Dissociated Insulinotropic Sensitivity to Glucose and Carbachol in High-Fat Diet–Induced Insulin Resistance in C57BL/6J Mice

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To study islet function following reduced insulin sensitivity, we examined mice of the C57BL/6J strain, the genotype of which carries an increased propensity to develop insulin resistance when metabolically challenged. The mice received either a high-fat diet (58% fat on an energy basis) or a control diet (11% fat) for 12 weeks. The body weight of mice on the high-fat diet increased significantly more than that of mice on the control diet (25.8 ± 0.4 *v* 21.3 ± 0.2 g, $P < .001$). Already after 1 week on the high-fat diet, a significant hyperglycemia accompanied by hyperinsulinemia had evolved, indicative of insulin resistance. After 12 weeks, plasma glucose levels for high-fat diet–treated mice were 7.5 ± 0.1 mmol/L, versus 6.5 ± 0.1 mmol/L in controls ($P < .001$); corresponding values for plasma insulin were 248 ± 17 and 104 ± 7 pmol/L, respectively ($P < .001$). Mice given a high-fat diet also had elevated levels of total cholesterol, triglycerides, and free fatty acids (FFAs) compared with controls. After 4, 8, and 12 weeks, glucose (2.8, 8.3, or 16.7 mmol/kg) or the cholinergic agonist carbachol (0.16 or 0.53 μmol/kg) was injected intraperitoneally. The insulinotropic response to glucose was not different between the two groups after 4 or 8 weeks, whereas after 12 weeks, glucose-induced insulin secretion was markedly impaired in high-fat diet–treated mice ($P < .001$). In contrast, after 8 and 12 weeks on a high-fat diet, carbachol-stimulated insulin secretion was potentiated ($P < .01$), whereas carbachol-stimulated glucagon secretion was not significantly altered. Furthermore, after 12 weeks on the high-fat diet, insulin secretion from isolated islets was impaired at glucose levels of 8.3, 11.1, and 16.7 mmol/L ($P \leq .05$). Moreover, islet morphology as examined by immunocytochemistry using insulin antibodies and islet innervation, as revealed by immunostaining of tyrosine hydroxylase (TH), neuropeptide Y (NPY), galanin, vasoactive intestinal polypeptide (VIP), and substance P (SP) were unaffected by the high-fat diet for 12 weeks. However, quantitative in situ hybridization showed a 3.5-fold upregulation of insulin gene expression in response to the high-fat diet ($P < .001$) despite unaltered B-cell mass and pancreatic insulin content. We conclude that as little as 1 week of treatment with a high-fat diet induces insulin resistance in C57BL/6J mice. This is accompanied later by hyperlipemia, potentiated carbachol-stimulated insulin secretion, and increased insulin gene expression but impaired glucose-stimulated insulin secretion. We suggest that after several weeks' duration, insulin resistance is accompanied by enhanced islet sensitivity to cholinergic activation and exaggerated insulin gene expression, whereas the failing islet sensitivity to glucose represents decompensation.

INSULIN RESISTANCE is compensated for by increased insulin secretion.[1-3] The mechanism of the islet compensation might involve a slight but continuous hyperglycemia, which stimulates basal insulin secretion and potentiates non–glucose-stimulated insulin secretion, as inferred from studies in humans.[1,2,4] The mechanism might also involve cholinergic hyperactivity, as indicated by studies in the glucose-infused rat, in which the potentiated glucose-stimulated insulin secretion was inhibited by atropine,[5] and in the insulin-resistant obese mouse, in which atropine reduced hyperinsulinemia.[6] Similarly, the frequency of diabetes induced by low-dose alloxan in mice has been shown to be increased by muscarinic blockade,[7] and the insulin secretory response to muscarinic activation by bethanechol has been shown to be exaggerated in the obese mouse.[8] Following long-standing insulin resistance, this compensation in islet function might fail, resulting in a decompensated state precipitating non–insulin-dependent diabetes mellitus. However, the islet consequences of long-standing insulin resistance are not established. To increase our understanding of the islet consequences of long-standing insulin resistance, we examined the C57BL/6J strain of mice, the genetic background of which carries a phenotype characterized by a high occurrence of insulin resistance when metabolically challenged.[9,10] It has, for instance, been demonstrated that when C57BL/6J mice are fed a high-fat diet, severe insulin resistance with hyperinsulinemia develops, in contrast to only a marginal hyperinsulinemia in another strain, the A/J mouse, given the same high-fat diet.[11] Furthermore, 4 months of treatment of C57BL/6J mice with a high-fat diet has been shown to induce hyperglycemia, hyperinsulinemia, slight obesity, and an impaired insulin secretory response to glucose.[11-13]

In the present study, we frequently examined islet function in C57BL/6J mice during 12 weeks of treatment with a high-fat diet. We studied insulin secretion in vivo and in vitro, and examined islets from C57BL/6J mice using immunocytochemistry and quantitative in situ hybridization for assessment of islet morphology and insulin gene expression. We paid particular attention to a potential difference in the islet response to glucose versus the cholinergic agonist, carbachol, in line with the hypothesis that cholinergic supersensitivity is a mechanism underlying the islet adaptation to an increased requirement for insulin that accompanies insulin resistance.[5-8]

MATERIALS AND METHODS

Animals

A total of 264 female mice of the C57BL/6J strain were obtained from Bomholtgaard Breeding and Research Centre (Ry, Denmark) at 4

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Submitted April 29, 1996; accepted August 20, 1996.

Supported by the Swedish Medical Research Council (14X-6834 and 12X-4499), Novo Nordic, the Albert Påhlsson, Crafoord, Ernhold Lundström, and Ester Ohlssons Foundations, the Diabetes Association of Malmö with surroundings, the Swedish Diabetes Association, Malmö University Hospital, and the Faculty of Medicine, Lund University.

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0026-0495/97/4601-0018$03.00/0

Table 1. Characteristics of Antisera Used in the Study

Antigen	Code	Raised Against	Raised In	Dilution	Source
Proinsulin	9003	Human proinsulin	Guinea pig	1:2,560	EuroDiagnostica (Malmö, Sweden)
$NPY_{(1-20)}$	8810	Protein-conjugated synthetic rat NPY	Rabbit	1:640	EuroDiagnostica
VIP	7852	Unconjugated porcine VIP	Rabbit	1:640	EuroDiagnostica
TH	P40101-0	Unconjugated rat TH	Rabbit	1:320	Pel-freez Biologicals (Rogers, AR)
SP	SP8	Protein-conjugated synthetic bovine SP	Rabbit	1:320	Dr P.C. Emson, MRC (Cambridge, UK)
Galanin	RAGS 5777	Unconjugated porcine galanin	Rabbit	1:640	Dr B.E. Dunning, Sandoz (E Hanover, NJ)

weeks of age. They were studied in batches of 50 to 55 mice each. At day 4 after arrival, half the mice in each batch started a high-fat diet (Research Diets, New Brunswick, NJ), and the other half received a standard rodent chow diet (Lactamin, Stockholm, Sweden). On a caloric basis, the high-fat diet consisted of 16.4% protein, 25.6% carbohydrates, and 58.0% fat (total, 23.4 kJ/g), whereas the control diet consisted of 25.8% protein, 62.8% carbohydrates, and 11.4% fat (total, 12.6 kJ/g). Throughout the study period of 12 weeks, the mice had free access to food and water. Four to five mice were kept per cage in a temperature-controlled (22° ± 1°C) room with a 12-hour light-dark cycle with lights on at 6:00 AM. The study was approved by the Animal Ethics Committee at Lund University.

Baseline Parameters

At day 4 after arrival, ie, when treatment with the various diets started, and then once every week for the study period of 12 weeks, all animals and food consumption were weighed, and a blood sample was taken from the intraorbital, retrobulbar plexus for measurement of plasma levels of insulin and glucose.

