Agouti Signaling Protein Stimulates Islet Amyloid Polypeptide (Amylin) Secretion in Pancreatic β-Cells

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Ectopic overexpression of the murine *agouti* gene results in yellow coat color, obesity, hyperinsulinemia, and type II diabetes. We have shown the human homologue of *agouti* (agouti signaling protein; ASP) to regulate human adipocyte metabolism and lipid storage via a Ca^{2+}-dependent mechanism. We have also demonstrated agouti expression in human pancreas, and that ASP stimulates insulin release via a similar Ca^{2+}-dependent mechanism. Plasma amylin is also elevated in agouti mutant mice. Amylin is cosecreted with insulin from β-cells, and overexpression of human amylin in β-cells in yellow agouti mutant mice resulted in accelerated pancreatic amyloid deposition, severely impaired β-cell function, and a diabetic phenotype. We report here that ASP stimulates amylin release in both the HIT-T15 β-cell line and human pancreatic islets in the presence of a wide range of glucose concentrations (0–16.7 mmol/L), similar to its effect on insulin release; this effect was blocked by 30 μmol/L nitrendipine, confirming a Ca^{2+}-dependent mechanism. Accordingly, ASP stimulation of amylin release may serve as a compensatory system to regulate blood glucose in yellow agouti mutants. [Exp Biol Med Vol. 226(6):565–569, 2001]

Key words: agouti; agouti signaling protein; amylin; calcium; diabetes; pancreatic islet

Ubiquitous overexpression of the mouse *agouti* gene results in a yellow coat color, as well as obesity, hyperinsulinemia, insulin resistance, and type II diabetes (1). The human homologue of *agouti* is expressed in adipose tissue (2). We have previously shown that recombinant agouti protein coordinately regulates adipocyte lipogenesis and lipolysis, thereby promoting lipid storage in adipose tissue (3, 4). We have also demonstrated that agouti is expressed in human pancreas, and stimulates both Ca^{2+}-signaling and insulin release in human pancreatic islets (5), thereby contributing to hyperinsulinemia.

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Islet amyloid polypeptide, also called amylin, is colocalized with insulin in β-cell secretory granules (6) and is cosecreted with insulin in response to glucose and nonglucose secretagogues (7). Recent data showed that amylin delayed the postprandial rise in plasma glucose levels by slowing the rate of gastric emptying (8), suggesting that amylin, as another β-cell hormone, may coordinate with insulin in regulating blood glucose homeostasis under physiological conditions.

However, amylin is the major component of islet amyloid deposits, which usually accumulate extracellularly between β-cells and capillaries in the islets of most of type II diabetic patients (9), and lead to diminished β-cell mass, impaired β-cell function, and hyperglycemia in the later stage of type II diabetes. Plasma amylin levels are elevated in viable yellow mice compared with wild-type mice (10). Furthermore, overexpression of human amylin in the pancreas of viable yellow mice results in accelerated islet amyloid deposition, severely impaired β-cell function, and diabetic phenotype (11). Since amylin release is regulated similarly to that of insulin, it is possible that agouti protein may directly stimulate amylin release, similar to its effect on insulin release.

Consequently, the present study was designed to investigate the effect of recombinant agouti protein on amylin release in pancreatic β-cells. We report here that recombinant agouti protein stimulated amylin release in both HIT-T15 pancreatic β-cell line and human pancreatic islets via a Ca^{2+}-dependent mechanism, similar to its effect on insulin release and adipocyte metabolism (3–5).

Materials and Methods

Isolation and Purification of Human Pancreatic Islets. Human pancreas was obtained from multiorgan donors and transported in cold University of Wisconsin solution. Islets were isolated using collagenase digestion and Ficoll gradient centrifugation as previously described (5). Briefly, the pancreas was distended with collagenase type V- (0.5 mg/ml) type XI (2 mg/ml) (Sigma, St. Louis, MO) in Hank's balanced salt solution and was digested in the same solution at 37°C with shaking for 30 min. The tissue digest was then washed and sifted through a 500-μm nylon

filter and purified by Ficoll gradient centrifugation (Fluka, Milwaukee, WI). Purified islets were maintained in RPMI-1640 medium supplied with 10 % fetal bovine serum (FBS, Hyclone, Logan, UT) and antibiotics (50 U/ml of penicillin and 5 μg/ml of streptomycin) for 48 hr. The use of human pancreas was approved by the University of Tennessee Institutional Review Board and by Tennessee Donor Services.

Cell Culture. HIT-T15 pancreatic β-cell (American Type Culture Collection, Rockville, MD) was cultured in F-12K nutrient mixture supplemented with 10% horse serum, 2.5% FBS, and antibiotics. Cell culture reagents were obtained from Life Technologies (Grand Island, NY) except for those mentioned above.

