Differential Changes in Islet Amyloid Polypeptide (Amylin) and Insulin mRNA Expression After High-Fat Diet-Induced Insulin Resistance in C57BL/6J Mice

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Islet amyloid, derived from islet amyloid polypeptide (IAPP or amylin), frequently occurs in type 2 diabetes. Availability of this peptide for amyloid formation may be enhanced by increased islet expression of IAPP. In the insulin resistant state, euglycemia is maintained by hypersecretion of insulin. Whether IAPP expression, which is regulated by glucose, or its ratio to that of insulin is altered by the metabolic perturbations associated with insulin resistance is not known. Therefore, we studied islet expression of IAPP and insulin mRNA in insulin resistance-prone C57BL/6J mice. Thus, after a long-term (48 weeks) challenge with a high-fat diet (58% fat on a caloric base compared with 11% in the control diet) hyperglycemia, hyperinsulinemia, hyperlipidemia, and hyperleptinemia evolved in the mice. An intraperitoneal (IP) glucose tolerance test showed a marked impairment of glucose disposal. Also, plasma IAPP levels were elevated in high-fat fed mice (11.3 ± 1.2 *v* 7.1 ± 0.6 pmol/L, $P < .001$). Quantitative in situ hybridization showed increased β-cell mass in high-fat fed mice, as evidenced by approximately 50% increase in area labeled for islet IAPP and insulin mRNA. IAPP mRNA expression per islet cell remained unchanged in both groups. In contrast, insulin mRNA expression per cell was significantly decreased in the high-fat fed mice ($P < .001$). We therefore conclude that glucose intolerance after long-term high-fat feeding in C57BL/6J mice is accompanied by reduced cellular expression of insulin, but not of IAPP. The increased ratio of IAPP versus insulin expression might underlie the amyloidogenicity of high-fat diet in species carrying an amyloidogenic form of IAPP.

OBESITY IS ASSOCIATED with insulin resistance, which elicits a compensatory increase in insulin secretion to preserve normoglycemia. If islet dysfunction exists, however, the islet adaptation is inadequate and glucose intolerance and type 2 diabetes may develop.[1-3] Many factors may be involved in the adaptation and, possibly, ultimately the impairment of islet function in obesity. One important mechanism may be elevated levels of circulating free fatty acids, which increase the β-cell content of triglycerides, reduce insulin biosynthesis and secretion, and induce apoptosis.[4-6] Another potentially important factor is the β-cell hormone islet amyloid polypeptide (IAPP; also known as amylin),[7] which forms amyloid deposits in islets of type 2 diabetic patients. This presumably plays a role in the development of the disease,[7-9] because IAPP aggregates, and fibrils have been found to be cytotoxic.[10-12]

The role of IAPP-derived islet amyloid and hyperlipidemia in the pathogenesis of diabetes has recently been addressed experimentally in transgenic mice expressing the human amyloid-forming IAPP-species; it was found that amyloid forms when the mice are either challenged with a high-fat diet[13] or are inbred onto an obese genetic background.[14,15] Although the mechanism of islet amyloidosis in these models remains unresolved, a hypothetical model is that high-fat diet elevates circulating levels of free fatty acids. These may increase IAPP expression resulting in enhanced amyloid formation and secretion of IAPP. In addition, IAPP overexpression may be an intraislet inhibitor of insulin secretion.[16,17] Whether IAPP expression is actually increased by high-fat diet is, however, not known. Therefore, we have examined whether long-term (48 weeks) feeding of a high-fat diet to mice of the insulin resistant-prone C57BL/6J strain alters the expression and circulating levels of IAPP; these alterations were compared with concomitant changes in expression and circulating levels of insulin.

