An Insulin-Degrading Enzyme Inhibitor Decreases Amylin Degradation, Increases Amylin-Induced Cytotoxicity, and Increases Amyloid Formation in Insulinoma Cell Cultures

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Amylin (islet amyloid polypeptide) is the chief component of the islet amyloid found in type 2 diabetes, and amylin fibril precursors may be cytotoxic to pancreatic β-cells. Little is known about the prevention of amylin aggregation. We investigated the role of insulin-degrading enzyme (IDE) in amylin degradation, amyloid deposition, and cytotoxicity in RIN-m5F insulinoma cells. Human ^{125}I-labeled amylin degradation was inhibited by 46 and 65% with the addition of 100 nmol/l human amylin or insulin, respectively. ^{125}I-labeled insulin degradation was inhibited with 100 nmol/l human amylin, rat amylin, and insulin (by 50, 50, and 73%, respectively). The IDE inhibitor bacitracin inhibited amylin degradation by 78% and insulin degradation by 100%. Amyloid staining by Congo red fluorescence was detectable at 100 nmol/l amylin and was pronounced at 1,000 nmol/l amylin treatment for 48 h. Bacitracin treatment markedly increased staining at all amylin concentrations. Bacitracin with amylin caused a dramatic decrease in cell viability compared with amylin alone (68 and 25%, respectively, at 10 nmol/l amylin). In summary, RIN-m5F cells degraded both amylin and insulin through a common proteolytic pathway. IDE inhibition by bacitracin impaired amylin degradation, increased amyloid formation, and increased amylin-induced cytotoxicity, suggesting a role for IDE in amylin clearance and the prevention of amylin aggregation. *Diabetes* 52:2315–2320, 2003

Type 2 diabetes is characterized by the loss of pancreatic β-cell mass, accompanied by the presence of islet amyloid deposits in the pancreas (1). The major component of islet amyloid is amylin (also known as islet amyloid polypeptide), a polypeptide copackaged and cosecreted with insulin by pancreatic β-cells (2). It is presently unclear whether islet amyloid merely is a consequence of diabetes or actually contributes to the progression of the disease. However, there is evidence that intermediate precursors of amylin fibrils are cytotoxic to β-cells, whereas mature amyloid fibrils are not toxic, suggesting that β-cell toxicity may precede deposition of islet amyloid (3,4). Because amylin is continually produced in individuals without type 2 diabetes, the mere presence of amylin is insufficient to induce amylin-induced β-cell toxicity and islet amyloid. Transgenic animals producing human (amyloidogenic) amylin fail to develop islet amyloid (5–7), but when additional diabetic traits are introduced, islet amyloid is detected (8–10). Therefore, there is a protective mechanism that prevents amyloid deposition under normal conditions, but is impaired under diabetic conditions. Because proteolysis is a major pathway in the regulation of protein levels, degradation of amylin may contribute to the prevention of islet amyloid aggregation. β-cell toxicity and islet amyloid may therefore result from an imbalance in amylin levels because of impaired degradation of amylin. At present, our understanding of the proteolytic pathways that contribute to the degradation of amylin is incomplete.

The enzyme responsible for the cellular proteolysis of insulin is insulin-degrading enzyme (IDE) (11). The substrate specificity of IDE is for amyloidogenic polypeptides, including atrial natriuretic peptide (ANP), glucagon, and the β-amyloid peptide found in Alzheimer's disease, and therefore may act as a scavenger that clears amyloidogenic peptides to prevent amyloid deposition (11,12). Recently, we showed that amylin was also an IDE substrate (13). Therefore, IDE may be involved in controlling the levels of amylin by degradation, similar to its role in regulating the levels of the Alzheimer's-related β-amyloid peptide (14). Both the GK rat model of type 2 diabetes, in which IDE has been identified as a diabetes susceptibility gene, and the recently described IDE-null mice show impaired glucose metabolism. In both models, cellular insulin-degrading activity has been found to be impaired. Therefore, it is possible that in diabetes, IDE dysfunction also results in impaired amylin degradation, increased amylin aggregation, and, eventually, islet amyloid deposition. The present study was performed to address the following questions: *1*) Are amylin and insulin degraded in β-cells by a common pathway? *2*) Does inhibition of IDE alter amylin degradation and islet amyloid formation? and

3) Does IDE inhibition affect the cytotoxic properties of amylin toward β-cells? To answer these questions, a pancreatic β-cell–derived cell line was used to characterize amylin degradation.