In Vivo Experiments

After 4, 8, and 12 weeks of treatment with a high-fat/control diet, all mice underwent one in vivo study. The animals were injected intraperitoneally with D-glucose (Sigma Chemical, St Louis, MO) at a dose level of 2.8, 8.3, or 16.7 mmol/kg, with the cholinergic agonist carbachol (Sigma) at a dose level of 0.16 or 0.53 μmol/kg or saline. The volume load was 10 μL/g body weight. Blood samples were taken at 6 and 10 minutes after the respective injection; initial experiments showed that peak levels of plasma insulin after intraperitoneal injection of glucose or carbachol in this strain of mice occurred within this period (data not shown). The samples were immediately centrifuged, and the plasma was stored at −20°C until assayed. The mean plasma levels of glucose, insulin, and glucagon were calculated for the 6- and 10-minute values, and the mean value obtained in the control saline-injected group in the same series was subtracted from the mean 6- to 10-minute value obtained in each experimental animal.

In Vitro Experiments

After 4, 8, and 12 weeks of treatment with a high-fat or control diet, pancreatic islets were isolated from four mice in each batch with a collagenase isolation technique modified from the study by Lacy and Kostianovsky.[14] In brief, the pancreas was retrogradely filled with 3 mL Hanks balanced salt solution (Sigma) supplemented with 0.3 mg/mL Collagenase P (activity, 1.86 U/mg; Boehringer, Mannheim, Germany). The pancreas was subsequently removed and incubated in the same solution for 20 minutes at 37°C. After rinsing, the islets were handpicked under a stereomicroscope and incubated overnight in RPMI 1640 medium supplemented with 10% fetal calf serum, 2.05 mmol/L L-glutamine, 2.5 μg/mL amphotericin B (GIBCO BRL, Paisley, Scotland), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Biol Ind, Beit Haemek, Israel) at 37°C in humidified air equilibrated with 5% CO_2. Following overnight incubation, the islets were washed three times and then preincubated for 60 minutes at 37°C in a HEPES medium (pH 7.36) supplemented with 0.1% human serum albumin (Sigma) and 3.3 mmol/L glucose. The medium consisted of the following (in mmol/L): 125 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.28 $CaCl_2$ (all Sigma), and 25 HEPES (Boehringer). After the preincubation, groups of three islets were transferred to separate chambers containing 200 μL of the medium supplemented with glucose at various concentrations. Following incubation at 37°C for 60 minutes, 25 μL of the medium was collected from each chamber and stored at −20°C until analysis.

Pancreatic Insulin Extraction

For analysis of pancreatic insulin, approximately 50 mg of the pancreas was removed from each animal and weighed. The tissue was thereafter homogenized in acid ethanol (0.18 mol/L HCl in 95% ethanol) followed by radioimmunoassay for insulin.

Analysis of Insulin, Glucagon, and Glucose

Insulin in plasma, medium, and extracted tissue was determined radioimmunochemically[15] with a guinea pig anti–rat insulin antibody, ^{125}I-labeled porcine insulin as tracer, and rat insulin as standard (Linco Research, St Louis, MO). Free and bound radioactivity was separated

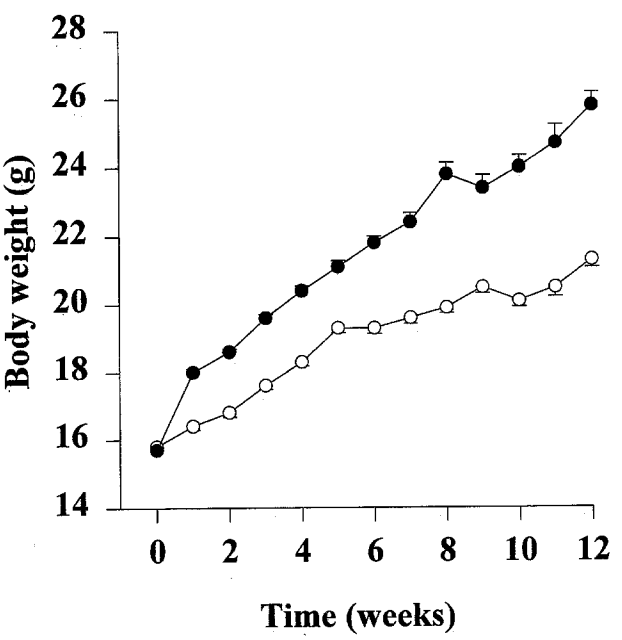

Fig 1. Body weight during treatment of C57BL/6J mice with a high-fat diet (●) or control diet (○) for a 12-week period. At initiation of the study, mice were 4 weeks old. The mean ± SEM is shown. The number of animals at each time point and group was 91 and 132. The difference between the 2 groups was highly significant ($P < .001$) at all time points from week 1 and onward.

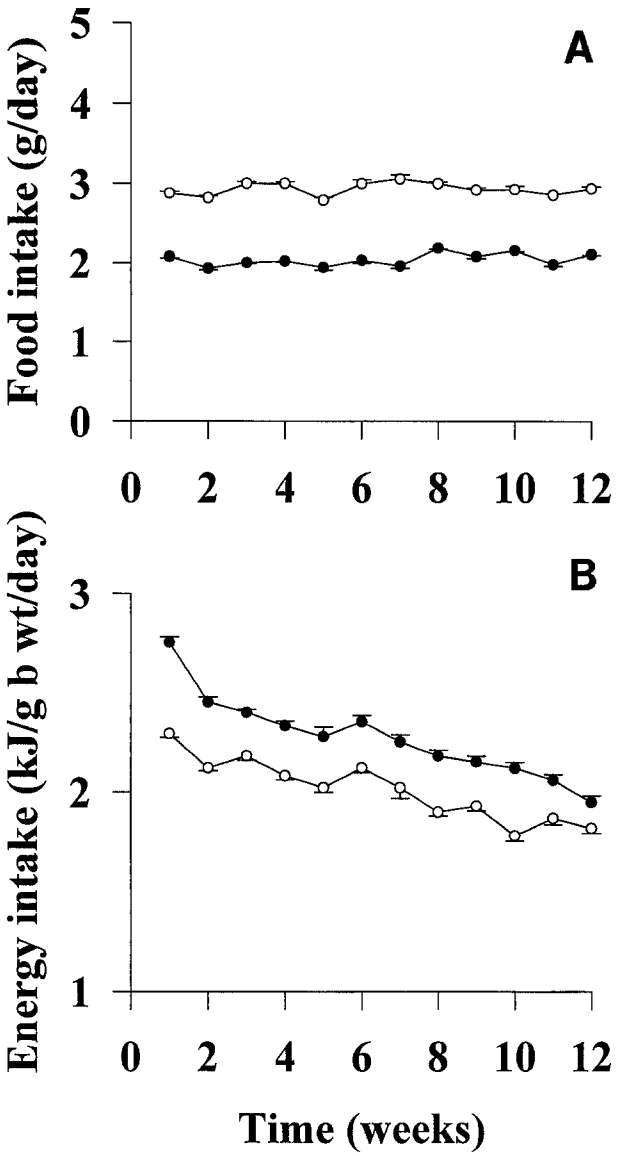

Fig 2. Daily food intake, expressed as total amount of food intake (A) and daily energy intake per g body weight (B) in C57BL/6J mice given a high-fat diet (●) or control diet (○) for 12 weeks, starting at age 4 weeks. The mean ± SEM is shown. The number of animals at each time point and group was 91 and 132. There was a highly significant difference ($P < .001$) between the 2 groups of mice at all time points.

by use of an anti-IgG (goat anti–guinea pig) antibody (Linco). The sensitivity of the assay is 12 pmol/L, and the coefficient of variation is less than 7% at both low and high levels. Analyses of glucagon concentrations were performed on unextracted plasma[16] with a double-antibody radioimmunoassay using guinea pig antiglucagon antibodies specific for pancreatic glucagon, ^{125}I-glucagon as tracer, and glucagon standard (Linco). The sensitivity of the assay is 7.5 pg/mL, and the coefficient of variation is less than 9% at both low and high levels. Plasma glucose was determined by the glucose oxidase method.[17]