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MATERIALS AND METHODS

Animals

Female mice of the C57BL/6J strain were obtained from Bomholtgaard Breeding and Research Centre (Ry, Denmark) at 4 weeks of age. Half of the mice received a high-fat diet, whereas the other half of the animals received an ordinary rodent chow diet (Research Diets, New Brunswick, NJ). On a caloric base, the high-fat diet consisted of 16.4% protein, 25.6% carbohydrates, and 58.0% fat from lard (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, the mice had free access to food and water. Four to 5 mice were kept per cage in a temperature-controlled (22 ± 1°C) room with a 12-hour light-dark cycle with light on at 6:00 AM. The study was approved by the Animal Ethics Committee at Lund University.

Blood Sampling

After 48 weeks on high-fat or control diets, body weight was determined, and a blood sample was taken from the nonfasted animal. The samples were taken from the intraorbital, retrobulbar plexus in heparinized tubes for the measurement of plasma levels of glucose, insulin, leptin, cholesterol, and triglycerides or in tubes to which EDTA had been added for the measurements of IAPP and free fatty acids. After centrifugation, plasma was stored at −20°C until assayed.

Analyses

Plasma levels of IAPP were determined with an immunoradiometric technique in unextracted plasma.[18] Plasma insulin was determined radioimmunochemically with the use of a guinea pig anti-rat insulin antibody, ^{125}I-labeled human insulin as tracer and rat insulin as standard

(Linco Research, St Charles, MO). Free and bound radioactivity were separated by use of an anti-immunoglobulin G (IgG) (goat anti-guinea pig) antibody (Linco). Plasma leptin levels were determined with a newly developed radioimmunoassay specific for mouse leptin.[19] The method uses a polyclonal rabbit antibody raised against highly purified recombinant mouse leptin, ^{125}I-labeled tracer prepared with recombinant mouse leptin, and mouse leptin as standard. Anti-rabbit IgG was used for separation of bound and free leptin. Plasma glucagon levels were analyzed with a radioimmunoassay on unextracted plasma[20] with the use of guinea pig antiglucagon antibody specific for pancreatic glucagon, ^{125}I-labeled glucagon as tracer and glucagon standard (Linco). Plasma levels of triglycerides and free fatty acids were determined colorimetrically, plasma cholesterol was determined enzymatically, and glucose was determined with the glucose oxidase method. For the various parameters, means ± SEM are shown. The mean ratio of circulating IAPP versus circulating insulin was calculated as the mean ± SEM of the ratios in each individual mouse. Statistical comparisons for the differences between high-fat and control diet-treated mice were performed by the use of Student's unpaired 2-tailed *t* test.

Glucose Tolerance Test

After 48 weeks on the respective diets, mice were anesthetized with an intraperitoneal (IP) injection of midazolam (Dormicum; Hoffman-La-Roche, Basel, Switzerland, 0.4 mg/mouse) and a combination of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm; Janssen, Beerse, Belgium). Thereafter, an orbital blood sample was taken, and D-glucose (Fluka Chemie AG, Buchs, Switzerland) was injected IP at the dose level of 1 g/kg. The volume load was 10 μL/g body weight. Retro-orbital blood samples were taken at 10, 30, 60, and 120 minutes. After centrifugation, plasma was stored at −20°C until assayed for its concentration of glucose and in samples from time 0, 10, and 120 minutes of insulin. The areas under the 120-minute glucose and insulin curves ($AUC_{glucose}$ and $AUC_{insulin}$, respectively) were determined by the trapezoid rule.

Quantitative In Situ Hybridization

After 48 weeks on the respective diets, the relative changes in IAPP and insulin mRNA levels were assessed by quantitative in situ hybridization as previously described in detail.[21] In brief, 2 to 3 paraffin sections from different depths of the pancreatic specimens were hybridized with ^{35}S-labeled IAPP and insulin oligodeoxynucleotide probes under identical conditions. The probes for IAPP and insulin mRNA have been described in detail.[22] Using computerized image analysis (Leica Q500MC 1.1; Leica, Cambridge, UK), levels of IAPP and insulin mRNA in islets were determined by measurement of the optical density (OD) in the dark field of the autoradiographic labeling by the respective probes. mRNA expression was given either as the mean OD of labeling, reflecting mRNA levels in individual cells, or integrated OD, reflecting the total abundance of mRNA in a labeled islet. To evaluate changes in β-cell mass, the total area of insulin mRNA-labeled cells in randomly chosen islets, in different parts of 2 sections from each animal cut at different depths, was determined using computerized image analysis. Each islet was considered as 1 observation. Statistical comparisons were made using an unpaired 2-tailed *t* test; a probability level of $P < .05$ was considered statistically significant.