RESEARCH DESIGN AND METHODS

Materials. Human and rat amylin were purchased from Bachem (Torrance, CA). Human amylin radiolabeled with ^{125}I at the COOH-terminal was purchased from Peninsula Labs (San Carlos, CA). The ^{125}I-insulin (mono-iodinated at the 14 position of the a-chain) and biosynthetic human insulin were provided by Lilly Research Laboratories. Bacitracin and Congo red were obtained from Sigma. The IDE-specific monoclonal antibody 9B12 (15) was kindly provided by Dr. Richard Roth (Stanford University, Stanford, CA).

Preparation of amylin solutions. Amylin solutions were prepared immediately before use from 1 mg/ml stock solutions into BSA-coated test tubes to prevent adhesion to plastic and minimize spontaneous amyloid fibril formation. Because of variable amounts of contaminating low-molecular-weight fragments and ^{125}I-labeled albumin, the ^{125}I-amylin was further purified by adsorption to a C_{18} Sep-Pak (Waters, Milford, MA) and eluted with 0.1% trifluoroacetic acid in acetonitrile. The eluent was lyophilized and resuspended in the original buffer (0.1 mol/l sodium phosphate [pH 7.4] containing 0.05 mol/l NaCl, 0.1% NaN_3, and 0.1% BSA) and frozen in aliquots until used.

Cell culture. Rat insulinoma (RIN-m5F) cells (16) were purchased from American Type Culture Collection and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1.5 g/l sodium bicarbonate, 2.0 g/l glucose, 1 mmol/l L-glutamine, and 10 μg/ml gentamicin. For experiments, cells were subcultured into 24-well plates at 2.5×10^5 cells/well; the medium was changed every 2 days until used.

Cellular degradation of amylin and insulin. The degradation of amylin and insulin in RIN-m5F cells was determined by the trichloroacetic acid (TCA) solubility assay using a variation of a method previously described (17). Briefly, RIN-m5F cells in 24-well plates were changed into serum-free medium containing 0.1% BSA for 1 h. The medium was removed and ^{125}I-amylin or ^{125}I-labeled insulin in serum-free medium containing 0.1% BSA was then added (20,000 cpm/ml), and the cells were incubated at 37°C for the times indicated. In some experiments, bacitracin was included at 1 mg/ml. The medium was removed and brought to 0.2% BSA and 16% TCA, chilled for 10 min on ice, and centrifuged at 3,000*g* for 15 min. The radioactivity in the supernatants and pellets was counted, and the degrading activity was expressed as the percent TCA soluble counts. In addition, the conditioned medium from the cells was removed and aliquots were tested for degrading activity by TCA solubility, as described above.

Immunodetection of IDE. Lysates were prepared from RIN-m5F cells in lysis buffer (50 mmol/l Tris [pH 7.4] containing 0.15 mol/l NaCl, 1% Triton X-100, and 0.1 mmol/l phenylmethylsulfonyl fluoride). Aliquots of 10 μg total protein were resolved in 7.5% SDS-PAGE then transferred to polyvinylidine fluoride membranes. For immunodetection, the membranes were probed with the 9B12 monoclonal antibody directed against IDE at 1:1,000 dilution for 1 h at room temperature. After being probed with a peroxidase-conjugated goat anti-mouse IgG (Jackson Immunochemicals, Temecula, CA) at 1:5,000 dilution for 1 h, immunoreactive proteins were detected by chemiluminescence.

Amyloid formation in RIN-m5F cultures. RIN-m5F cells were seeded onto poly-L-lysine-coated eight-well glass chamber slides at 10^5 cells/cm². The medium was changed to serum-free RPMI-1640 containing 0.1% BSA before treatment, then cells were treated with human amylin (0.01–1.0 μmol/l) for 48 h. In some experiments, bacitracin was included at 1 mg/ml. To determine the involvement of IDE-like proteolytic activity in islet amyloid formation, some cells were also treated with the IDE inhibitor bacitracin (1 mg/ml) at the same time as the amylin addition. After treatment, the cells were washed with PBS, fixed for 5 min in 4% buffered formalin, and then stained for amyloid using a modified Congo red staining procedure (18). Briefly, the samples were counterstained with Weigert iron hematoxylin for 15 s, rinsed, then placed in 0.5% HCl in 70% ethanol for 5 s. After being washed, the samples were stained with Congo red for 20 min. The samples were immediately dehydrated to xylene and mounted. The degree of amyloid formation was determined by fluorescence microscopy using a tetramethyl rhodamine isothiocyanate filter set.

