In Vivo Glucose-Stimulated Amylin Secretion Is Increased in Nondiabetic Patients With Pancreatic Cancer

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The incidence of diabetes is increased in patients with pancreatic cancer, but the mechanisms underlying this association are not clear. Alterations in β-cell function, such as formation of amyloid from excessive production of amylin and reduced expression of GLUT2, have been suggested to be possible mechanisms. We compared in vivo secretory responses of amylin and insulin (n = 37) and expression of GLUT2 in pancreata (n = 10) obtained at surgery between diabetic and nondiabetic patients with and without pancreatic tumors. Fourteen had pancreatic adenocarcinoma, 7 had diabetes (duration 6 ± 3 years) and a pancreatic tumor, 8 had type 2 diabetes (duration 6 ± 2 years), and 8 were normal subjects. First (0 to 10 minutes) and second (10 to 120 minutes) phase insulin and amylin secretion were characterized using the hyperglycemic clamp technique. Both amylin and insulin concentrations followed a biphasic pattern in nondiabetic subjects. In nondiabetic patients with pancreatic cancer, total, as well as nonglycosylated amylin concentrations, were increased compared with nondiabetic subjects without pancreatic cancer. Both first- and second-phase plasma amylin and serum immunoreactive insulin concentrations were low in all patients with diabetes, ie, both in type 2 diabetes and in those patients with diabetes and pancreatic tumors. At surgery, specimens were obtained for characterization of GLUT2 expression in β cells, which was unaltered in nondiabetic (n = 7) and diabetic (n = 3) patients. Amyloid staining was similarly negative in diabetic and nondiabetic pancreata independent of pancreatic carcinoma. In conclusion, plasma amylin, but not insulin concentrations, are increased in nondiabetic patients with pancreatic cancer, but low in all patients with diabetes. These data support the potential of using an increase in the ratio of circulating amylin to insulin as a marker for pancreatic cancer in nondiabetic patients.

THE INCIDENCE OF DIABETES is slightly increased in patients with pancreatic cancer,[1-5] and hyperglycemia may occasionally be its first sign.[6,7] The mechanisms underlying this association are not clear. Diabetes does not seem to be explained by destruction of the islet β cells by the tumor mass.[8-11] Alterations in β-cell function and synthesis,[12,13] insulin antibodies,[14] and release of diabetogenic mediators by tumor cells[11,15,16] have been suggested to contribute to hyperglycemia in these patients. On the other hand, according to some studies, β-cell function is normal,[9] and the hyperglycemia has even been exclusively attributed to peripheral insulin resistance.[7,17-20]

Amylin is a 37-amino acid polypeptide produced and secreted from pancreatic β cells.[21] High local amylin concentration may be toxic to β cells,[22,23] and amyloid fibrils accumulate in pancreata of patients with type 2 diabetes.[24-29] Circulating concentrations of amylin are, however, lower in patients with type 2 diabetes than in nondiabetic subjects.[30,31] In contrast, fasting plasma amylin concentrations have been reported to be increased in patients with pancreatic cancer and diabetes,[32,33] but normal[32] or slightly increased[33] in nondiabetic patients with pancreatic cancer. During hyperglycemia, increases in plasma amylin concentrations have been seen only in nondiabetic and postoperative pancreatic cancer patients.[33] However, no characterization of first- and second-phase total and nonglycosylated amylin secretory responses have been performed hitherto.

A low-affinity glucose transporter, GLUT2, is present in the plasma membrane of pancreatic β cells.[34] Reduced expression of the glucose transporter, GLUT2, in β cells is closely coupled to reduced insulin secretion in several diabetic animal models.[34-37] Mice lacking GLUT2 develop early diabetes and abnormal islets.[38] Mice overexpressing GLUT2 antisense RNA in β cells have impaired insulin secretion and are hyperglycemic.[37] A reduction in GLUT2 could therefore contribute to defective insulin secretion in pancreatic cancer. GLUT2 expression has not been examined in pancreata from patients with hyperglycemia secondary to pancreatic disease.

The present study was designed to compare first- and second-phase insulin and amylin secretion between diabetic and nondiabetic patients with pancreatic cancer and in subjects with normal and impaired glucose tolerance without cancer. For this purpose, a hyperglycemic clamp was performed in all subjects. At surgery, pancreatic tissue was obtained from nondiabetic and diabetic patients with pancreatic tumors and those subjects with normal pancreas to characterize the intensity of insulin, glucagon, and GLUT2 immunohistologic staining and amyloid staining by Congo red.