Analysis of Cholesterol, Triglycerides, and Free Fatty Acids

At 6 weeks after the start of treatment with a high-fat or control diet, blood was collected and plasma separated for determination of total cholesterol, triglycerides, and free fatty acids (FFAs). Total cholesterol level was measured enzymatically,[18] and triglycerides and FFAs were determined colorimetrically.[19,20]

Immunocytochemistry

After 12 weeks of treatment with a high-fat or control diet, pancreatic specimens were removed and fixed overnight by immersion in Stefanini's solution (2% paraformaldehyde and 1.5% saturated solution of picric acid in 0.1 mol/L phosphate-buffered saline, pH 7.2). After rinsing in sucrose-enriched (10%) buffer, the specimens were frozen on dry ice. Sections were cut at 10 μm thickness in a cryostat and mounted

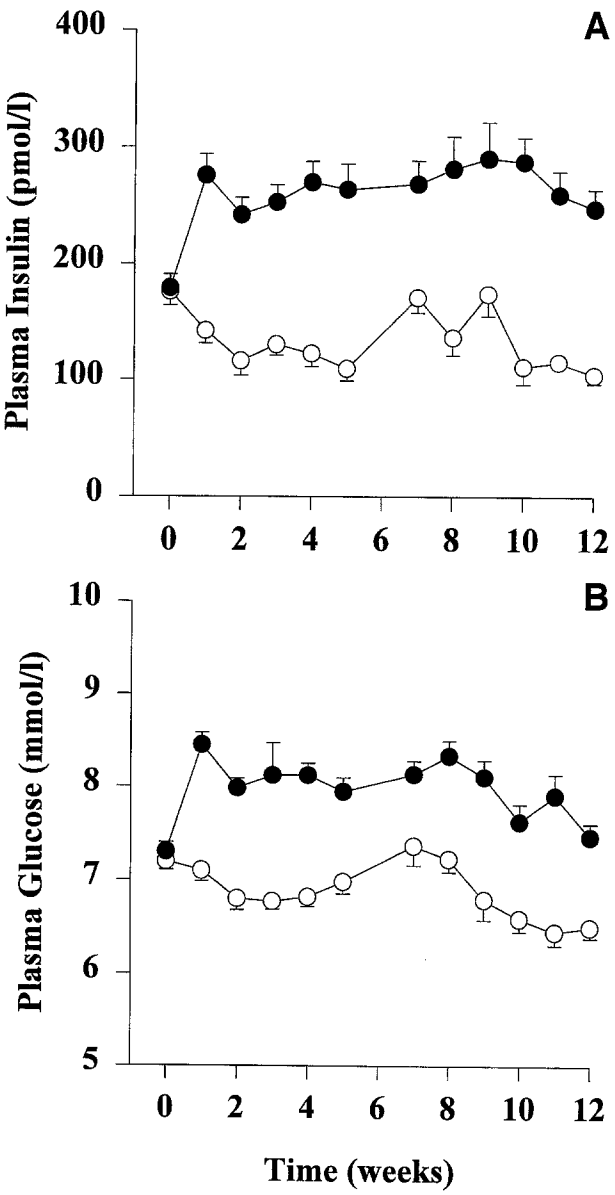

Fig 3. Baseline plasma levels of insulin (A) and glucose (B) during treatment of C57BL/6J mice with a high-fat diet (●) or control diet (○) for a 12-week period. At initiation of the study, mice were 4 weeks old. The mean ± SEM is shown. The number of animals at each time point and group was 91 and 132. The difference between the 2 groups was highly significant ($P < .001$) for both plasma insulin and plasma glucose at all time points from week 1 and onward.

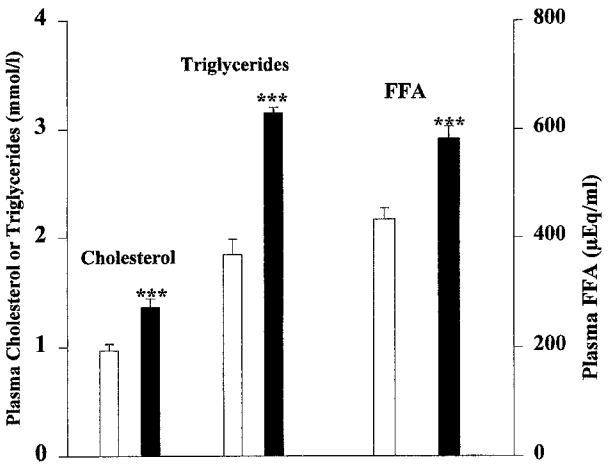

Fig 4. Plasma levels of cholesterol, triglycerides, and FFA after 6 weeks of treatment of C57BL/6J mice with a control diet (□) or high-fat diet (■). The mean ± SEM is shown. There were 20 to 25 animals per group. Random difference between groups, ***$P < .001$.

on chromalum-coated slides. The sections were then processed for immunocytochemical detection of insulin, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), tyrosine hydroxylase (TH), galanin, and substance P (SP) using the indirect immunofluorescence technique.[21] Details of the antisera are shown in Table 1. Fluorescein isothiocyanate–labeled porcine antisera against rabbit or guinea pig IgG were used as secondary antisera (dilution 1:80; EuroDiagnostica, Malmö, Sweden). Control sections were incubated with the antisera (in working dilution) preabsorbed with the respective antigen in excess (obtained from Peninsula Europe, Merseyside, England; 10 to 100 μg/mL). The relative frequency of peptide-containing nerve fibers was assessed semiquantitatively by two independent observers. The final score was the mean of these estimations, and was graded as follows: 0, no fibers; 1, occasional nerve fibers; 2, moderate number of nerve fibers; and 3, numerous nerve fibers. At least seven sections were examined for each type of nerve fiber (NPY, VIP, TH, galanin, and SP, respectively).

In Situ Hybridization

Insulin mRNA was detected in situ in islets using a mix of oligonucleotide probes complementary to rat insulin mRNA I and II (BPR 236; R&D Systems, Abingdon, Oxon, UK); the probes were 3′-end-tailed with ^{35}S as previously described.[22] The protocol for in situ hybridization has previously been described in detail.[22] Briefly, the pancreatic sections (5 μm thickness) were deparaffinized, rehydrated, and permeabilized in 0.01% Triton X-100. Before hybridization, the sections were treated with proteinase K (10 μg/mL; Sigma) and acetylated by 0.25% acetic anhydride in 0.1 mol/L triethanolamine. Hybridization was performed overnight at 37°C in sealed moisturizing chambers, using a probe concentration of 200 fmol/mL. Following stringent posthybridization washing, the slides were dipped in Ilford K-5 emulsion (Ilford Anitec, Göteborg, Sweden), exposed for 7 days, and developed in Kodak D-19 (Kodak, Stockholm, Sweden). Levels of insulin mRNA were determined by quantification of probe labeling of islets, using Q 500 MC Leica (Leica Cambridge, Cambridge, UK) as previously described in detail.[23] Briefly, before analysis, the system was calibrated by conversion of the grey values in a standard section to optical density (OD), yielding an arbitrary scale from 0 to 5. The calibration and illumination of the sections in the microscope were checked repeatedly during analysis to ensure that mRNA levels were determined under identical conditions. Dark-field images of islets were captured and digitized, and the polarity was reversed. The outline of the islets was interactively defined by the investigator. Subsequently, the