Recombinant Agouti Production. Recombinant agouti polypeptide was produced by subcloning agouti cDNA into a baculoviral expression vector, which was then used to infect *Trichiphisia ni* (*T. ni*) cells (4, 5). Medium containing the secreted agouti was collected 48 hr post-infection and was purified as previously described (4, 5).

Amylin Release Experiment. Amylin release experiments were conducted using a similar procedure to that previously described for insulin release (5). Briefly, HIT-T15 pancreatic β-cells and human pancreatic islets were incubated in glucose-free, serum-free medium for 2 hr on the day of experiment and were then incubated with test agents indicated under each figure for 2 hr. Medium was then removed and stored at −80°C for amylin measurement. Cells were sonicated in sucrose buffer (250 mmol/L sucrose, 1 mmol/L dithiothreitol [DTT], 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 mmol/L ethylenediaminetetraacetic acid [EDTA], Sigma) and stored at −80°C for protein measurement.

Amylin level in the medium was measured by a human radioimmunoassay (RIA) kit (Peninsular, Belmont, CA). The antibody in the kit has been shown to cross-react with both human and hamster amylin (7). The sensitivity of the assay is 2 pg/tube and the coefficiency of variation is less than 10%. The total amount of protein in the cells/islets was determined by a modified Bradford method using Coomassie Blue dye (Pierce, Rockford, IL).

Statistics. All data are expressed as means ± SE. Data were analyzed by one-way Analysis of Variance (ANOVA) using the procedures of the SPSS Inc. (Chicago, IL). A P value < 0.05 is considered significant.

Results

As shown in Figure 1A, glucose dose-dependently stimulated amylin release in HIT-T15 pancreatic β-cells (39% and 98% increase at 2.5 and 10 mmol/L glucose, respectively; $P < 0.05$). The addition of 100 nmol/L agouti protein further enhanced amylin release in these cells, with 34%, 79%, and 34% further increase at 0, 2.5, and 10 mmol/L glucose concentrations, respectively ($P < 0.05$).