RESULTS

Body Weight and Baseline Plasma Levels of IAPP, Insulin, Glucose, Glucagon, Leptin, Triglycerides, Cholesterol and Free Fatty Acids

Table 1 shows that 48 weeks of challenging the mice with a high-fat diet markedly increased the body weight ($P < .001$), plasma levels of insulin (by 3.3-fold, $P < .001$), and plasma levels of IAPP (by ≈60%, $P < .001$). The circulating ratio of IAPP versus insulin was 0.11 ± 0.02 in mice given the control diet versus only 0.06 ± 0.01 in mice given the high-fat diet ($P < .001$). Furthermore, the 48-week challenge with the high-fat diet increased plasma levels of glucose, triglycerides, cholesterol, free fatty acids, glucagon, and leptin ($P = .002$ or less).

Table 1. Baseline (nonfasted) Plasma Levels of IAPP, Insulin, Glucose, Cholesterol, Triglycerides, Free Fatty Acids, Glucagon, and Leptin and $AUC_{glucose}$ and $AUC_{insulin}$ During 120 Minutes After IP Administration of Glucose (1 g/kg) in C57BL/6J Mice Given a High-Fat or a Control Diet for 48 Weeks

Parameter	Control Diet	*P* Value	High-Fat Diet
Body weight (g)	24.8 ± 0.3 (36)	<.001	32.7 ± 1.1 (30)
IAPP (pmol/L)	7.1 ± 0.6 (36)	<.001	11.3 ± 1.2 (30)
Insulin (pmol/L)	140 ± 19 (36)	<.001	473 ± 106 (30)
Glucose (mmol/L)	6.9 ± 0.3 (36)	<.001	7.9 ± 0.3 (30)
Leptin (ng/mL)	7.4 ± 0.6 (12)	<.001	30.8 ± 5.1 (11)
Glucagon (pg/mL)	82 ± 5 (9)	<.001	155 ± 8 (12)
Cholesterol (mmol/L)	0.93 ± 0.05 (12)	<.001	1.38 ± 0.09 (12)
Triglycerides (mmol/L)	1.63 ± 0.09 (12)	<.001	3.03 ± 0.14 (12)
Free fatty acids (mEq/L)	0.63 ± 0.03 (12)	.002	0.94 ± 0.08 (12)
$AUC_{glucose}$ (mmol/L × 120 min)	854 ± 56 (12)	<.001	1526 ± 145 (12)
$AUC_{insulin}$ (nmol/L × 120 min)	31.2 ± 2.6	<.001	61.4 ± 5.6

NOTE. Mean ± SEM are shown. *P* indicates the probability level of random difference between the groups. Number within parenthesis is the number of animals.

Glucose Tolerance

After 48 weeks of the high-fat diet in the C57BL/6J mice, a severe glucose intolerance had evolved, because plasma levels of glucose were higher than in control diet-fed mice at 30, 60, and 120 minutes after the IP glucose administration ($P < .001$). This is also evident by the marked increase in $AUC_{glucose}$ in high-fat–fed mice (Table 1). Similarly, the reduction in plasma glucose from minute 10 to minute 30 was 55% ± 6% in control diet-fed animals versus only 34% ± 4% in high-fat diet-fed animals ($P < .001$). Plasma insulin levels were higher in high-fat–fed animals both at baseline and at 10 and 120 minutes after the glucose administration ($P < .001$), and, similarly, $AUC_{insulin}$ was elevated in high-fat–fed mice (Table 1).