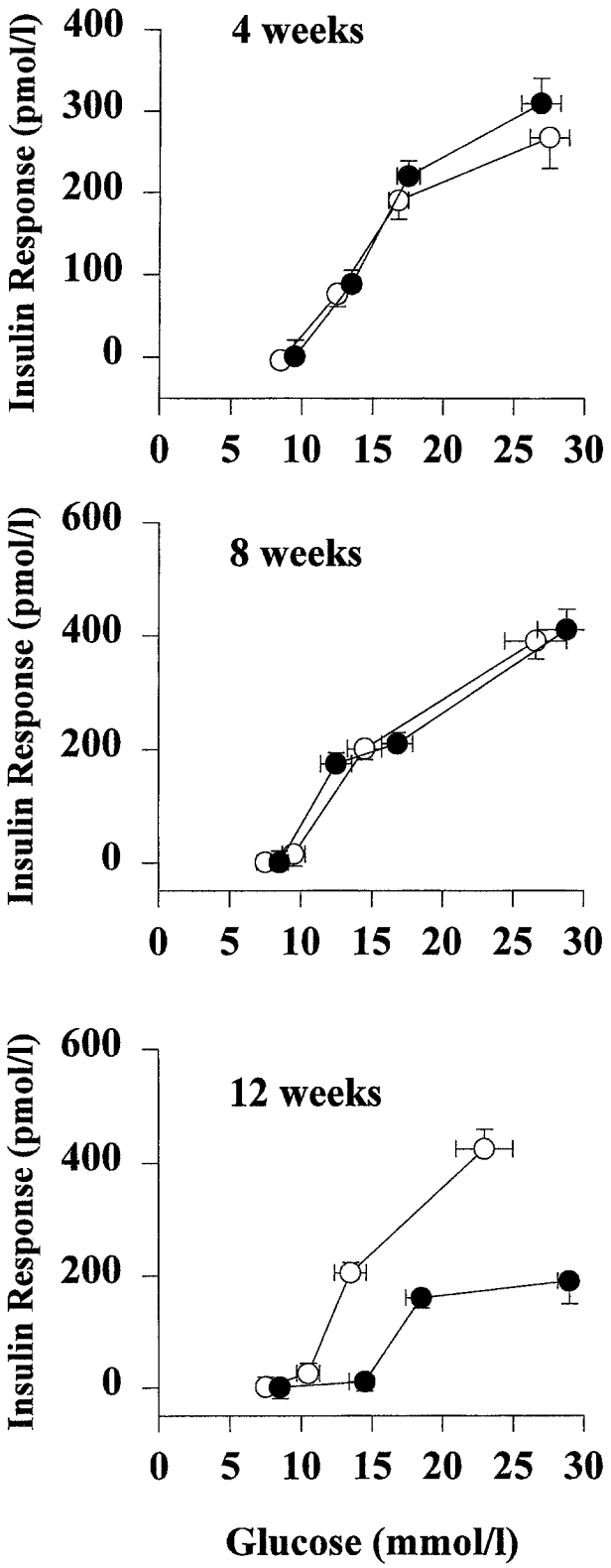

Fig 5. Glucose-stimulated insulin secretion in vivo at 4, 8, and 12 weeks after start of treatment with a high-fat diet (●) or control diet (○) in C57BL/6J mice as a function of plasma glucose levels. Glucose was injected at 2.8, 8.3, or 16.7 mmol/kg, and plasma was sampled after 6 and 10 minutes. The mean ± SEM is shown. There were 12 to 24 animals per group.

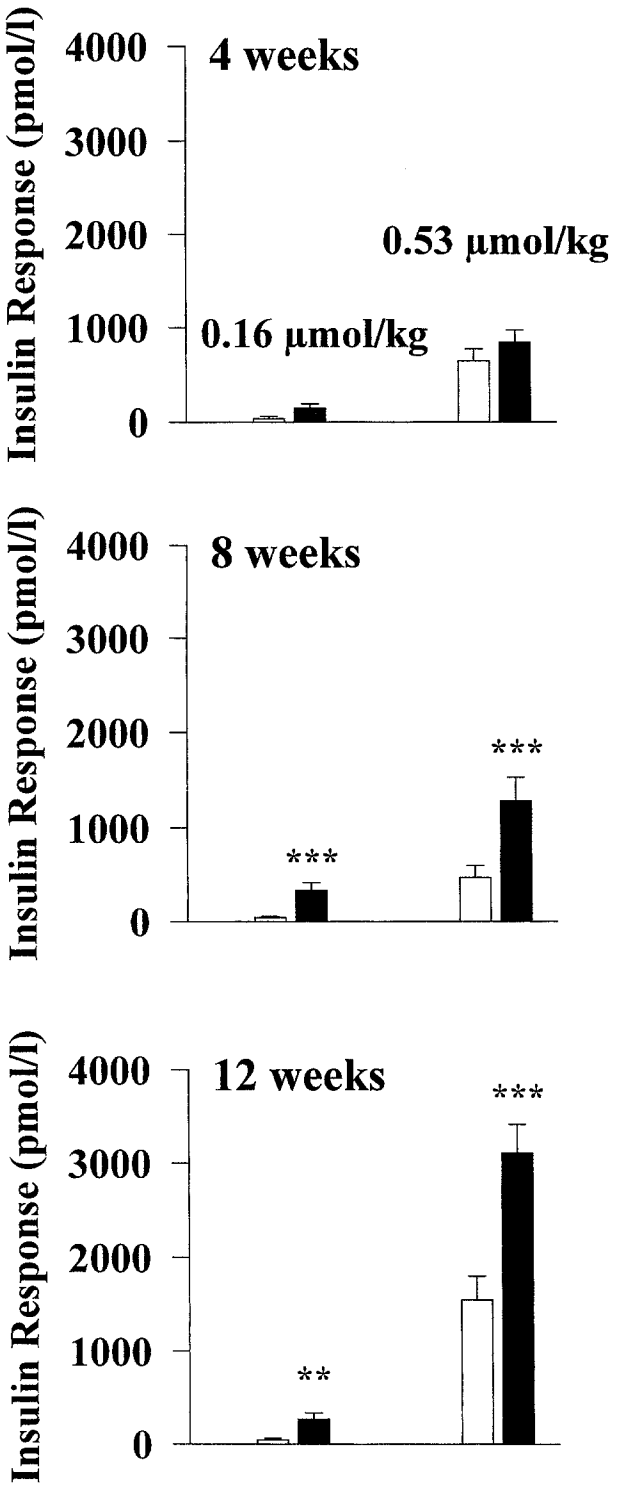

Fig 6. Carbachol-stimulated insulin secretin in vivo at 4, 8, and 12 weeks after start of treatment with a control diet (□) or high-fat diet (■) in C57BL/6J mice. Carbachol was injected at 0.16 or 0.53 µmol/kg, and plasma was sampled after 6 and 10 minutes. The mean ± SEM is shown. There were 14 to 16 animals per group. **$P <$ .01, ***$P <$ .001.

Table 2. Plasma Glucagon Levels After Intraperitoneal Injection of Carbachol or Saline in C57BL/6J Mice Given a High-Fat or Control Diet for 4, 8, or 12 Weeks

Diet	Carbachol 0.16 µmol/kg	Carbachol 0.53 µmol/kg	Saline
4 weeks			
High-fat	98 ± 17 (15)	196 ± 9 (8)	48 ± 6 (29)
P	NS	NS	.005
Control	92 ± 8 (16)	196 ± 29 (8)	26 ± 2 (21)
8 weeks			
High-fat	115 ± 10 (14)	164 ± 26 (7)	59 ± 7 (27)
P	NS	NS	.006
Control	121 ± 14 (13)	167 ± 25 (6)	36 ± 4 (15)
12 weeks			
High-fat	273 ± 54 (15)	261 ± 23 (23)	63 ± 4 (43)
P	NS	NS	NS
Control	294 ± 67 (12)	269 ± 20 (21)	59 ± 3 (37)

NOTE. Values are the mean ± SEM for the 6- and 10-minute plasma samples. Carbachol was injected at either of 2 dose levels, and samples were taken 6 and 10 minutes after injection. Number of animals is in parentheses.

mean OD within the islets was determined, correlating with the amount of probe hybridized to an islet and thus to the insulin mRNA abundancy. In addition, the total area of insulin probe–labeled cells in the islets was determined, thereby yielding data on B-cell mass. Sections from each individual animal were analyzed in duplicate; 10 randomly selected islets from different parts of the sections were analyzed.

