Rapid Publications

Effects of Meal Ingestion on Plasma Amylin Concentration in NIDDM and Nondiabetic Humans

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Recent interest has focused on the potential role of amylin in the pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM). This 37–amino acid peptide is found in extracellular amyloid deposits in ~50% of pancreatic islets of patients with NIDDM and has been shown to inhibit skeletal muscle glycogen synthesis in vitro. Immunocytochemical studies have colocalized amylin and insulin within β-cell secretory granules in nondiabetic humans, provoking the following questions. Is amylin cosecreted with insulin? Are circulating amylin concentrations higher in patients with NIDDM either before or after food ingestion? To answer these questions, we developed a sensitive and specific immunoassay to measure plasma concentrations of amylin in humans. Use of this assay indicated that, in lean nondiabetic subjects, glucose ingestion resulted in an increase ($P < 0.001$) in the plasma concentration of amylin (from 2.03 ± 0.22 to 3.78 ± 0.39 pM) and insulin (from 48.3 ± 3.1 to 265 ± 44 pM). There was a significant correlation between the concentrations of insulin and amylin ($r = 0.74$, $P < 0.001$) and the increase in insulin and amylin concentration ($r = 0.65$, $P < 0.005$). Fasting concentrations of amylin did not differ in diabetic and weight-matched nondiabetic subjects and showed a similar pattern of change after ingestion of a mixed meal. We conclude that amylin is secreted in response to ingestion of either glucose or a mixed meal and circulates at concentrations that do not differ in patients with NIDDM and nondiabetic subjects. It remains to be determined whether amylin at physiological concentrations influences carbohydrate metabolism and if so whether its effects differ in diabetic and nondiabetic humans. *Diabetes* 39:752–56, 1990

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Pancreatic tissue of some but not all patients with non-insulin-dependent diabetes mellitus (NIDDM) contains extracellular amyloid deposits (1). Similar deposits have been noted at a lower percentage in islets of pancreatic tissue from nondiabetic patients (1). Two groups purified and sequenced the constitutive peptide of these amyloid deposits from diabetic cats and human insulinoma tissue (2,3) and pancreatic tissue from patients with NIDDM (3,4). The 37–amino acid peptide was initially named insulinoma- or islet-associated polypeptide (2) and subsequently referred to as diabetes-associated peptide (4) and amylin (5). By use of a specific antiserum, immunohistochemical studies have localized amylin and insulin to the islet β-cells in pancreatic tissue of nondiabetic humans (6–8). These observations are supported by RNA hybridization studies, which have shown abundant amylin mRNA in islet tissue only (9). In contrast, calcitonin gene–related peptide (CGRP), which shares ~46% homology with amylin (4), is predominantly localized to neural tissue (10). This tissue specificity and the presence of amylin in insulin secretory granules within islets of nondiabetic subjects (8) has lead to the suggestion that amylin is involved in the post-translational processing of insulin and that a defect in this process results in the accumulation of amylin excess in patients with NIDDM (11). Based on the assumptions that amylin is secreted from the islet and that circulating CGRP has effects similar to amylin, it has been suggested that amylin may also be involved in the pathogenesis of insulin resistance of NIDDM, because CGRP (and amylin) caused a decrease in insulin-stimulated glycogen synthesis in rat skeletal muscle (5,12). However, it remains to be determined whether amylin is cosecreted with insulin into the circulation and whether concentrations differ in patients with NIDDM and nondiabetic humans.

Therefore, we sought to develop a specific assay for amylin in plasma to address the following questions. First, does amylin circulate in the plasma of nondiabetic humans, and

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if so, do plasma amylin and insulin concentrations increase concordantly after ingestion of glucose or a mixed meal in nondiabetic humans? Second, do plasma amylin concentrations differ in patients with NIDDM and nondiabetic subjects before and after ingestion of a mixed meal?