MATERIALS AND METHODS

Subjects and Study Design

A total of 37 hyperglycemic clamp studies were performed. Twenty-one of these subjects were referred to the Department of Surgery at Helsinki University Hospital because of a pancreatic disease. Fourteen of these patients had pancreatic adenocarcinoma (CA), and 7 patients had diabetes and a pancreatic tumor (DM+P) (Table 1). The diagnosis was verified by histology or cytology, but no glucose tolerance test was performed. In addition, 8 patients with type 2 diabetes and 8 normal subjects with normal glucose tolerance (NGT), according to an oral glucose tolerance test,[39] were studied. Clinical characteristics of the subjects are given in Table 2.

All subjects participated in a hyperglycemic clamp study prior to

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Table 1. Diagnoses of Patients With Diabetes and a Pancreatic Tumor and Duration of Diabetes at the Time of Diagnosis of the Pancreatic Tumor

Patient No.	Diagnosis	Duration of Diabetes (yr)
1	Carcinoma adenomatosum	2
2	Carcinoma adenomatosum	5
3	Carcinoma adenomatosum	20
4	Carcinoma male differentatum	12
5	Cystadenoma mucinosum	2
6	Cystadenoma mucinosum	2
7	Cystadenoma microcystica	2

surgery to characterize first- and second-phase serum insulin secretion.[40] This technique was also used to examine whether a first- and a second-phase secretory pattern characterizes plasma total amylin and nonglycosylated amylin concentrations in humans. Immunohistologic staining of GLUT2, insulin, and glucagon and Congo red staining for amyloid deposits were performed in pancreatic β cells from pancreatic tissue samples obtained from 3 patients with pancreatic CA, 3 patients with diabetes and pancreatic tumor, and 4 patients with histologically normal pancreatic tissue (Table 3). The nature, risks, and potential benefits of the study were explained to all subjects prior to obtaining their written informed consent to participate in the study. The experimental protocol was designed and performed according to the principles of the Helsinki Declaration and was approved by the Ethical Committee of the Helsinki University Central Hospital.

Hyperglycemic Glucose Clamp

The hyperglycemic clamp technique was used for quantitation of first- and second-phase insulin responses.[40] The study was started after a 12-hour overnight fast. A dorsal hand vein was cannulated retrogradely with an 18-gauge catheter (Venflon;, Viggo-Spectramed, Helsingborg, Sweden), and the hand was kept in a thermoregulated box at 60°C for arterialization of venous blood.[41] An ipsilateral antecubital vein was cannulated with an 18-gauge catheter for infusion of glucose. The arterialized venous plasma glucose concentration was raised to 5.6 mmol/L (100 mg/dL) over the measured basal glucose concentration and maintained at this level for 2 hours.[40] This was accomplished by infusing a 2-minute intravenous priming dose of a 50% glucose solution.[42] Thereafter, a 20% glucose solution was infused intravenously at a rate needed to maintain the plasma glucose concentration at the desired plateau and adjusted based on plasma glucose measurements, which were performed at 5-minute intervals throughout the study. The type 2 diabetic patients received an intravenous infusion of insulin for normalization of plasma glucose concentrations prior to the study as previously described.[42] The insulin infusion was discontinued for 60 minutes prior to start of the hyperglycemic clamp to avoid any suppressive effects of exogenous insulin on insulin or amylin secretion. Samples for measurements of serum insulin and plasma amylin concentrations were taken at 0, 2.5, 5.0, 7.5, 10, 20, 30, 60, 70, and 120 minutes, and in addition, at 15, 40, 50, 80, 90, and 100 minutes for insulin. The areas under the curve for the concentrations of insulin and amylin during 0 to 10 minutes and 10 to 120 minutes were defined as first- and second-phase insulin and amylin secretion.