Statistics

The mean ± SEM are reported. Statistical comparisons for differences between mice treated with a high-fat or control diet in body weight, food intake, and baseline plasma levels of insulin, glucose, and lipids were performed by Student's *t* test. Differences with regard to insulin, glucagon, or glucose responses to glucose or carbachol in vivo or to insulin secretion in vitro were evaluated by two-way ANOVA and the Newman-Keul post hoc test. Insulin mRNA levels, areas of insulin probe–labeled cells, insulin cell numbers, and the relative frequency of NPY and VIP nerves were compared using a two-tailed Mann-Whitney *U* test.

RESULTS

Body Weight

Already after 1 week of treatment with the high-fat diet, body weight was significantly higher compared with control levels (18.0 ± 0.1 *v* 16.8 ± 0.1 g, $P < .001$; Fig 1). After 12 weeks, body weight in mice given the high-fat diet was 25.8 ± 0.4 g (n = 109), versus 21.3 ± 0.2 g in controls (n = 91, $P < .001$).

Food Intake

Daily food intake was stable in the two groups of mice throughout the study (Fig 2). Daily energy intake was slightly but significantly higher in mice given the high-fat diet than in controls (Fig 2; $P < .01$ between groups at all time points).

Baseline Glucose and Insulin

After 1 week on the high-fat diet, hyperinsulinemia was seen that persisted throughout the study period (Fig 3). After 12 weeks of treatment, plasma insulin levels in mice given the high-fat diet were 248 ± 17 pmol/L (n = 109), versus 104 ± 7

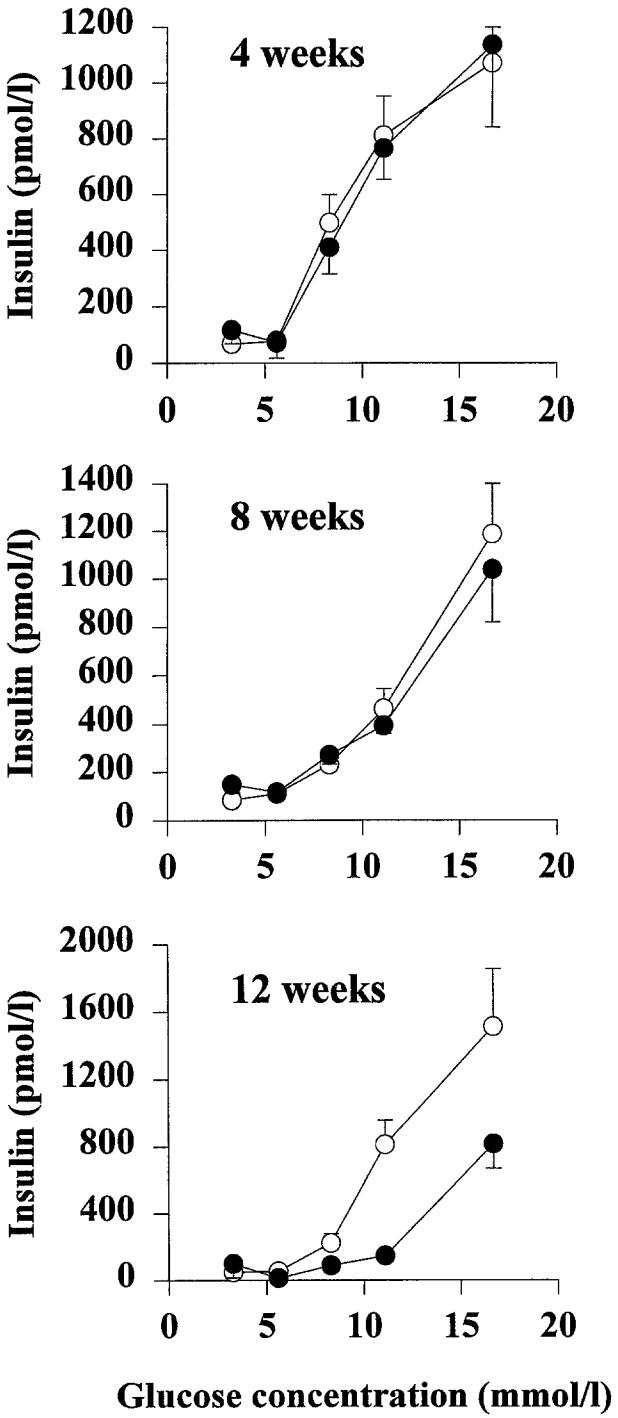

Fig 7. Glucose-stimulated insulin secretion from islets isolated from C57BL/6J mice after 4, 8, or 12 weeks of treatment with a high-fat diet (●) or control diet (○) after incubation for 60 minutes in different concentrations of glucose. The medium glucose concentration is shown on the x-axis, and the medium insulin secretion is shown on the y-axis. The mean ± SEM is shown. For each separate group and time, 3 to 4 different experiments with a total of 13 to 29 observations at each point are shown.

pmol/L in control mice (n = 91, $P < .001$). Also, plasma glucose levels were increased after 1 week of treatment with the high-fat diet; this difference of approximately 1 mmol/L was present throughout the study period. After 12 weeks, plasma glucose levels in mice given the high-fat diet were 7.5 ± 0.1 mmol/L (n = 109), versus 6.5 ± 0.1 mmol/L in controls (n = 91, $P < .001$; Fig 3).

Plasma Lipids

After 6 weeks of treatment with the high-fat diet, plasma levels of cholesterol, triglycerides, and FFAs were all significantly elevated compared with levels in mice given the control diet (Fig 4).

Glucose-Stimulated Insulin Secretion In Vivo

At 4 and 8 weeks after starting the high-fat or control diet, glucose-stimulated insulin secretion was not different between the two groups of animals (Fig 5). However, after 12 weeks of treatment, the plasma insulin response to glucose injected at 16.7 mmol/kg was impaired after the high-fat diet, since it was 446 ± 56 pmol/L in control animals (n = 14), versus only 203 ± 62 pmol/L in animals given the high-fat diet (n = 15, $P = .008$; Fig 5).

Carbachol-Stimulated Insulin and Glucagon Secretion In Vivo

After 4 weeks of treatment with the two diets, there was no significant difference between the two groups with regard to carbachol-stimulated insulin secretion (Fig 6). However, after both 8 and 12 weeks of treatment, carbachol-stimulated insulin secretion was exaggerated in mice given the high-fat diet compared with the control diet (Fig 6). Thus, after 12 weeks of treatment, carbachol at 0.53 μmol/kg increased plasma insulin by 3,105 ± 308 pmol/L in mice given the high-fat diet, versus an increase of only 1,544 ± 256 pmol/L in controls ($P = .002$). In contrast, carbachol-stimulated glucagon secretion did not differ significantly between the two groups at any of the studied time points (Table 2).

Glucose-Stimulated Insulin Secretion In Vitro

After 4 and 8 weeks of treatment, no significant difference was evident between the two groups regarding glucose-stimulated insulin secretion in vitro (Fig 7). However, after 12 weeks of treatment, glucose-stimulated insulin secretion was markedly impaired in mice given the high-fat diet, since the medium insulin concentration was lower after incubation of islets from these animals at glucose concentrations of 8.3 mmol/L ($P = .038$), 11.1 mmol/L ($P = .021$), and 16.7 mmol/L ($P < .001$).

Cellular Expression of Insulin

No visible difference was observed between the two groups of mice with regard to insulin-like immunofluorescence, as illustrated in representative islets from mice given the control or high-fat diet for 12 weeks (Fig 8). Similarly, pancreatic insulin content after 12 weeks of treatment did not differ between the groups (4.0 ± 1.1 pmol/mg wet weight in pancreas from high-fat diet–treated mice, n = 6, *v* 3.9 ± 0.8 in controls, n = 6, NS).