RESEARCH DESIGN AND METHODS

Ten lean healthy subjects (aged 36 ± 2 yr, body mass index 24 ± 1 kg/m^2) with no family history of diabetes mellitus were studied after an overnight fast. No subject was taking medication. At 0800, a blood sample was collected from a forearm vein. The subjects then ingested 50 g chilled flavored glucose solution, and a second blood sample was collected 30 min later. On a separate occasion, 10 healthy patients with NIDDM (aged 48 ± 2 yr, body mass index 30 ± 2 kg/m^2) and 5 healthy subjects (aged 52 ± 5 yr, body mass index 31 ± 3 kg/m^2) ingested a solid mixed meal (472 cal: 45% carbohydrate, 40% fat, and 16% protein). Six diabetic patients were maintained on diet alone, and 3 of them were taking oral sulfonylureas. Glycosylated hemoglobin concentration measured by affinity chromatography averaged 9.5 ± 0.5% in the diabetic patients (normal range 4–7%). The three subjects who were taking sulfonylureas discontinued them 2 wk before study. Blood was collected at 30-min intervals for 3 h after meal ingestion. Blood for measurement of insulin, CGRP, and amylin was immediately placed in glass tubes on ice containing EDTA and 1 ml 5 M benzamidine/10 ml blood. Blood was immediately centrifuged (4000 rpm for 10 min at 4°C), and the plasma was stored at −20°C. Blood for measurement of glucose was collected into tubes containing fluoride oxalate.

Insulin was measured by radioimmunoassay (RIA) as previously described (13). CGRP was assayed with the method of Conlon et al. (14). Glucose concentrations were measured by the glucose oxidase method (YSI, Yellow Springs, OH).

Human amylin acid and human amylin amide were synthesized by solid-phase methodology. The purity of peptides was checked by analytical high-performance liquid chromatography (HPLC), thin-layer chromatography, electrophoresis, and amino acid analysis, and fast atomic bombardment spectrum was >98%.

Ten milligrams human amylin acid was conjugated with 50 mg bovine thyroglobulin (Sigma, St. Louis, MO) through 10 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma). The reaction mixture was incubated in 8 ml 0.01 M sodium phosphate saline at 4°C for 16–24 h. Thin-layer chromatography showed that 80–90% of amylin was conjugated to the thyroglobulin. A mixture of 200 μl conjugate and 300 μl 0.9% NaCl was emulsified with 500 μl Freund's complete adjuvant and injected intramuscularly into a New Zealand White rabbit once a week for 2 wk. This was boosted 4 wk later, followed by subcutaneous boosting every 2 wk; antiserum against human amylin acid was obtained at the fourth immunization. Human amylin acid and human amylin amide bound to the antibody with similar affinity (Fig. 1*A*). Serial dilution of extracted plasma resulted in parallel displacement. The antibody did not cross-react with human CGRP or various other peptides.

Human amylin was iodinated with ^{125}I-sodium iodide (Amersham, Arlington Heights, IL) by the ioodogen method. Specific activity was >1000 Ci/mmol. Iodinated peptides were purified by Sephadex G-10 (Sigma) or reverse-phase HPLC with a C-18 column (Vydac, Hesperia, CA).

Five milliliters human plasma was centrifuged with equal amounts of 0.1% trifluoroacetic acid (TFA) in H_2O at 17,500 *g* at 4°C for 20 min. The supernatant was loaded on a Sep-Pak C-18 cartridge, which was equilibrated initially with 3 ml 60% acetonitrile in 0.1% TFA and followed twice with 3 ml 0.1% TFA in H_2O. Three milliliters 60% acetonitrile in 0.1% TFA was then eluated on the cartridge. The eluates were lyophilized, and the extracts were kept at −20°C. Extraction as assessed by addition of ^{125}I-labeled amylin or ^{125}I-amylin amide to plasma averaged 88 and 90%, respectively. Immunoreactive amylin in extracted plasma eluted as a sin-

