 2 diabetes. Important effects of hyperglycemia and NEFAs should therefore be demonstrable some time after normalization of either one of the metabolic abnormalities. The lingering effects of hyperglycemia *per se* have been well studied (1–3) but the effects of NEFAs less so. Therefore we decided to test for the effects of elevated NEFAs some time after the normalization of this parameter. To this end, diabetic rats with transplants received either a high-fat or a low-fat diet for 7 weeks. Tests of transplant function were then performed 1 week after diet-normalization. The results have been reported, in part, in abstract form (11).

Materials and methods

Animals

Female and male Wistar–Furth rats were obtained from B&K Universal, Sollentuna, Sweden. Female rats served as donors, and male syngeneic rats served as recipients in the transplantation experiments. Male rats were made diabetic by an intravenous injection with streptozotocin (60 mg/kg; Sigma, St Louis, MO, USA). Diabetes was induced at least 2 weeks before the experiments, and was confirmed by blood-glucose levels (>20 mmol/l).

Isolation and transplantation of islets

Islets of Langerhans were isolated by collagenase treatment (12) from 12–15-week-old female syngeneic rats. Islets were cultured overnight in RPMI 1640 (Life Technologies, Paisley, UK). Then two islet grafts with 200 and 20 islets respectively were transplanted under the left kidney capsule as previously described (13, 14). The smaller graft was included for measurements of insulin content that could not, for technical reasons, be performed on the larger graft. Islets isolated from a single donor rat were divided into two equal portions: one portion was transplanted into a rat belonging to the high-fat treatment group; the other was transplanted into a rat belonging to the low-fat treatment group. This procedure served to reduce variations in test parameters between treatment groups.

Experimental protocols

The Stockholm Ethics Committee approved the experimental protocols for the animal experiments. After transplantation, the recipient rats were fed either a high-fat diet or a low-fat diet (Analysen, Lidköping, Sweden) for 7 weeks. The diets contained lard, glucose,

cornstarch, cellulose powder, mineral premix, soya oil and vitamin prefix. The high-fat diet contained 35.9% fat as lard, whereas the percentage was only 7% in the low-fat diet. The energy concentration of the high-fat diet was 17.8 MJ/kg and that of the low-fat diet was 13.5 MJ/kg. For both diets, the treatment period was followed by a 1-week period during which all of the rats received normal laboratory chow (B&K, Stockholm, Sweden), to exclude acute effects of elevated NEFAs on β -cell function. The normal laboratory chow contained 4.4% fat.

Two protocols were employed. In the first protocol (Protocol 1), the rats were allowed to feed *ad libitum*; in the second (Protocol 2), food was restricted for the rats fed with the low-fat diet, in order to match glucose levels with those of the rats fed with the high-fat diet. Both protocols were followed by 1 week of normal laboratory chow (see above). Isolation and perfusion of the graft-bearing kidneys terminated all the experiments.

Blood-glucose levels were measured weekly at 0900 h throughout the experimental period. In Protocol 2, blood was also sampled 1, 7 and 8 weeks after transplantation for later assays of plasma C-peptide and NEFAs.

Kidney perfusion

Graft-bearing kidneys were isolated and perfused, and grafts were retrieved as described previously (13, 14). Each kidney was perfused with Krebs–Ringer bicarbonate buffer supplemented with BSA (20 g/l; Sigma), dextran T-70 (20 g/l; Pharmacia, Uppsala, Sweden), and glucose (3.3 mmol/l). After a 10 min equilibration period, grafts were stimulated for 30 min with 27.8 mmol/l glucose. This stimulation was followed by an equivalent period of perfusion with 3.3 mM glucose. Finally, grafts were stimulated with 10 mmol/l arginine in a background of 3.3 mmol/l of glucose. Effluents were collected every minute and stored at -20°C until the insulin assay. After the perfusions, grafts were excised and stored for measurement of preproinsulin mRNA (larger grafts) and insulin content (smaller grafts).

Assays

Levels of blood glucose were assayed by a glucose oxidase method (Accutrend Sensor Glucose; Boehringer Mannheim, Mannheim, Germany). Plasma levels of C-peptide were assayed by radioimmunoassay (Linco Research, St Charles, MO, USA). Free fatty acids were assayed by a calorimetric method (NEFA C; Wako Chemicals, Neuss, Germany). The insulin content of the smaller grafts (containing 20 islets) was extracted as reported previously (15). Immunoreactive insulin in effluents and extracts was measured by radioimmunoassay (16).