Analytical Procedures

Plasma samples for amylin measurement were collected in EDTA tubes and analyzed using 2 separate immunoassays that use monoclonal antibodies (Amylin Pharmaceuticals, San Diego, CA) as recently described in detail.[43,44] The cross-reactivity of these antibodies with calcitonin gene-related peptides I and II, calcitonin, and insulin were less than 0.01%. The total amylin assay measures both nonglycosylated and glycosylated forms of amylin.[45] These glycosylated forms have O-linked oligosaccharides attached at threonine residues near the N-terminus.[45] This type of glycosylation is enzymatic and occurs during biosynthesis of amylin. The other amylin assay measures specifically nonglycosylated amylin. The minimum detective concentration is 0.5 pmol/L for both amylin assays, and the intra-assay and interassay coefficients of variation less than 10% and less than 15%. Serum-free insulin immunoactivity was determined by double antibody radioimmunoassay (Pharmacia Insulin RIA kit; Pharmacia, Uppsala, Sweden) after precipitation with polyethylene glycol.[46] The cross-reactivity of insulin antibody is, by weight, 41% with proinsulin, less than 0.1% for insulin-like growth factors 1 and 2, and less than 0.1% for C-peptide.

Table 2. Characteristics of the Study Groups

	NGT (n = 8)	CA (n = 14)	DM + P (n = 7)	Type 2 DM (n = 8)
Females/males	4/4	11/3	1/6	3/5
Age (yr)	54 ± 4	60 ± 2	62 ± 5	55 ± 4
Body mass index (kg/m^2)	26.7 ± 0.7	23.6 ± 1.1*	24.3 ± 1.3	27.9 ± 1.1
Fasting plasma glucose (mmol/L)	5.4 ± 0.1	5.9 ± 0.2	9.8 ± 1.0†	9.1 ± 0.5†
Fasting serum insulin (pmol/L)	37 ± 4	45 ± 4	52 ± 12	71 ± 9‡
Fasting serum C-peptide (nmol/L)[a]	0.70 ± 0.16	0.68 ± 0.09	0.44 ± 0.12§	0.82 ± 0.08
Fasting plasma total amylin (pmol/L)	3.9 ± 0.9	7.5 ± 1.8‖¶	2.3 ± 0.6	3.2 ± 0.6
HbA_{1c} (%)[b]	4.9 ± 0.2	5.4 ± 0.1	10.3 ± 0.8†	7.4 ± 0.3†
Serum creatinine (μmol/L)[c]	92 ± 15	67 ± 3	82 ± 7	87 ± 3
Serum cortisol (nmol/L)[d]	411 ± 70	518 ± 48	566 ± 87	447 ± 49
Duration of diabetes (yr)			6 ± 3	6 ± 2

NOTE. Values are mean ± SE.

Abbreviations: NGT, subjects with normal glucose tolerance; CA, pancreatic cancer; DM + P, diabetes and pancreatic tumor; type 2 DM, type 2 diabetes.

*$P < .05$ for CA *v* type 2 DM.

†$P < .001$ for DM + P and type 2 DM *v* other groups.

‡$P < .05$ for type 2 DM *v* NGT.

§$P < .05$ for DM + P *v* NGT and type 2 DM.

‖$P < .01$ for CA *v* DM + P.

¶$P < .05$ for CA *v* type 2 DM.

[a]Reference range for normal subjects 0.33 to 0.67 nmol/L, [b] 4.0 to 6.0%, [c] <115 μmol/L, [d] 150 to 650 nmol/L.

For free insulin immunoreactivity, the minimum detectable concentration is 2.5 mU/L, and the intra-assay and interassay coefficients of variation less than 5.3% and 7.6%. Plasma glucose was measured in duplicate using the glucose oxidase method (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Hemoglobin A_{1c} (HbA_{1c}) was measured by high-pressure liquid chromatography using the fully automated Glycosylated Hemoglobin Analyzer System (Bio-Rad, Richmond, CA). Fasting serum C-peptide concentrations were determined by radioimmunoassay.[47] Serum creatinine concentrations were assessed using kinetic Jaffe methods (Böhring-Mannheim, Mannheim, Germany). Serum cortisol concentrations were determined by an enzyme immunologic method (Bayer, München, Germany).