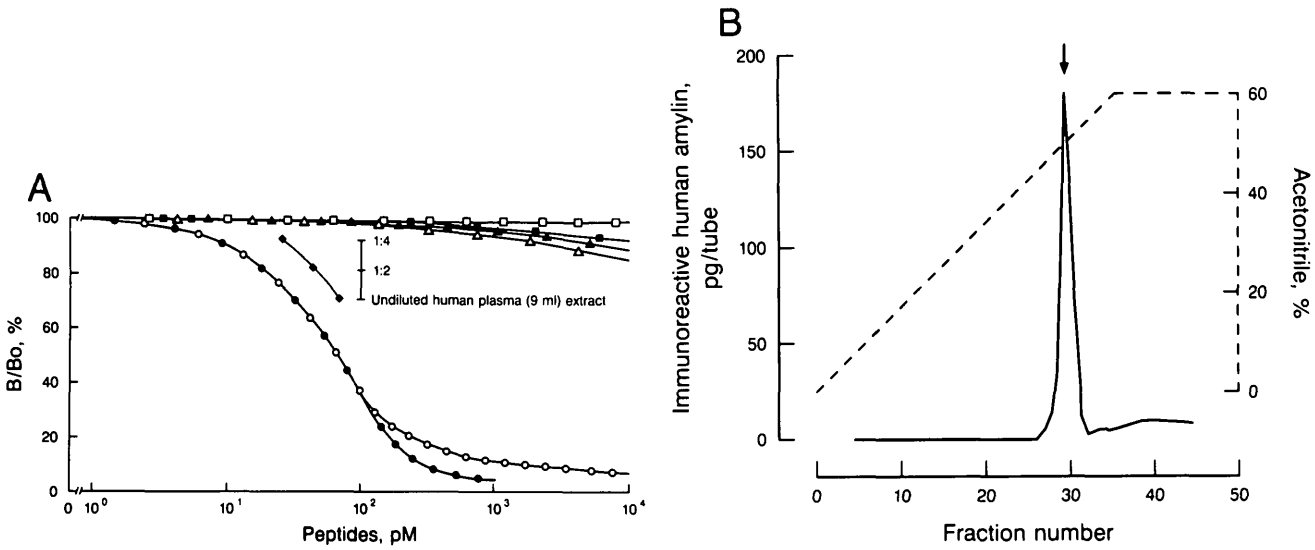

FIG. 1. *A*: competitive binding (B/B$_0$) curves for antibody vs. human amylin (●), human and rat amylin amide (○), rat calcitonin gene–related peptide (CGRP) and CGRP II (▲), human CGRP (■), human CGRP II (△), and human insulin (□). In addition, pork neuropeptide Y; human, pork, or rat vasoactive intestinal peptide; pancreastatin; human glucagon; somatostatin-28; somatostatin-14; and pancreatic polypeptide showed no cross-reactivity. *B*: immunoreactivity of plasma extract. *Arrow*, elution site of synthetic human amylin.

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gle peak on HPLC that coincided with synthetic human amylin (Fig. 1*B*).

One hundred microliters of either standard amylin or plasma extracts in RIA buffer (0.1 M sodium phosphate [pH 7.4], 0.05 M NaCl, 0.1% bovine serum albumin, 0.01% NaN_3, and 0.1% Triton X-100) were mixed with 100 μl antiserum and incubated at 4°C for 16–24 h. One hundred microliters tracer (~10,000–15,000 counts/min) was then added, and the mixture was incubated at 4°C for a further 16–24 h. Final dilution of antiserum was 1:42,000. One hundred microliters second antibody (goat anti-rabbit γ-globulin diluted 1:10) and 100 μl normal rabbit serum (diluted 1:16; both from Scantibodies, Lakeside, CA) were then added, and the mixture was incubated for 90 min at room temperature. After adding 500 μl cold RIA buffer, the mixture was centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was aspirated, and the pellet of labeled amylin bound to antiserum was counted with an LKB 1277 γ-counter. Samples were routinely assayed in duplicate.