Measurement of preproinsulin mRNA

Total RNA of the islet grafts was extracted by an acid phenol method (17). Levels of preproinsulin mRNA were determined by a non-saturated solution hybridization assay, using an RNA probe radio-labeled with ^{35}S -UTP (18). An *in-vitro*-synthesized 58 bp oligonucleotide corresponding to the last part of exon 3 of the rat preproinsulin II gene flanked by BamHI and KpnI restriction sites was inserted into pGEM-3Zf(+). The resulting vector was linearized by EcoRI and transcribed *in vitro* with SP6 RNA polymerase in the presence of ^{35}S -UTP (3 $\mu\text{mol/l}$) for synthesis of the probe. Unlabeled sense RNA was obtained by transcription with T7 RNA polymerase after linearization with XbaI. The DNA template was removed by RQI deoxyribonuclease I (Promega GmbH, Mannheim, Germany), 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 $\times$ SET (1 $\times$ SET contains 1% SDS, 20 mmol/l Tris-HCl, pH 7.5, and 10 mmol/l EDTA) were mixed with 20 μl 2 $\times$ hybridization solution (20 000 c.p.m. 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 $^{\circ}\text{C}$ for 18 h, 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 min at 37 $^{\circ}\text{C}$ in a volume of 1 ml. Protected probe was precipitated with 100 μl 100% trichloroacetic acid. Precipitates were collected on glass-fiber filters, and the radioactivity was counted in a scintillation counter (Perkin-Elmer Life Sciences, Turku, Finland). Parallel hybridizations with increasing amounts of unlabeled sense RNA allowed the construction of a standard curve. The amount of preproinsulin mRNA was calculated by comparison with 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 1–50 $\times$ background. All quantifications of preproinsulin mRNA in graft extracts are based on at least three serial dilutions within the linear range of the assay.

Statistical analysis

Data in tables and figures are expressed as means $\pm$ S.E.M. Incremental secretion of insulin in response to secretagogues was calculated as the areas under the curves after subtraction of the values registered before the stimulation periods. Tests of significance were done using a Student's *t*-test (two-tailed). A probability value of <0.05 was considered significant.

Results

Body weights and blood glucose of recipient rats

In the first series of experiments (Protocol 1), all rats were allowed free access to their respective food. Body weights after 7 weeks of dieting did not differ significantly between the rats fed on high-fat diets and those fed on low-fat diets (Fig. 1). However, the level of hyperglycemia of rats fed on the high-fat diet fell below that of rats on the low-fat diet (Fig. 2).

The differences in blood-glucose levels between the rats on the high-fat diet and those fed on the low-fat diet could potentially affect the end-point parameters of β -cell function. These considerations prompted a second series of experiments (Protocol 2) in which the food intake of rats on the low-fat diet was restricted in order to reduce blood-glucose values to levels comparable with those of the high-fat-diet group. During Protocol 2, the mean body weight of the low-fat-diet group fell below that seen after the high-fat diet (Fig. 1), whereas blood-glucose levels were comparable between the groups (Fig. 2).

NEFAs

Plasma levels of NEFAs were assayed at different time-points during Protocol 2. Levels of NEFAs were elevated almost threefold at the end of the 7-week period of the high-fat diet (Table 1). This significant elevation had

Figure 1 Body weight of rats fed on a low-fat diet (○) or a high-fat diet (●) in Protocol 1 (upper panel) and Protocol 2 (lower panel). #*P* < 0.05 relative to low-fat diet diabetic rats.

Figure 2 Blood-glucose levels of rats fed on a low-fat diet (○) or a high-fat diet (●) in Protocol 1 (upper panel) and Protocol 2 (lower panel). # $P < 0.05$ relative to low-fat diet diabetic rats.

diminished to a non-significant 32% elevation 1 week after cessation of the high-fat diet, i.e. at the end of the study (Table 1).

C-peptide

At the end of the 7-week treatment period, plasma levels of C-peptide were elevated in the high-fat diet group vs the low-fat diet group. This elevation had diminished 1 week after the cessation of the high-fat diet (Table 1).

Insulin secretion in vitro

The data on perfusion of islet grafts are shown in Fig. 3 and Table 2. High-fat feeding tended to increase insulin

Figure 3 Insulin secretion from graft-bearing kidneys of rats fed on a low-fat diet (○) or a high-fat diet (●) after 7 weeks of the diet according to Protocol 1 (upper panel) and Protocol 2 (lower panel).

release during basal (3.3 mmol/l) glucose conditions ($P = 0.19$ for Protocol 2). Insulin responses to 27.8 mmol/l glucose were absent after both dietary protocols, this absence being due to chronic hyperglycemia (18). After a return to 3.3 mmol/l glucose, grafts from low-fat diet rats showed an increase in insulin secretion (off-response) which was absent from the grafts from the high-fat diet group; however, this difference between groups again was not significant.