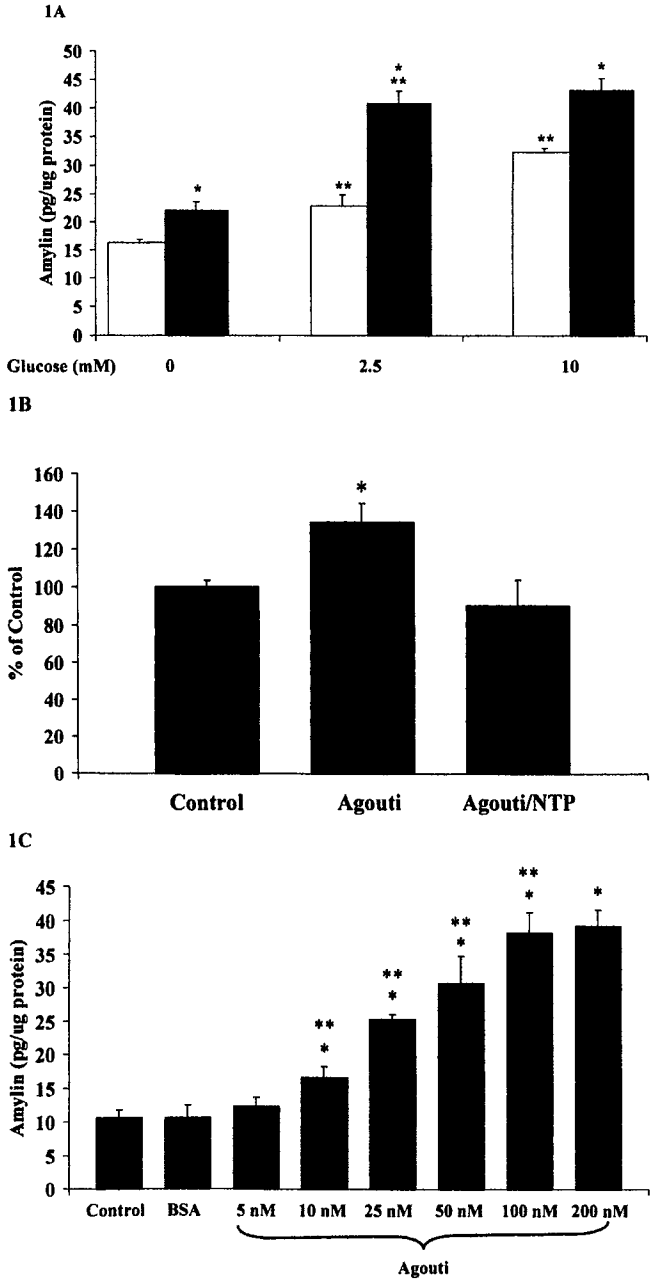

Figure 1. Effects of recombinant agouti protein on amylin release in HIT-T15 pancreatic β-cell line. HIT-T15 cells were pretreated with glucose-free, serum-free medium for 2 hr. Cells were then incubated in the same medium with agouti (100 nmol/L) in the presence of different concentrations of glucose (0–10 mmol/L) (A), with agouti (100 nmol/L) and nitrendipine (30 μmol/L) in the absence of glucose (B), or with agouti (0–200 nM) to establish a dose-response relationship in the absence of glucose (C) for 2 hr. At the end of incubation, medium was removed and stored at −80°C before amylin measurement. Cells were sonicated and stored at −80°C for protein correction. Data are means ± SE for three to four experiments. (A) □ Control, ■ Agouti, $^*P < 0.05$ versus control, $^{**}P < 0.05$ versus previous agouti or glucose treatment. (B) $^*P < 0.05$ versus control. (C) $P < 0.001$ versus control; $^{**}P < 0.05$ versus preceding concentration.

Figure 1B shows that agouti-stimulated amylin release in HIT-T15 cells was blocked by 30 μmol/L nitrendipine, suggesting that this effect of agouti protein is mediated via a Ca^{2+}-dependent mechanism, similar to our previous re-

ports of agouti regulation of adipocyte metabolism and β-cell insulin release (3–5). Figure 1C demonstrates the dose-responsive nature of this relationship, with a 60% increase elicited by 10 nM agouti protein, a 2-fold increase with 25 nM, and a maximal effect noted with 100 nM agouti protein, which produced nearly a 4-fold increase.

Similar results were observed in primary cultured human pancreatic islets (Fig. 2). However, in contrast to HIT-T15 cells, glucose exerted its stimulatory effect on amylin release at a higher concentration, 16.7 mmol/L, and 100 nmol/L agouti protein further enhanced amylin release under these glucose concentrations ($P < 0.05$, Fig. 2A). Such differences between pancreatic β-cell line and primary cultured pancreatic islet responses to glucose have been previously documented (12). Agouti-stimulated amylin release in human pancreatic islets was blocked by nitrendipine (Fig. 2B), similar to the observed effects in HIT-T15 cells noted above.

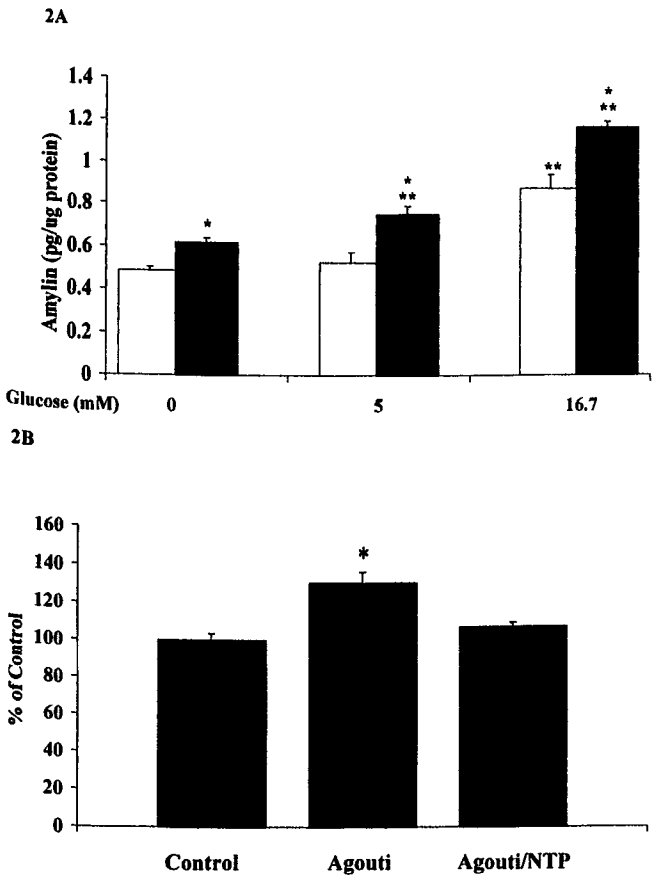

Figure 2. Effects of recombinant agouti protein on amylin release in human pancreatic islets. Human pancreatic islets were isolated via a collagenase digestion and Ficoll gradient centrifugation method. Islets were pretreated with glucose-free, serum-free medium for 2 hr. Islets were then incubated in the same medium with agouti (100 nmol/L) in the presence of different concentrations of glucose (0–16.7 mmol/L) (A) or with agouti (100 nmol/L) and nitrendipine (30 μmol/L) in the absence of glucose (B) for 2 hr with the 0 glucose value from (A) also serving as the 100% value for this panel. At the end of incubation, medium was removed and stored at −80°C before amylin measurement. Islets were sonicated and stored at −80°C for protein correction. Data are means ± SE for three to four experiments. (A) □ Control, ■ Agouti, $^*P < 0.05$ versus control, $^{**}P < 0.05$ versus previous agouti or glucose treatment. (B) $^*P < 0.05$ versus control.