Histological Staining

Pancreatic tissue samples were fixed in formaldehyde, embedded in paraffin, and cut into 5-μm thick sections for staining. Prior immunostaining endogenous peroxidase was blocked with 0.5% H_2O_2 in methanol followed by phosphate-buffered saline (PBS) washes. Nonspecific binding was blocked with normal goat serum. The sections were incubated with the polyclonal GLUT-2 antiserum (1:1,000 dilution, Chemicon, Temecula, CA) overnight at room temperature followed by PBS washes. Sections were then incubated with biotinylated goat antirabbit immunoglobulin G (IgG) antibody for 30 minutes at room temperature followed by PBS washes and exposed to avidin-biotin-peroxidase complex for 30 minutes at room temperature. Goat sera, biotinylated antibodies, and avidin-biotin-peroxidase complexes were purchased from Vector Laboratories (Burlingame, CA). The binding site was visualized with 3-amino-9-ethyl carbazole (AEC) (Sigma, St Louis, MO) for 15 minutes at room temperature, then counterstained with Mayer's hematoxylin solution. The sections were also similarly incubated and immunostained with the polyclonal insulin (1:800) and glucagon (1:3,000) antiserums (Dako, Carpinteria, CA) to localize Langerhans' islets in pancreatic tissue. Amyloid deposits in Langerhans' islets were observed by polarization microscopy after Congo red staining.[48] Sections were observed on an Olympus BH-2 microscope (Olympus, Tokyo, Japan) by a pathologist in a blinded fashion.

Statistical Analyses

Data between the study groups were analyzed using analysis of variance followed by pairwise comparison using Fischer's least-significant-difference test. All calculations were made using the SYSTAT statistical package (SYSTAT, Evanston, IL). Areas under curves were calculated using GraphPad Prism, version 2.01 (GraphPad Software, San Diego, CA). All data are expressed as mean ± SEM.

RESULTS

Serum-Free Immunoreactive Insulin and Plasma Total Amylin Concentrations

Serum-free immunoreactive insulin and plasma amylin concentrations were both characterized by 2 distinct phases in all nondiabetic subjects. The profiles of the 2 phases were different. In individuals with NGT, the maximal first-phase serum-free immunoreactive insulin concentration was approximately twice as high as the highest concentration during the second phase (Fig 1, upper panel on the left), while plasma total amylin concentrations were higher during the second phase compared with the first phase (Fig 1, lower panel on the left). In patients with diabetes with or without pancreatic tumor, no first-phase peak could be discerned for either free immunoreactive insulin or total amylin concentrations, and second-phase secretory responses were also markedly lower than in the other groups (Fig 1).

In nondiabetic patients with pancreatic cancer, the maximal first-phase serum-free immunoreactive insulin concentration was approximately 50% reduced compared with subjects with NGT, while first-phase amylin concentrations were similar in both nondiabetic groups (Fig 1). Second-phase insulin concentrations were comparable between subjects with NGT and nondiabetic patients with pancreatic cancer (Fig 1). In contrast, second-phase amylin concentrations were significantly higher in the nondiabetic patients with pancreatic cancer than in the subjects with NGT (Fig 1). HbA_{1c} was inversely correlated with the total ($r = -.67$, $P < .01$), first phase ($r = -.77$, $P < .001$), and second phase ($r = -.53$, $P < .05$) area under the plasma amylin concentration curve in nondiabetic patients with pancreatic cancer.

Plasma Nonglycosylated Amylin Concentrations

Figure 2 shows plasma nonglycosylated amylin concentrations in the different groups. The profile of nonglycosylated amylin was comparable to that of total amylin (Figs 1 and 2). As with total amylin, a first-phase peak characterized glucose-induced secretion of nonglycosylated amylin in all nondiabetic groups, but in neither of the diabetic groups (Fig 2). A second-phase peak was highest in the nondiabetic patients with pancreatic cancer (Fig 2) similar to total amylin. During the 120-minute hyperglycemic clamp, plasma nonglycosylated amylin concentrations averaged 57% ± 9%, 59% ± 10%, 52% ± 10%, and 87% ± 30% (nonsignificant [NS] between groups) of total amylin in individuals with NGT, pancreatic carcinoma, type 2 diabetes, and diabetic patients with pancreatic tumor (Fig 2).

Histochemistry

Positive GLUT2 immunoreactivity was observed in all preserved islets of Langerhans and was colocalized to insulin-positive β cells. Alpha-cell areas of the islets were glucagon-positive. There were no differences in the intensity of GLUT2, insulin, or glucagon staining between the study groups (Table 3). Amyloid staining was not observed in any of the pancreata (Table 3).