Perfusion with 10 mM arginine induced a marked increase in insulin secretion from grafts into rats fed a low-fat diet (Fig. 3 and Table 2). This response was markedly smaller from grafts of high-fat diet rats. The inhibitory effect of the high-fat diet was seen in both

Table 1 Plasma free fatty acid and C-peptide levels of rats in Protocol 2.

	<i>n</i>	Weeks of diet		
		1	7	After wash-out
Free fatty acid level (mEq/l)				
Low-fat diet diabetic rats	4	0.50 ± 0.14	0.26 ± 0.02	0.47 ± 0.10
High-fat diet diabetic rats	5	0.89 ± 0.17	0.73 ± 0.05*	0.62 ± 0.17
C-peptide level (pmol/l)				
Low-fat diet diabetic rats	4	86 ± 25	87 ± 15	71 ± 17
High-fat diet diabetic rats	5	97 ± 29	204 ± 21*	63 ± 15

* $P < 0.05$ vs diabetic rats fed a low-fat diet.

Table 2 Insulin secretion from islet grafts.

	<i>n</i>	Insulin secretion (fmol/min)		
		3.3 mM glucose	27.8 mM glucose	10 mM arginine
Protocol 1				
Low-fat diet diabetic rats	6	9.2 ± 7.2	−0.8 ± 10.3	109.2 ± 39.2
High-fat diet diabetic rats	6	12.5 ± 2.7	−1.2 ± 8.0	13.4 ± 14.6*
Protocol 2				
Low-fat diet diabetic rats	4	0.0 ± 0.0	3.8 ± 1.8	94.8 ± 17.9
High-fat diet diabetic rats	5	9.7 ± 6.1	−2.9 ± 9.9	31.9 ± 5.1*

* $P < 0.05$ vs diabetic rats fed a low-fat diet.

protocols. Arginine-induced insulin release was reduced by 88% in Protocol 1 and by 66% in Protocol 2.

Graft content of insulin and preproinsulin mRNA

In contrast to the marked effect on insulin secretion, the high-fat diet failed to affect insulin content or preproinsulin mRNA as measured 1 week after the cessation of the diet. This absence of an effect was observed both after Protocol 1 and after Protocol 2 (Table 3).

Discussion

Our results are restricted to effects after the cessation of a high-fat diet rather than the effects during such a diet. With this restriction in mind, we have obtained evidence that a high-fat diet does not produce a lingering effect on the insulin content or the preproinsulin mRNA of transplanted islets. On the contrary, we found that there was a negative effect of the previous high-fat diet on arginine-induced insulin secretion. These results constitute our main findings. The results are strengthened by similar findings from two different protocols.

In our first protocol, we found a significant difference in blood-glucose levels between rats fed with the two diets. This difference could be due to a higher intake of energy from the high-carbohydrate diet than that from the high-fat diet. Such an effect has been documented

in other studies in diabetic rats (19). The present effect of a high-fat diet on blood glucose is, however, different from that obtained in diabetes models with a positive energy balance and obesity, such as the diabetic Zucker fa/fa rat (7, 9). In our study, the differences in blood-glucose levels obtained with the two diets prompted a second protocol in which blood glucose levels were kept comparable between groups. The results of the second protocol were very similar to those of the first. The similarities demonstrate that differences in blood-glucose levels cannot provide an explanation for the effects of the high-fat diet on β -cell parameters. To our knowledge, this *in vivo* study is the first one in which an influence of putative 'lipotoxicity' can be interpreted separately from 'glucotoxicity'.

The negative effects of chronic hyperglycemia of β -cell function are well documented (1–3). Of particular significance are observations in transplantation models demonstrating that chronic hyperglycemia causes irreversible loss of β -cells (20). Also, in the present transplantation model we demonstrated continuous β -cell deterioration (14). As to the effects of fatty acids, several reports indicate that exposure to fatty acids *in vitro* reduces insulin content, reduces insulin mRNA and inhibits [H^3]leucine incorporation into proinsulin biosynthesis (5, 21). Furthermore, in mice, a high-fat diet reduces insulin biosynthesis (6). The effects of fatty acids on β -cell replication may be dual. Long-term-elevated fatty acids can lead to hyperplasia of β -cells (22). However, elevated fatty acids *in vivo*, with a resulting increase in islet triglycerides, have been linked

Table 3 Insulin and preproinsulin mRNA content of islet grafts.