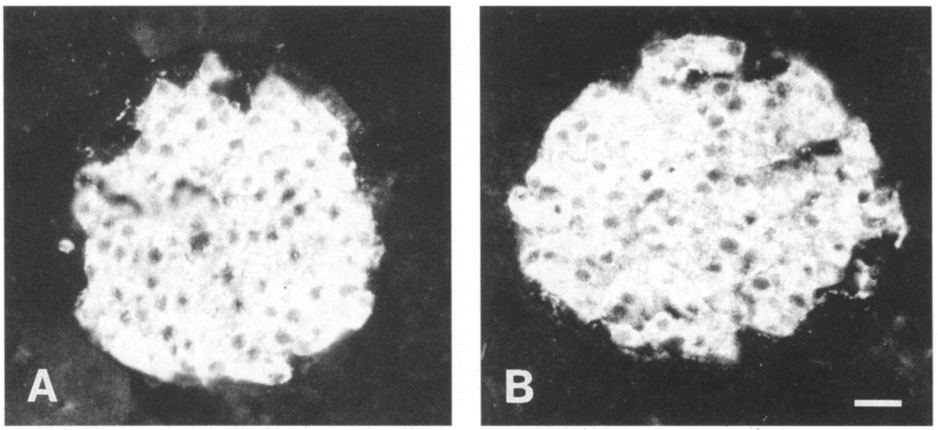

Fig 8. Immunofluorescence for insulin in pancreatic islets from C57BL/6J mice given a control diet (A) or high-fat diet (B) for 12 weeks. No difference in the pattern of immunostaining was observed between groups (bar = 20 μm).

Insulin Gene Expression

Figure 9 illustrates representative in situ hybridizations of islets from the two groups of mice, hybridized with oligonucleotides complementary to proinsulin mRNA. Insulin gene expression was markedly increased after the high-fat diet. Quantitative in situ hybridization showed that insulin probe labeling was increased approximately 3.5-fold in mice given the high-fat diet for 12 weeks compared with the controls (Fig 10; $P < .001$). In contrast, B-cell mass, as shown by the total area of the insulin probe–labeled cells, was not different between the groups: 14,516 ± 1,862 μm^2 in mice given the high-fat diet (n = 61 islets from nine animals) compared with 14,656 ± 1,653 μm^2 in controls (n = 60 islets from eight animals, NS).

Neural Expression of Islet Neuropeptides

Pancreatic tissues from control and high-fat diet–treated mice were immunostained for markers of islet nerves. Figure 11 shows representative islet immunostaining in the two groups of mice for two different neuropeptides: NPY, a marker for pancreatic adrenergic nerves, and VIP, a marker for pancreatic parasympathetic, presumably cholinergic, nerves.[24] In both groups of mice, islet innervation with nerves harboring these two neuropeptides was evident. However, there was no difference in the relative frequency of innervation with NPY and VIP fibers between the two groups of mice (Fig 12). Furthermore, in both groups of mice, islet innervation with nerves immunoreactive for TH and galanin (neuropeptide in pancreatic adrenergic nerves[24]) and SP (marker for sensory nerves[25]) was evident. However, similarly as for NPY and VIP, there was no difference in the relative frequency of TH, galanin, and SP fibers between the two groups.

DISCUSSION

In this study, we confirm previous reports by Surwit et al[11,12] that a high-fat diet increases body weight and induces insulin resistance in mice of the C57BL/6J strain, as evident by slight hyperglycemia and marked hyperinsulinemia already after 1 week. We also found that this insulin resistance was accompanied later (12 weeks) by impaired glucose-stimulated insulin secretion, which enforces the idea that this strain of mice carries a genetic predisposition to develop type II diabetes mellitus.[11] We focused our interest on the first 12 weeks of treatment with a high-fat diet to establish the consequences for the islet adaptation of reduced insulin resistance after several weeks, but before diabetes evolves.

Reduced insulin sensitivity is compensated for by hyperinsulinemia to avoid hyperglycemia.[1-3] Previous studies in the glucose-infused rat and the *ob/ob* mouse have suggested that increased sensitivity to cholinergic activation might be a mechanism underlying the compensatory hyperinsulinemia.[5-8] Thus, in the *ob/ob* mouse, muscarinic blockade by atropine reduces plasma insulin levels,[6] and similarly, *ob/ob* mice exhibit an increased insulinotropic action of the muscarinic agonist bethanechol.[8] Cholinergic activation by means of vagal nerve activation or administration of muscarinic agonists is known to stimulate insulin secretion.[8,26-28] Increased vagal activity or muscarinic sensitivity as a compensatory response to peripheral insulin resistance would therefore increase insulin secretion. We

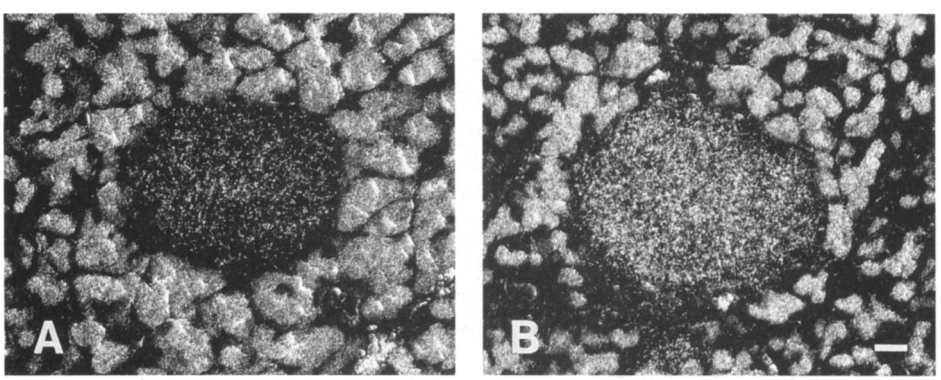

Fig 9. In situ hybridization with radiolabeled insulin probes in islets from C57BL/6J mice given a control diet (A) or high-fat diet (B) for 12 weeks. The high-fat diet markedly increased the abundance of insulin mRNA (bar = 50 μm).

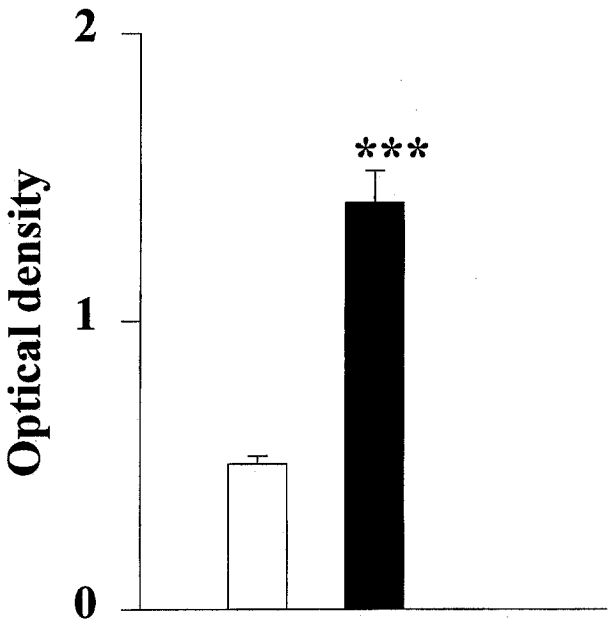

Fig 10. Proinsulin mRNA levels in pancreatic islets from C57BL/6J mice given a control diet (□) or high-fat diet (■) for 12 weeks, as determined by quantitative in situ hybridization and shown as OD. The mean ± SEM from analysis of 60 to 61 islets from 9 to 11 animals per group is shown. ***Random difference between groups, $P < .001$.

examined this by challenging the mice with the cholinergic agonist carbachol at 4, 8, and 12 weeks after the start of a high-fat diet. We found an increased insulinotropic response to the cholinergic agonist carbachol after 8 weeks of treatment with the high-fat diet. This result suggests that increased muscarinic activity develops as a compensation for insulin resistance. Increased B-cell cholinergic sensitivity might therefore be of importance for maintenance of long-term hyperinsulinemia. Vagal nerve activation and muscarinic agonism also stimulate glucagon secretion.[28,29] However, in contrast to the potentiated insulinotropic action of carbachol in high-fat diet–treated mice, the glucagonotropic effect of carbachol was found not to be augmented after treatment with a high-fat diet. This shows that the increased sensitivity to muscarinic activation is restricted to insulin secretion, rather than being an unspecific hyperactivity in the vagal system.