Discussion

Data from this study demonstrate that agouti signaling protein (ASP) directly stimulated amylin release in both pancreatic β-cell line and human pancreatic islets in the presence of a wide range of glucose concentrations. Therefore, ASP stimulation of amylin release may contribute to the elevated amylin level present in yellow agouti mutant mice (10). In addition, we have recently shown that agouti is expressed in human pancreas (5). As ASP is a paracrine factor that does not enter the general circulation (15), these data suggest that agouti may stimulate amylin release in humans in a paracrine/autocrine fashion, similar to its paracrine/autocrine actions on β-cell insulin release (5) and adipocyte lipid metabolism (3, 4).

Recently, several studies have demonstrated that administration of amylin dose-dependently delayed the rise in plasma glucose levels after oral glucose load by slowing the rate of gastric emptying, with an ED_{50} of 20 pmol/L (8). Amylin also dose-dependently suppressed arginine-induced glucagon secretion in rats, with an EC_{50} of 18 pmol/L (16). As these effects occur at close to physiological concentrations of amylin, it is hypothesized that amylin may serve to delay glucose input into blood and inhibit hepatic glucose output after meals, thereby coordinating with insulin to modulate blood glucose level under physiological conditions. Consistent with this, the human amylin analogue pramlintide has been shown to improve postprandial glycaemic control in both type I and II diabetic patients (17). In addition, amylin has been shown to suppress food intake in rats and mice after either peripheral or central administration (18, 19). Thus, amylin may also act as a peripheral satiety factor to modulate food intake, as do insulin, cholecystokinin and glucagon-like peptide-1.

Consequently, ASP stimulation of amylin release may serve as a compensatory mechanism along with insulin in regulating blood glucose homeostasis. Moreover, ASP stimulation of amylin release may also act as a peripheral satiety factor to modulate food intake, thereby representing a feedback mechanism in body weight regulation.

The association of islet amyloid deposition with β-cell failure suggests that amylin may have an important role in the pathogenesis of type II diabetes. Because of the difference in the composition at amino acids 20 and 29, amylin from rodents is not amyloidogenic, whereas islet amyloid formation does occur in humans, cats, and racoons (20). Although humans express an amyloidogenic species of amylin, amyloid deposition rarely occurs in nondiabetic individual (9). In addition, most lines of transgenic mice overexpressing human amylin in β-cells do not spontaneously develop islet amyloid deposits and hyperglycemia (21–23). One strain of homozygotic transgenic mice overexpressing human amylin did develop hyperglycemia (24). However,

this is associated with small intra- and extracellular amorphous islet amyloid polypeptide (IAPP) aggregates, resulting in cytotoxicity and β-cell death, instead of large extracellular amyloid deposits that are commonly seen in most of the transgenic animals and type II diabetes patients. In addition, two recently developed immortal mouse pancreatic β-cell lines overexpressing human amylin also do not spontaneously develop islet amyloid (25).

On the other hand, introducing the agouti gene into these transgenic animals did result in extensive islet amyloid formation, leading to reduced β-cell mass and type II diabetes (11). Similarly, feeding these transgenic mice a high-fat diet, or introducing the *ob* gene into these animals, both of which induce insulin resistance, similarly resulted in accelerated amyloidogenesis and profound hyperglycemia (26, 27).

These data suggest that production of an amyloidogenic amylin by itself is necessary, but not sufficient, to induce amyloid deposit formation, whereas the interaction between obesity/insulin resistance status and amylin expression appears to be important in islet amyloidogenesis. Islet amyloid formation may be both a consequence of obesity/insulin resistance and a cause of β-cell failure and subsequent hyperglycemia in the later stage of type II diabetes.

ASP-induced increases in amylin release may predispose individuals to the development of islet amyloid deposit, which under conditions that induce obesity/insulin resistance status, may accelerate the process of amyloidogenesis, and ultimately result in hyperglycemia and type II diabetes. We have also shown ASP to stimulate insulin release, thereby contributing to hyperinsulinemia (5). Moreover, adipocyte ASP and agouti mRNA levels are positively correlated to fatty acid synthase activity and mRNA level in human adipose tissue (28), consistent with agouti regulation of fatty acid synthase *in vitro* (3, 4), indicating a potential role for agouti in inducing human obesity *in vivo*. Thus, ASP-induced hyperamylinemia, combined with its effects in promoting obesity and hyperinsulinemia, may contribute to the risk factors that may lead to increased amyloid deposition and type II diabetes.

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