		Insulin content	Preproinsulin mRNA content
	<i>n</i>	(pmol/graft)	(pg/graft)
Protocol 1			
Low-fat diet diabetic rats	6	20.3 ± 3.3	97.7 ± 11.4
High-fat diet diabetic rats	6	22.5 ± 4.6	89.6 ± 22.7
Protocol 2			
Low-fat diet diabetic rats	4	22.8 ± 3.0	61.1 ± 14.1
High-fat diet diabetic rats	5	22.4 ± 1.7	53.3 ± 5.3

to β -cell apoptosis in the diabetic Zucker fa/fa rat (9, 23). All in all, the available data indicate that elevated NEFAs can, at least under certain conditions, enhance a hyperglycemia-induced β -cell deterioration.

We have found that both insulin and preproinsulin mRNA content in the recovered transplants was similar between groups 1 week after the cessation of the high-fat or low-fat diets. These results argue against either a positive or a negative effect of the high-fat diet on β -cell mass. Our study attempted to obtain further evidence in the form of morphometric measurements but yielded insufficient data for a statistical analysis (results not shown). We are, however, unaware of a situation, similar to the present one in terms of hyperglycemia, in which parallel findings for the parameters of insulin content and insulin mRNA would not correspond with a direct assessment of β -cell mass. Hence, our results do not support a deleterious effect on β -cell mass (other than that of chronic hyperglycemia), from a high-fat diet under the present experimental conditions. Such a conclusion is also compatible with levels of C-peptide *in vivo* being enhanced (as a result of ambient elevated NEFAs) (24–27) during (but not after) the period of the high-fat diet.

It should be emphasized that our study was limited to the effects of elevated NEFAs and ensuing increased fatty acid metabolism in overt diabetes. The study was not designed to address any influence of NEFAs on the evolution of diabetes. Animal models other than the present one are needed for the study of this important question.

In our experiments, the effects of the high-fat diet on glucose-induced insulin secretion could not be evaluated. This is because of the well-known complete desensitization of this aspect of secretion by hyperglycemia *per se* (1–3). To test for a lingering effect of the high-fat diet on insulin secretion, arginine was used as the stimulus. Arginine, which is a non-nutrient secretagogue, potentially stimulates insulin secretion also during diabetic conditions (2). In our diabetic transplantation model, arginine-induced insulin secretion was approximately equal to the insulin secretion of transplants in normal rats over the same time period (8 weeks) (14).

We have found that a previous high-fat diet diminished arginine-induced insulin secretion from the perfused transplant-containing kidney. These results were unexpected in view of the fact that a 48 h lipid infusion in normal rats reduced glucose-induced insulin secretion but not the secretion induced by non-nutrient secretagogues (4). However, the regulation of non-nutrient-induced insulin secretion may be different in diabetes. Hence, after chronic hyperglycemia, the insulin secretory responses to non-nutrient secretagogues are sometimes enhanced when tested *ex vivo* (2). The endogenous supply of nutrients, notably glycogen, is increased in β -cells by hyperglycemia and could serve to enhance non-nutrient-induced

insulin secretion *in vitro* (28). In our experiments, it is possible that a high-fat diet would lower glycogen stores in the transplants in comparison with a low-fat and carbohydrate-rich diet. Such a situation would persist even for some time after a switch to a normal diet, perhaps as a result of reduced uptake and metabolism of glucose (29). A difference in glycogen stores could be relevant also to the difference between the present results and the observations of normal or exaggerated arginine-induced insulin secretion in ZDF rats (30), since the positive energy balance in the latter diabetic model should favor the accumulation of endogenous nutrients.

Also of interest in relation to our results on arginine-induced insulin secretion are observations on leptin levels. An increase in leptin levels after a high- vs a low-fat diet in rats with streptozotocin-induced diabetes has been reported (19). In theory, such an increase could affect insulin secretion from the transplants. Overall, it is obvious that further studies are needed to establish the mechanisms behind diet-induced effects on arginine-induced insulin secretion.

In conclusion, the present study has provided evidence, in a rat transplantation model, that elevated NEFAs during hyperglycemia can induce insulin secretory abnormalities but does not enhance hyperglycemia-induced loss of β -cell mass. These findings strengthen the notion that cellular damage, by fatty acids, to pancreatic β -cells in diabetic conditions is limited to situations with a positive energy balance.

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