DISCUSSION

The present study was undertaken to characterize in detail plasma amylin secretion in nondiabetic and diabetic patients with pancreatic cancer and to test the hypothesis that chronic hyperglycemia is associated with loss of GLUT2 expression in the pancreas. We found plasma amylin concentrations to be very low both in patients with common type 2 diabetes and in those with diabetes and pancreatic tumors. Plasma amylin concentrations were, however, increased in nondiabetic patients with pancreatic cancer, although the insulin concentrations of these patients were similar to those in normal subjects (Fig 1). We also found, in contrast to rat islets in which GLUT2 is abundantly expressed,[49] expression of GLUT2 to be low in the human pancreas and similar between diabetic and nondiabetic subjects.

The present data are the first to characterize first- and second-phase amylin secretion in any group of human subjects. It has been suggested that insulin and amylin are cosecreted from

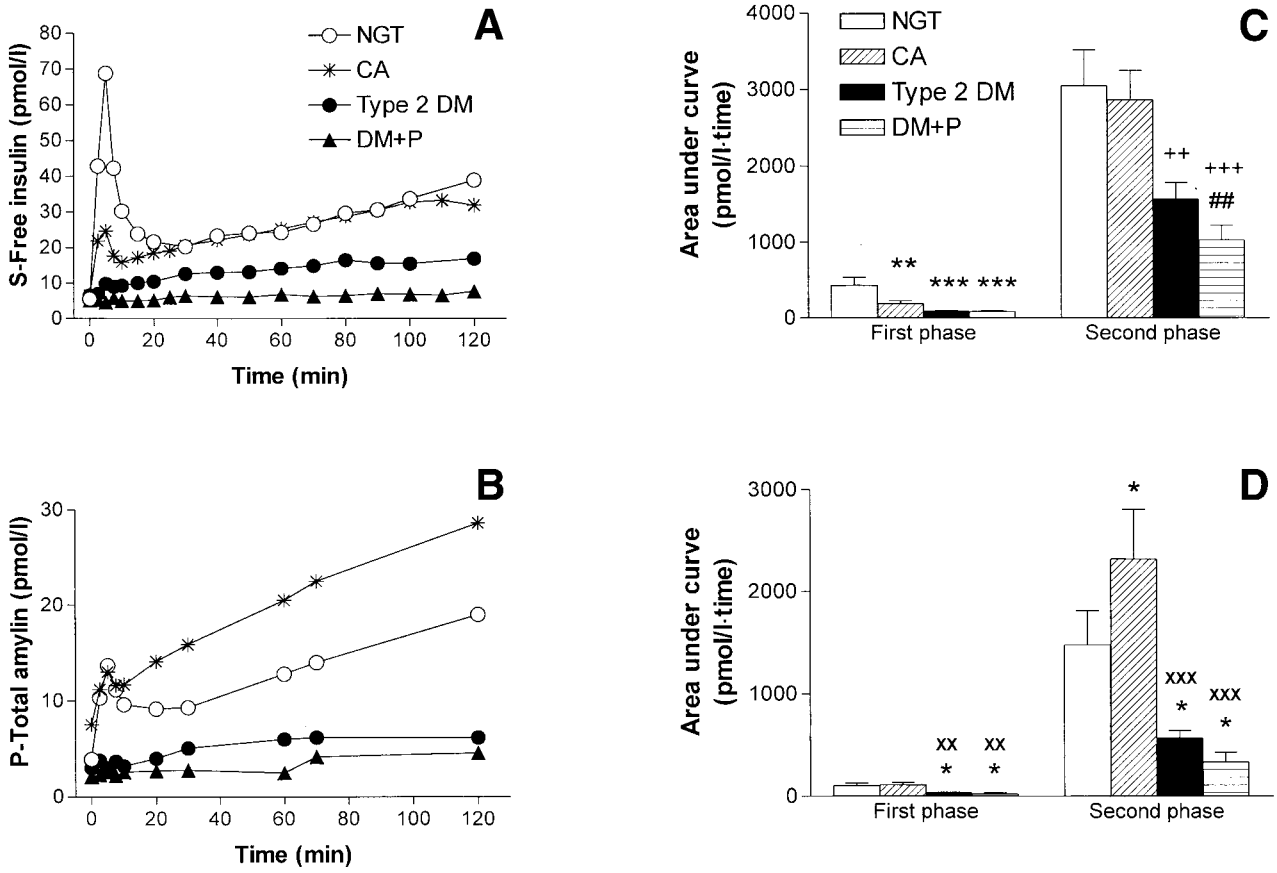

Fig 1. Plasma total amylin (A) and serum-free insulin (B) concentrations as a function of time during the hyperglycemic clamp in patients with NGT, pancreatic CA, type 2 diabetes (type 2 DM), and diabetic patients with pancreatic tumor (DM+P); (C) and (D) depict first (0 to 10 minutes) and second (10 to 120 minutes) phase secretory responses as areas under the curve respectively. $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$ for other groups *v* NGT. $^{+++}P < .001$ for DM+P *v* NGT and CA, $^{++}P < .01$ for type 2 DM *v* other groups. $^{\#\#}P < .01$ for type 2 DM *v* DM+P. $^{XXX}P < .001$, $^{XX}P < .01$ for type 2 DM or DM+P *v* CA.