All results are given as means ± SE. Integrated responses above baseline and postprandial integrated responses were calculated with the trapezoidal rule. Student's paired and unpaired *t* tests were used to test statistical significance within and between groups with $P < 0.05$ as significant.

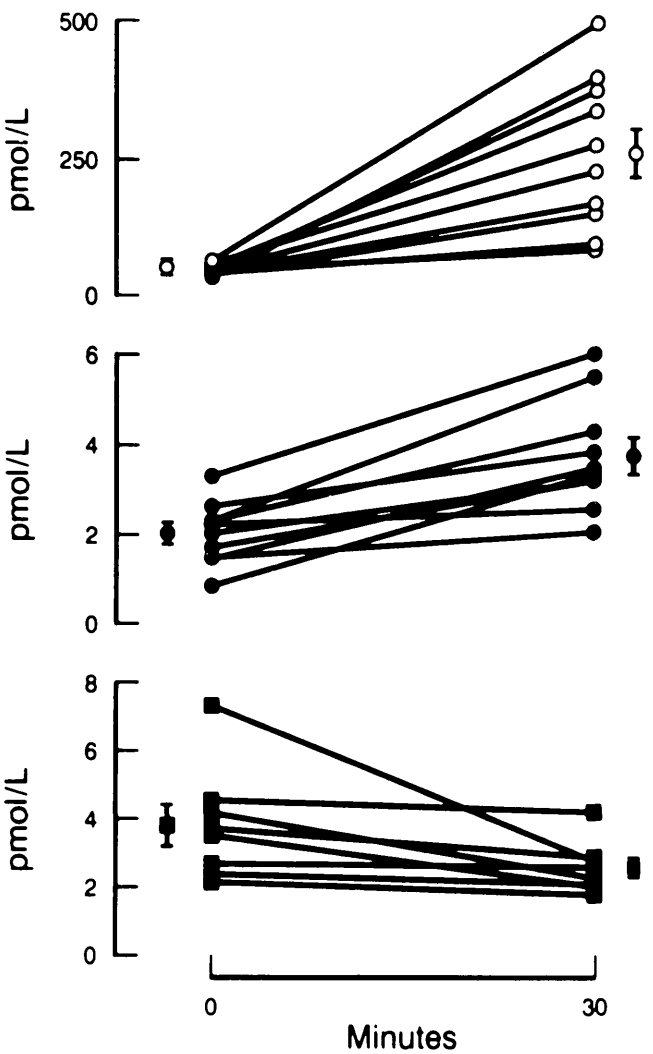

FIG. 2. Plasma insulin (*top*), amylin (*middle*), and calcitonin gene–related peptide (*bottom*) concentrations before and 30 min after ingestion of 50 g glucose.

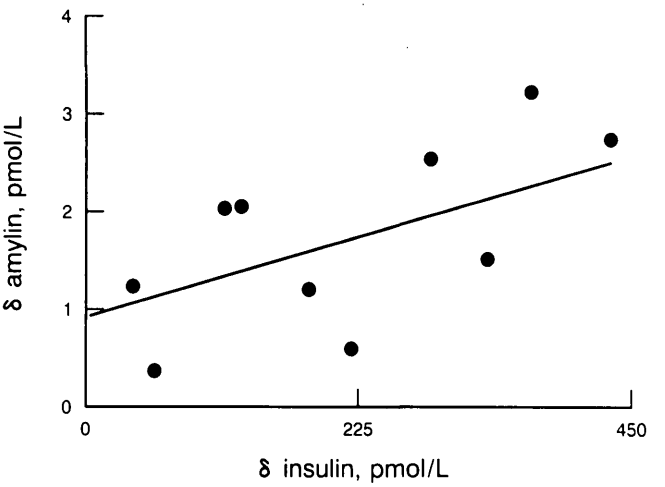

FIG. 3. Correlation between change in plasma insulin and change in plasma amylin concentrations 30 min after ingestion of 50 g glucose. $r = 0.65$, $P < 0.05$.