In contrast to the exaggerated insulinotropic response to cholinergic stimulation, C57BL/6J mice given a high-fat diet developed impaired glucose-stimulated insulin secretion under both in vivo and in vitro conditions after 12 weeks of treatment. This shows that the marked hyperinsulinemia at this stage is not dependent on a potentiated insulinotropic response to glucose. Further, the hyperinsulinemia is not caused by islet hypertrophy, since B-cell mass was not altered by the high-fat diet. Moreover, mice given the high-fat diet had increased insulin mRNA levels despite the unaffected B-cell mass and pancreatic insulin content. This indicates that the insulin biosynthetic machinery is

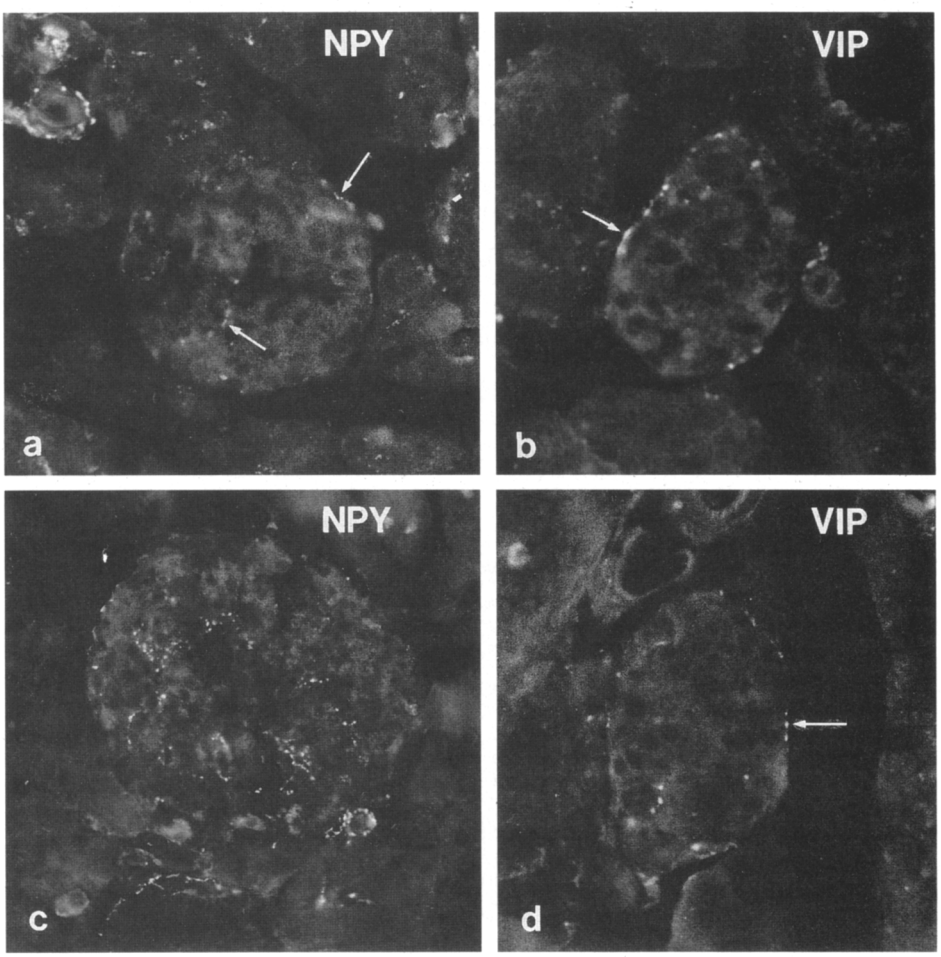

Fig 11. Immunofluorescence for NPY (a and c) or VIP (b and d) in pancreatic islets from C57BL/6J mice given a control diet (a and b) or high-fat diet (c and d) for 12 weeks. No difference in the pattern or degree of immunostaining was observed between groups. (×80.)

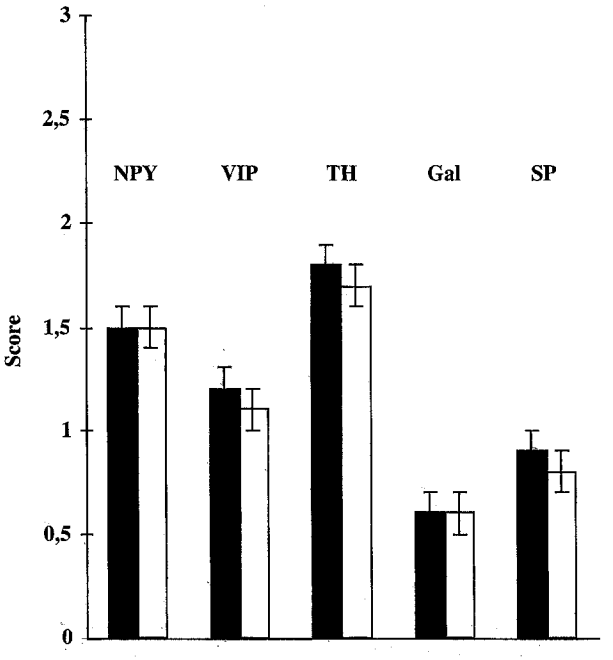

Fig 12. Relative frequency (mean ± SEM) of NPY, VIP, TH, galanin (gal), and SP nerve fibers in islets from C57BL/6J mice after 12 weeks' treatment with a control diet (□) or high-fat diet (■). The mean ± SEM for semiquantitative estimation of the relative frequency is shown; n = 7 to 10 for each bar.

highly activated in insulin resistance and that insulin turnover is increased, causing increased insulin release. Thus, it seems that long-standing insulin resistance for 8 to 12 weeks induces increased insulin gene expression and increased B-cell sensitivity to cholinergic activation together with impairment of glucose-stimulated insulin secretion.

We found that following long-term insulin resistance of 12 weeks, the insulin secretory response to glucose failed. This phenomenon may represent an early decompensation in islet function that might be a mechanism of importance in diabetes pathogenesis. Several explanations may be offered for the failing glucose-stimulated insulin secretion following treatment with a high-fat diet for several weeks. One possible explanation is increased sympathoadrenal activity, which has been demonstrated in C57BL/6J mice given a high-fat diet showing increased glycemic responses to stress and epinephrine.[11,12] Such an increased sympathoadrenal activation would diminish insulin secretion.[24] Another explanation is that the hyperglycemia, although slight but chronic, impairs islet function due to a long-standing B-cell glucose toxicity.[30,31] However, this explanation is unlikely in these mice, since it previously has been demonstrated that a 7-day exposure to high glucose (28 and 56 mmol/L) did not cause any deterioration in islets isolated from C57BL/6J mice.[32] A third explanation is that the hyperlipidemia, particularly the increased circulating levels of triglycerides and FFAs, that were evident in our mice, caused the deteriorated glucose-stimulated insulin secretion. In fact, it has previously been demonstrated that lipid infusion in rats inhibits glucose-stimulated insulin secretion,[33] and several groups have shown that islets incubated during a long period with fatty acids exhibit inhibited glucose-stimulated insulin secretion.[34-36] However, in the present study, this explanation was not explored in detail, since plasma lipid levels were determined only after 6 weeks on the high-fat diet. Finally, a fourth explanation is that a high-fat diet induced impaired signaling mechanisms crucial to insulin secretion, in analogy with recent findings in the Zucker diabetic fatty rat.[37] However, the exact nature of the mechanism of the impaired glucose-stimulated insulin secretion in C57BL/6J mice on a high-fat diet remains to be clarified. It should be emphasized that we found the impaired insulin secretion under both in vitro and in vivo conditions, which indicates that long-term alterations of islet function rather than in vivo conditions determine the failing insulinotropic action of glucose.