Expression of IAPP and Insulin mRNA

As shown in Table 2 and illustrated in Fig 1, 48 weeks of a high-fat diet increased the β-cell mass, evidenced by approximately 50% increase in area labeled for insulin mRNA ($P = .025$); a similar increase was observed for IAPP mRNA ($P = .017$), which in mice is predominantly expressed in β cells, but also in the smaller population of somatostatin cells.[22] IAPP expression per cell (mean OD of in situ hybridization labeling) was similar in both groups of mice. In contrast, insulin mRNA expression per cell was significantly decreased in the high-fat diet-fed mice ($P < .001$). However, due to the increase

Table 2. Data From Morphometrical and Quantitative In Situ Hybridization Analyses

	Islet Area	Integrated OD	Mean OD
IAPP			
Control diet	11,573 ± 1,140	37,940 ± 5,240	2.23 ± 0.13
High-fat diet	17,007 ± 2,016	57,177 ± 10,600	1.98 ± 0.13
P	.017	.097	.17
Insulin			
Control diet	10,113 ± 850	32,558 ± 2,920	2.33 ± 0.12
High-fat diet	15,465 ± 2,500	29,632 ± 4,210	1.49 ± 0.13
P	.025	.66	<.001

NOTE. Islet areas are given as μm², whereas integrated OD and mean OD are arbitrary values. Mean ± SEM are shown. *P* indicates the probability level of random difference between the groups. For IAPP, the number of islets analyzed in mice fed a control diet was 68 *v* 63 in high-fat diet-fed mice; for insulin, these numbers were 61 *v* 44.

in β-cell mass, the total level of insulin mRNA was similar in both groups of mice ($P = .66$). Accordingly, total levels of IAPP mRNA increased by approximately 50%, although this did not reach statistical significance ($P = .097$). Thus, concomitantly with increased islet mass, long-term high-fat diet and hyperlipidemia differentially affected IAPP and insulin mRNA expression, in that the cellular expression of insulin, but not that of IAPP, was reduced.

DISCUSSION

Previous studies have shown impacted glucose metabolism, impaired islet function, increased circulating levels of lipids and hyperleptinemia after challenging C57BL/6J mice with a high-fat diet.[19,23-30] The present study shows that these perturbations are accompanied by reduction of insulin expression, but not of IAPP expression. This shows that a relative overexpression of IAPP versus insulin exists in the islets after a high-fat diet. Such imbalance in expression of IAPP versus insulin may be involved in the development of glucose intolerance.

The high-fat diet in the C57BL/6J mice resulted in insulin resistance, indicated by basal hyperinsulinemia, in combination with impaired glucose disposal during glucose challenge, despite exaggerated insulin release. Notwithstanding this protracted burden of insulin resistance and hyperlipidemia on the islets, the mice did not develop overt diabetic baseline hyperglycemia; rather, the glucose level was only moderately elevated (≈1 mmol/L). The results, however, are nevertheless suggestive of islet dysfunction, because the insulin response, although greater than in controls, was not sufficient to prevent the impaired glucose disposal. We previously encountered a similar finding in the same strain of mice at 12 and 24 weeks and 1.5 years of an identical diet.[19,27,29,30] The stability and reproducibility of these changes make this model useful for studies on