RESULTS

After glucose ingestion, we found increased concentrations of plasma glucose (from 5.3 ± 0.1 to 7.2 ± 0./3 mM, $P < 0.001$) and insulin (from 48.3 ± 3.1 to 265 ± 44 pM, $P < 0.001$). The plasma amylin concentration also increased whether measured by the amylin assay (from 2.03 ± 0.22 to 3.78 ± 0.39 pM, $P < 0.001$, $n = 10$) or the amylin amide assay (from 2.17 ± 0.36 to 5.29 ± 0.69 pM, $P < 0.001$, $n = 8$) after glucose ingestion. In contrast, plasma CGRP concentrations decreased (Fig. 2), although nonsignificantly, after glucose ingestion (from 3.81 ± 0.59 to 2.57 ± 0.27 pM, $P = 0.06$). Plasma amylin and insulin concentrations before and after glucose ingestion were significantly correlated ($r = 0.74$, $P < 0.001$). The changes in plasma insulin and amylin concentration after glucose ingestion were also significantly correlated ($r = 0.65$, $P < 0.05$; Fig. 3). There was no significant correlation between the change in plasma insulin and plasma CGRP concentrations ($r = 0.002$, $P = 0.99$).

To determine whether amylin concentrations vary under conditions of daily living, plasma amylin was measured after ingestion of a mixed meal. As observed with glucose ingestion, both amylin and insulin concentrations increased after the meal ($P < 0.01$; Fig. 4). Amylin concentrations were slightly but not significantly higher in the diabetic than nondiabetic subjects before meal ingestion (6.7 ± 0.7 vs. 4.8 ± 2.0 pM, $P = 0.3$). After meal ingestion, neither integrated response above basal (378 ± 89 vs. 471 ± 173 pM/3 h, $P = 0.65$) nor postprandial integrated response (1578 ± 194 vs. 1348 ± 455 pM/3 h, $P = 0.66$) differed in the diabetic and nondiabetic subjects.

DISCUSSION

We present a method for plasma extraction and an RIA suitable for measurement of plasma amylin concentration. A

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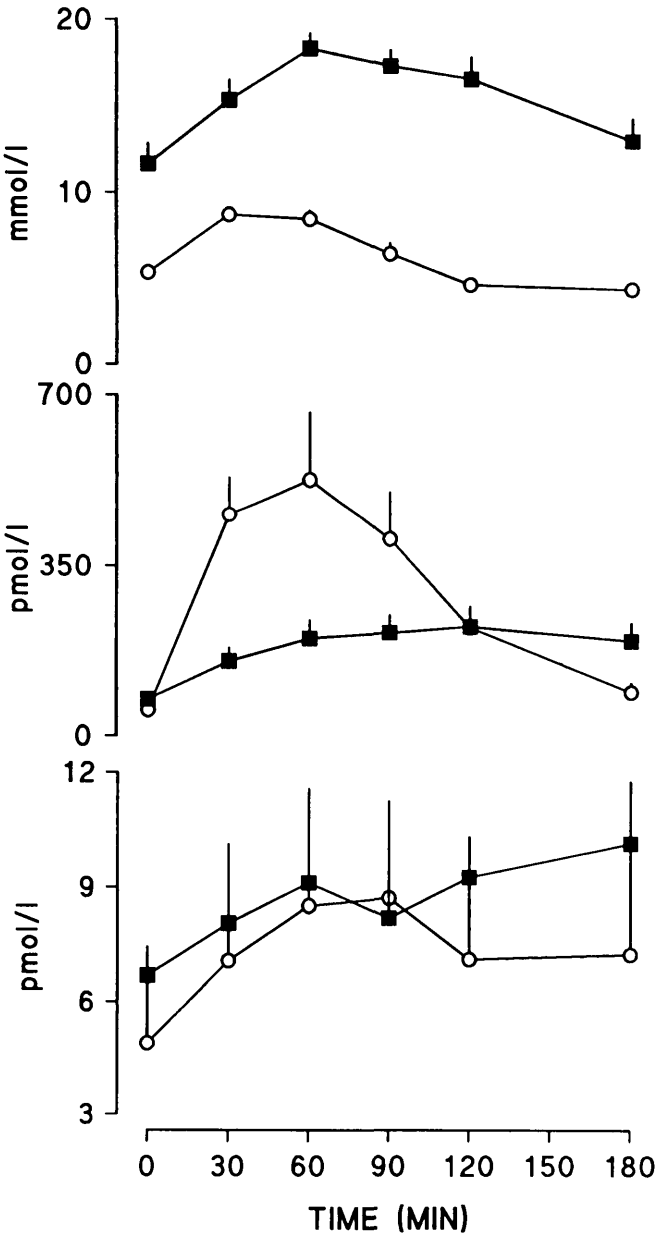