As demonstrated by our immunocytochemical analysis of the islets, the pattern of islet innervation was not altered by a high-fat diet. We studied the innervation pattern of islets with regard to TH, NPY, and galanin, which are associated with sympathetic adrenergic nerves, with VIP, which is associated with parasympathetic cholinergic nerves, and with SP, which is associated with sensory nerves.[24,25] However, all these different fibers in the islets displayed a similar distribution pattern and relative frequency in mice given the high-fat and control diets. This suggests that changes in insulin secretion after treatment with the high-fat diet are not related to an altered pattern of innervation from a morphologic point of view, which does not exclude functional alterations in the neural regulation of islet hormone secretion.

In conclusion, we have demonstrated that treatment of the C57BL/6J strain of mice with a high-fat diet induces insulin resistance already after 1 week, as shown by marked hyperinsulinemia and slight hyperglycemia. Furthermore, after 12 weeks of treatment, glucose-induced insulin secretion is impaired both in vivo and in vitro, whereas carbachol-stimulated insulin secretion is potentiated. Moreover, the insulin gene is upregulated, but the islet size is not altered. Based on these results, we suggest that cholinergic supersensitivity and an increased B-cell turnover of insulin is a mechanism compensating for the peripheral insulin resistance with increased insulin secretion, whereas the failing glucose-stimulated insulin secretion represents an early decompensation in islet function.

ACKNOWLEDGMENT

The authors are grateful to Lilian Bengtsson, Lena Kvist, Ann-Christin Lindh, Doris Persson, and Iréne Reimertz for technical assistance.

REFERENCES

1. Beard JC, Halter JB, Best JD, et al: Dexamethasone-induced insulin resistance enhances B cell responsiveness to glucose level in normal men. Am J Physiol 247:E592-E596, 1984

2. Kahn SE, Beard JC, Schwartz MW, et al: Increased B-cell secretory capacity as mechanism for islet adaptation to nicotinic acid–induced insulin resistance. Diabetes 38:562-568, 1989

3. Bergman RN, Phillips LS, Cobelli C: Physiologic evaluation of factors controlling glucose tolerance in man. Measurement of insulin sensitivity and β-cell glucose sensitivity from the response to intravenous glucose. J Clin Invest 68:1456-1467, 1981

4. Porte D Jr: B cells in type 2 diabetes mellitus. Diabetes 40:166-180, 1991

5. Balkan B, Dunning BE: Muscarinic stimulation maintains in vivo insulin secretion in response to glucose after prolonged hyperglycaemia. Am J Physiol 268:R475-R479, 1995

6. Ahrén B, Lundquist I: Modulation of basal insulin secretion in the obese, hyperglycemic mouse. Metabolism 31:172-179, 1982

7. Ahrén B, Sundkvist G, Mulder H, et al: Blockade of muscarinic transmission increases the frequency of diabetes after low-dose alloxan challenge in the mouse. Diabetologia 39:383-390, 1996

8. Fukudo S, Virnelli S, Kuhn CM, et al: Muscarinic stimulation and antagonism and glucoregulation in nondiabetic and obese hyperglycemic mice. Diabetes 38:1433-1438, 1989

9. Coleman DL: Obese and diabetes: Two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia 14:141-148, 1978

10. Coleman DL, Hummel KP: The influence of genetic background on the expression of the obese (*ob*) gene in the mouse. Diabetologia 9:287-293, 1973

11. Surwit RS, Kuhn CM, Cochrane C, et al: Diet-induced type II diabetes in C57BL/6J mice. Diabetes 37:1163-1167, 1988

12. Surwit RS, Seldin MF, Kuhn CM, et al: Control of expression of insulin resistance and hyperglycemia by different genetic factors in diabetic C57BL/6J mice. Diabetes 40:82-87, 1991

13. Lee SK, Opara EC, Surwit RS, et al: Defective glucose-stimulated insulin release from perifused islets of C57BL/6J mice. Pancreas 11:206-211, 1995

14. Lacy PE, Kostianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 16:35-39, 1967

15. Herbert V, Lau KS, Gottlieb CW, et al: Coated charcoal immunoassay of insulin. J Clin Endocrinol Metab 25:1375-1384, 1965

16. Ahrén B, Lundquist I: Glucagon immunoreactivity in plasma from normal and dystrophic mice. Diabetologia 22:258-263, 1982

17. Bruss ML, Black AL: Enzymatic microdetermination of glycogen. Anal Biochem 84:309-312, 1978

18. Allain CC, Poon LS, Chan CSG, et al: Enzymatic determination of total serum cholesterol. Clin Chem 20:470-475, 1974

19. Fossati P, Prencipe L: Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem 28:2077-2080, 1982

20. Antonis A: Semiautomated method for the colorimetric determination of plasma free fatty acids. J Lipid Res 6:307-312, 1965

21. Coons AH, Leduc EM, Connolly JM: Studies on antibody production. I. A method for the histochemical determination of specific antibodies and its application to a study of the hyperimmune rat. J Exp Med 102:49-59, 1955

22. Mulder H, Lindh AC, Sundler F: Islet amyloid polypeptide gene expression in the endocrine pancreas of the rat: A combined in situ hybridization and immunocytochemical study. Cell Tissue Res 274:467-474, 1993

23. Mulder H, Ahrén B, Sundler F: Islet amyloid polypeptide (amylin) and insulin are differentially expressed in chronic diabetes induced by streptozotocin in rats. Diabetologia 39:649-657, 1996

24. Ahrén B, Taborsky GJ Jr, Porte D Jr: Neuropeptidergic versus cholinergic and adrenergic regulation of islet hormone secretion. Diabetologia 29:827-836, 1986

25. Karlsson S, Sundler F, Ahrén B: Neonatal capsaicin-treatment in mice: Effects on pancreatic peptidergic nerves and 2-deoxy-D-glucose–induced insulin and glucagon secretion. J Autonom Nerv Syst 39:51-60, 1992

26. Kaneto A, Kajinuma H, Kosaka K, et al: Stimulation of insulin secretion by parasympathomimetic agents. Endocrinology 83:651-658, 1968

27. Ahrén B, Lundquist I: Effects of autonomic blockade by methylatropine and optical isomers of propranolol on plasma insulin levels in the basal state and after stimulation. Acta Physiol Scand 112:57-63, 1981

28. Ahrén B, Taborsky GJ Jr: The mechanism of vagal nerve stimulation of glucagon and insulin secretion in the dog. Endocrinology 118:1551-1557, 1986

29. Ahrén B, Lundquist I: Interaction of vasoactive intestinal peptide (VIP) with cholinergic stimulation of glucagon secretion. Experientia 38:405-406, 1982

30. Weir GC, Leahy JL, Bonner-Weir S: Experimental reduction of β-cell mass: Implications for the pathogenesis of diabetes. Diabetes Metab Rev 2:125-161, 1986

31. Rosetti L, Giaccari A, DeFronzo RA: Glucose toxicity. Diabetes Care 13:610-630, 1990

32. Svensson C, Sandler S, Hellerström C: Lack of long-term beta-cell glucotoxicity in vitro in pancreatic islets isolated from two mouse strains (C57BL/6J; C57BL/KsJ) with different sensitivities of the beta-cells to hyperglycaemia in vivo. J Endocrinol 136:289-296, 1993

33. Sako Y, Grill VE: A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. Endocrinology 127:1580-1589, 1990

34. Capito K, Hansen SE, Hedeskov CJ, et al: Fat-induced changes in mouse pancreatic islet insulin secretion, insulin biosynthesis and glucose metabolism. Acta Diabetol 28:193-198, 1992

35. Zhou YP, Grill VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. J Clin Invest 93:870-876, 1994

36. Lee Y, Hirose H, Ohneda M, et al: Beta-cell lipotoxicity in the pathogenesis of non–insulin-dependent diabetes mellitus in obese rats: Impairment in adipocyte–beta-cell relationships. Proc Natl Acad Sci USA 91:10878-10882, 1994

37. Tokuyama Y, Sturis J, DePaoli AM, et al: Evolution of β-cell dysfunction in the male Zucker diabetic fatty rat. Diabetes 44:1447-1457, 1995