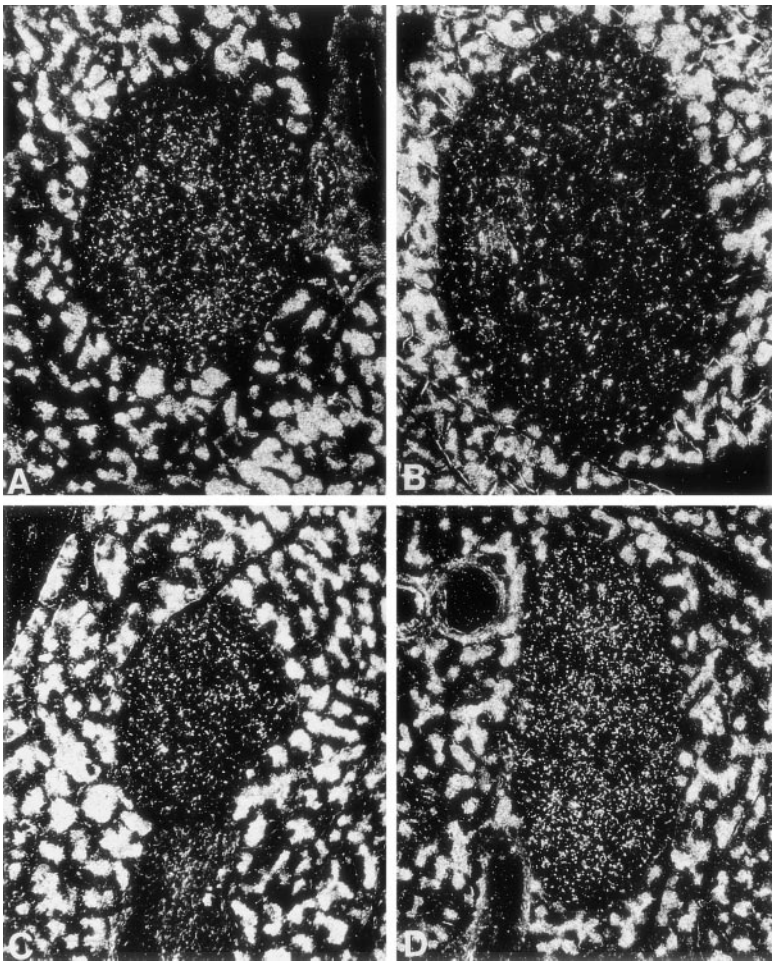

Fig 1. In situ hybridization with radiolabeled insulin (A, B) and IAPP (C, D) probes in islets from C57BL/6J mice given a control diet (A, C) or a high-fat diet (B, D) for 48 weeks. The high-fat diet had increased the size of the islets concomitantly with reduced cellular expression of insulin mRNA without affecting the cellular expression of IAPP mRNA. (Original magnification × 250.)

pathogenetic mechanisms for development of glucose intolerance and type 2 diabetes.

Here, we show an increase in β-cell mass in the high-fat diet-fed mice, but not in the controls, suggesting that it evolves as an adaptation to hyperlipidemia and insulin resistance. Similar observations have been made in Zucker diabetic fatty rats,[31] as well as in the same genetic background in mice that we have used here, but carrying an additional mutation in the leptin gene; these ob/ob mice display an extreme hypertrophy of islets.[32] We also found that IAPP mRNA expression was unaffected at the cellular level, whereas that of insulin mRNA had decreased. At 12 weeks of the same diet,[27] we have previously found an upregulation of cellular insulin mRNA expression without any signs of islet cell hypertrophy. Conceivably, at this early time point, the long-term impact of a high-fat diet has not yet structurally impacted islets, whereas at 48 weeks of a high-fat diet, islets are clearly subject to (lipo)toxicity, manifested as decreased insulin mRNA expression. Interestingly, we have found a similar regulation of insulin mRNA expression in a model of diet-induced obesity in rats[33]; here, insulin mRNA expression was decreased already at 2 weeks of a high-fat diet, while changes in β-cell mass were lacking. The mechanism underlying the dissociated action of a high-fat diet on IAPP versus insulin mRNA expression remains to be established. Involvement of lipids is, however, plausible, because the mice experienced long-term hyperlipidemia, and lipids may exert toxic effects in the islets.[4-6]

The relative increase in IAPP expression versus insulin is a key event in amyloid formation, because its rate is increased when IAPP production and release are increased relative to insulin, such as under hyperglycemic conditions in vitro.[34] Also in vivo, amyloid formation and diabetes occur when the demands on β cell function increase, such as when hIAPP transgenic mice are fed either a high-fat diet[13] or when they have been inbred onto an obese genetic background.[14,15] Under these conditions, the release of IAPP is increased, but the cellular IAPP expression has not been examined.