FIG. 4. Glucose (*top*), insulin (*middle*), and amylin (*bottom*) concentrations after ingestion of mixed meal at t = 0. ○, Nondiabetic subjects; ■, diabetic subjects.

similar assay has recently been reported that gave similar values for fasting amylin concentration in humans (15).

We report that the plasma amylin concentration increases in parallel with plasma insulin after ingestion of either glucose or a mixed meal. This supports the prediction of Lukinius et al. (8) that the amylin in β-cell insulin secretory granules is cosecreted with insulin (6–8). Because the hepatic and extrahepatic clearance of amylin is unknown, it is not possible from the available data to determine whether the ~20- to 40-fold lower plasma amylin versus insulin concentration is due to differences in rates of secretion and/or clearance. However, more abundant insulin mRNA in islets suggests that this difference is at least in part due to lower rates of secretion (9). Further studies are required to determine whether all β-cell secretagogues and inhibitors result in concordant release of insulin and amylin.

In contrast to amylin, the CGRP concentration fell, although nonsignificantly, after glucose ingestion. The opposite regulation of CGRP and amylin after glucose ingestion stresses the importance of separate specific assays for measurement of circulating concentrations of these peptides. Because CGRP is present in class 4 sensory afferent nerve fibers (16), which are abundant in skeletal muscle (17), the local concentration of CGRP in skeletal muscle may be considerably higher than the simultaneous plasma concentration. It is therefore possible that, although CGRP is only present in picomolar concentrations in plasma, it reaches local concentrations in excess of the 10^{-9} M reported to be required to alter skeletal muscle carbohydrate metabolism (5,12). Thus, the role of CGRP as a physiological regulator of skeletal muscle glycogen synthesis remains to be determined.

In contrast, because amylin is secreted by β-cells, the concentration influencing muscle presumably is equal to or less than the circulating amylin concentration. Our study and that of Nazakato et al. (15) indicate that plasma amylin concentrations in healthy humans are 1000-fold lower than the lowest concentration of amylin yet reported to decrease glycogen synthesis in skeletal muscle (5,12). Of perhaps the greatest importance, the plasma concentrations of amylin are not significantly different in diabetic and weight-matched nondiabetic subjects either before or after meal ingestion, making it extremely unlikely that amylin is the cause of insulin resistance in NIDDM. However, our data do not exclude the possibility that amylin concentrations are higher in earlier stages of diabetes mellitus or that subtle differences in amylin concentrations may exist in diabetic and nondiabetic subjects at other times of the day.

In conclusion, application of a sensitive specific assay for measurement of plasma amylin indicates that amylin is cosecreted with insulin after glucose or mixed-meal ingestion and that plasma concentrations do not differ in diabetic and nondiabetic subjects. Now that concentrations present under conditions of daily living have been defined, further studies will be required to determine whether amylin is a physiological regulator of carbohydrate metabolism in humans.

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