the pancreas, although even data based on isolated rat pancreas preparations have been controversial in this regard.[50,51] In the present study, the 2 phases were clearly different for amylin and insulin. The second phase of plasma amylin concentrations was markedly higher than that of the first, while the opposite was true for insulin (Fig 1). Whether these differences reflect slower clearance of amylin than insulin[52] or lack of cosecretion of the 2 peptides in humans, cannot be determined based on these data. The finding of low concentrations of insulin and amylin in the type 2 diabetic patients is consistent with previous reports using oral,[30,31] and intravenous[30] glucose tolerance tests to compare type 2 diabetic patients and normal subjects. The low concentrations of amylin in plasma do not exclude the presence of amyloid deposits in the pancreas. Indeed, several studies have found amyloid deposits in 76% to 92% of pancreata from patients with type 2 diabetes and in 0% to 55% of those of nondiabetic subjects.[24-29] In the present study, which included a small number of subjects (Table 3), amyloid deposits were not found in any of the specimens examined. Since formation of amyloid deposits increases with the duration of diabetes,[28,29] the relatively short duration of diabetes (Table 2) may have contributed to lack of amyloid in the pancreata from our patients with diabetes.

In previous studies by Permert el al,[32,33] patients with typical type 2 diabetes had lower plasma amylin concentrations than normal subjects, as was also found in the present study. However, the absolute concentrations of fasting plasma amylin were normal in nondiabetic patients with pancreatic cancer, but 3-fold (non–insulin-treated patients) to 6-fold (insulin-treated patients) increased in patients with diabetes and pancreatic cancer compared with normal subjects. These data may seem to contrast our data in which diabetic patients with pancreatic tumors had barely detectable levels of amylin just like the type 2 diabetic patients without pancreatic tumors. Importantly, in our group with diabetes and pancreatic tumors, only 3 had pancreatic CA which makes it difficult to compare the result to the previous studies. In addition, in the present study, the nondiabetic patients with pancreatic cancer had disproportionately high plasma amylin concentrations compared with patients with other pancreatic disease and nondiabetic subjects. Thus, while we confirmed the observation of increased plasma amylin concentrations in patients with pancreatic cancer, it was found to characterize nondiabetic rather than diabetic patients. On the other hand, altough all our nondiabetic patients with pancreatic cancer had fasting plasma glucose in the nondiabetic range and normal HbA_{1c}, oral glucose tolerance tests were not