An important point in relation to amyloid function is that it has been shown that insulin can inhibit IAPP fibrillogenesis in vitro.[35,36] This implies that an increased ratio of IAPP to insulin may facilitate amyloid formation. Therefore, it was interesting to note in our present study that this ratio was increased. Similar alterations occur also in other experimental models for the pathogenesis of type 2 diabetes mellitus, involving insulin resistance[22] and β-cell stress.[37-39] This would suggest that a high-fat diet may increase the likelihood of islet amyloid formation during development of glucose intolerance and diabetes. In the C57BL/6J mice fed a high-fat diet, however, formation of amyloid is not possible. The mouse species of IAPP is not amyloidogenic, in contrast to IAPP species from human, monkey, and cat due to amino acid sequence variations in a critical amyloidogenic motif in the 24-28 position.[40] Nevertheless, the high-fat diet-challenged C57BL/6J mice may be better suited for studies on the expression of islet IAPP, because a potential influence of amyloid fibrils on islet function is avoided.

Plasma levels of IAPP increased by approximately 50% in the C57BL/6J mice fed a high-fat diet. Consequently, when applying a metabolic perspective, the restraining effects of IAPP on insulin release suggest that the increased ratio of IAPP to insulin mRNA in the high-fat diet-fed mice may pave the way for enhanced IAPP actions in islets. This could further contribute to the metabolic perturbations in hyperlipidemic mice. However, although plasma IAPP was elevated by approximately 50%, this degree of elevation was lower than that of circulating insulin. Therefore, the increased ratio of IAPP to insulin expression in response to the high-fat diet was not reflected by circulating hormone levels. This may be explained in several ways. First, the circulating hormone levels reflect more the islet β-cell secretion, rather than hormone expression in a long-standing condition, such as the high-fat challenge, a process which may be regulated differently than that of secretion. Thus, the long-standing insulin resistance might have induced a continuous stimulation of insulin secretion for adaptation to the resistance. This stimulus, the exact nature of which is yet to established, might be preferential for insulin over IAPP secretion. That IAPP expression is regulated differentially to that of insulin, eg, under conditions of β-cell stress, has been shown previously.[22,37-39] Second, the increased ratio of IAPP to insulin expression in islets may not translate into circulating levels because clearance of the hormones from the circulation is different; insulin is predominantly extracted from the circulation by the liver, whereas IAPP is renally excreted.[41,42] A previous study showed that high-fat fed mice of the NMRI (Naval Medicine Research Institute) strain displayed marked elevation of plasma IAPP without any clear hyperinsulinemia.[43] Other than differences in exact composition of diet and assays as potential explanations for the discrepant result in this study, an important difference is that the NMRI strain is more resistant to the glucose intolerant action of a high-fat diet than the C57BL/6J strain.[30] Whether the relative difference in circulating IAPP versus insulin in these 2 strains of mice after a high-fat diet is of relevance for their differential sensitivity to develop glucose intolerance is an intriguing question, which remains to be studied. On the other hand, in ob/ob mice, a similar change in the ratio of circulating IAPP versus insulin occurs.[44]

In conclusion, this study has shown that a high-fat diet challenge over 48 weeks to glucose intolerance-prone C57BL/6J mice is accompanied by a 50% increase in plasma levels of IAPP concomitantly with marked hyperinsulinemia, marked hyperlipidemia, and marked hyperleptinemia. At the islet level, increased β-cell mass is paralleled by reduced cellular insulin mRNA expression, whereas IAPP mRNA expression, per cell, is unaltered. Therefore, high-fat diet-induced glucose intolerance is accompanied by increased islet IAPP expression and an increased ratio of IAPP to insulin mRNA. These events in islets after a high-fat diet may be of importance for the development of islet dysfunction and may, in species carrying an amyloidogenic IAPP, be involved in the development of islet amyloidosis, thus further deteriorating islet function.

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