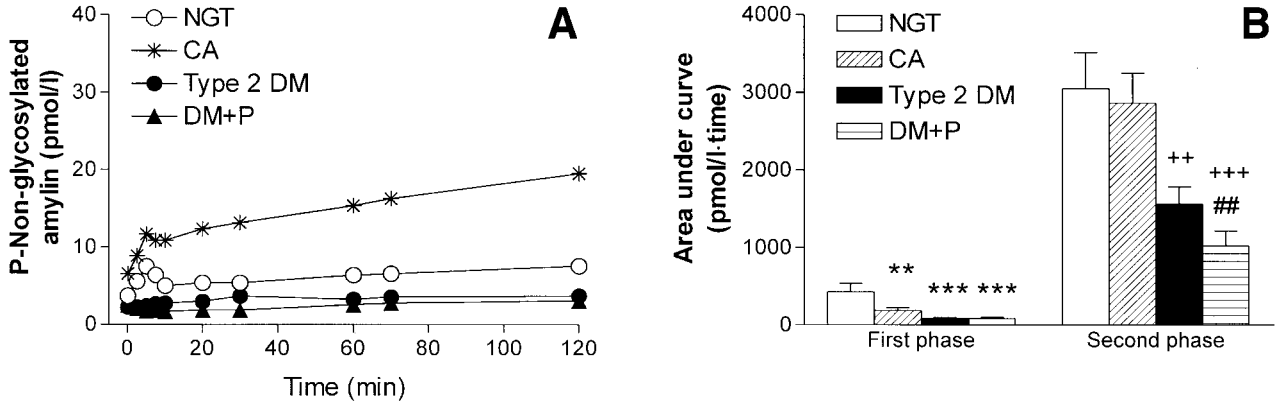

Fig 2. Plasma nonglycosylated amylin concentrations as a function of time during the hyperglycemic clamp (A) in subjects with NGT, pancreatic CA, type 2 diabetes (type 2 DM), and diabetic patients with pancreatic tumor (DM+P). (B) Depicts first (0 to 10 minutes) and second (10 to 120 minutes) phase secretory responses as areas under the curve, respectively. $^{***}P < .001$, $^{**}P < .01$ for DM+P *v* CA. $^{++}P < .01$, $^{+}P < .05$ for type 2 DM *v* CA. $^{xx}P < .01$ for CA *v* NGT, $^{x}P < .05$ for DM+P *v* NGT.

performed to exclude impaired or diabetic glucose tolerance. HbA_{1c} was, however, inversely correlated with the area under the plasma amylin concentration curve, suggesting that the increased plasma amylin concentration was not attributable to diabetes.

Taken together, both the studies of Permert et al[32,33] and the present study support the view that amylin concentrations are excessively increased relative to those of insulin in patients with pancreatic cancer. These in vivo observations support recent in vitro data, which have shown that exposure of β cells to pancreatic cancer-cell–conditioned medium selectively stimulate islet β cells to secrete amylin,[53] and that pancreatic CA is associated with hypersecretion of amylin relative to insulin on a molar basis.[54]

Several animal models including the Zucker diabetic rat,[55] the BB diabetic rat,[56] the neonatal streptozotocin-treated rat model of diabetes,[57] the glucocorticoid-induced diabetic rat,[58] and diabetic db/db mice[35] are characterized by under expression of GLUT2, the β-cell high K_m glucose transporter, and a reduction in glucose transport by islet cells in proportion to the reduction in GLUT2 expression.[55] GLUT2 mRNA and protein expression can also be decreased by acute exposure to hyperglycemia in rats.[59] These studies suggested that GLUT2 might be an important target for glucose-induced regulation of insulin secretion also in human β cells. Human β cells express, however, far less GLUT2 than those of rodents.[60] GLUT2 protein levels have been hitherto measured in 4 organ donors with type 2 diabetes, in which GLUT2 levels were similar to those in nondiabetic subjects.[61] In the present study, GLUT2 expression was unaltered in β cells in 3 patients who had diabetes secondary to pancreatic disease. Admittedly, immunohistochemistry is a semiquantitative technique at its best, but the lack of any difference in GLUT2 staining between the nondiabetic and diabetic specimens is in contrast to the GLUT2 data in diabetic animal models. Together these data strengthen the view that chronic hyperglycemia does not impair insulin secretion via changes in GLUT2 expression in humans.

We conclude that plasma amylin concentrations are increased in nondiabetic patients with pancreatic cancer, while serum insulin concentrations are nearly comparable between nondiabetic subjects with pancreatic cancer and healthy subjects. These data do support the in vitro data demonstrating that tumor-exposed media can stimulate β cells to produce amylin.

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Table 3. Expression of GLUT2, Insulin, Glucagon, and Amyloid in Pancreatic Tissue Samples

Patient No.	Study Group	Diagnosis	GLUT2	Insulin	Glucagon	Amyloid
1	CA	Carcinoma adenomatosum	+	+++	+	−
2	CA	Carcinoma adenomatosum	+	+++	+	−
3	CA	Carcinoma adenomatosum	+	+++	++	−
4	DM + P	Carcinoma adenomatosum	+	++	+	−
5	DM + P	Cystadenoma mucinosum	+	+++	+	−
6	DM + P	Cystadenoma microcystica	++	+++	++	−
7	NGT	Pancreatitis chronica	+	+++	++	−
8	NGT	Normal pancreas, Carcinoma mucocellulare oesophagi	++	+++	+++	−
9	NGT	Normal pancreas, Melanoma malignum metastaticum	+	+++	++	−
10	NGT	Normal pancreas, Dysplasia ducti choledochi	+	+++	++	−

NOTE. Intensity of grading: +++, very strong staining; +, low intensity of staining; −, no staining.

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