Cell toxicity assessment. For cell toxicity studies, RIN-m5F cells were cultured in 96-well plates and treated with human or rat amylin, without and with bacitracin as described above for 48 h. Cell viability was measured by the reduction of methylthiazolyldiphenyl-tetrazolium bromide (MTT), as previously described (19).

Statistics. All data are expressed as means ± SE. For degradation assays, comparisons for differences between groups were made by one-way ANOVA with Dunnett's posttest (degradation assays) or two-way ANOVA with Bon-
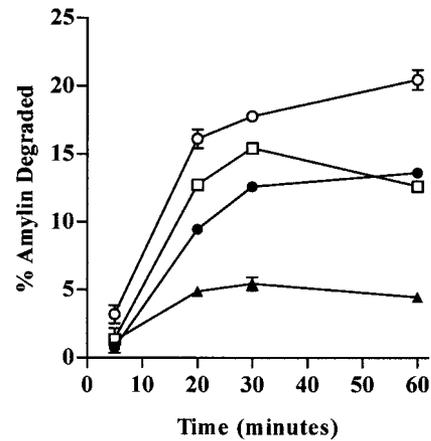

FIG. 1. Amylin degradation in RIN-m5F cells over time. The degradation of amylin by RIN-m5F cells over time was measured by the production of acid-soluble products from ^{125}I-amylin in cell culture media. Cells were also treated with 0.1 μmol/l unlabeled amylin or insulin or the IDE inhibitor bacitracin (1 mg/ml). Data are expressed as percent acid-soluble products produced (*n* = 3). ○, no treatment; ●, + amylin; □, + insulin; ▲, + bacitracin.

ferroni posttest (viability assay). *P* < 0.05 was interpreted as significant. Analyses were made using the GraphPad Prism3 software package.

RESULTS

Amylin and insulin degradation by RIN-m5F cells was examined over time. The amount of degradation was limited to ~20% to remain within the linear range of the assay. Degradation of ^{125}I-amylin reached ~20% by 30 min (Fig. 1). In contrast, ^{125}I-insulin degraded much more slowly, and required 120 min of incubation to reach 20% degradation (Fig. 2). The addition of either unlabeled amylin or insulin to the reaction decreased the degradation of both substrates, indicating a common degradative pathway. The addition of the IDE inhibitor bacitracin essentially eliminated the degradation of amylin and insu-
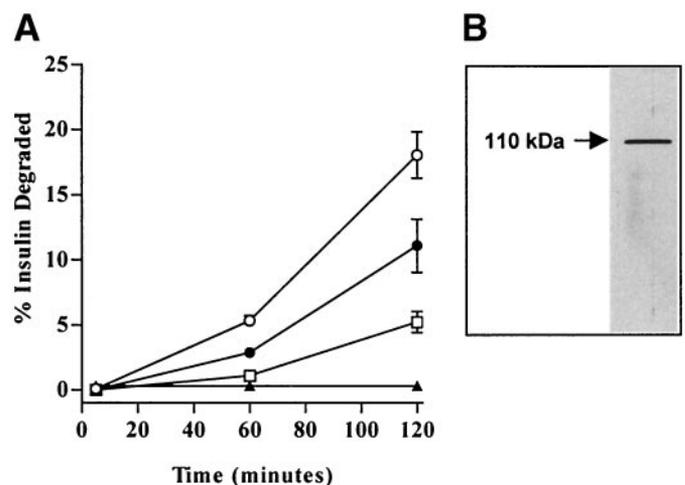

FIG. 2. Insulin degradation in RIN-m5F cells over time. *A*: The degradation of insulin by RIN-m5F cells over time was measured by the production of acid-soluble products from ^{125}I-insulin in cell culture media. Cells were also treated with 0.1 μmol/l unlabeled amylin or insulin or the IDE inhibitor bacitracin (1 mg/ml). Data are expressed as percent acid-soluble products produced (*n* = 3). ○, no treatment; ●, + amylin; □, + insulin; ▲, + bacitracin. *B*: Lysates from RIN-m5F were probed with the IDE-specific monoclonal antibody 9B12, and immunoreactive proteins were detected by chemiluminescence. A single band at 110 kDa was detected, consistent with the presence of IDE.
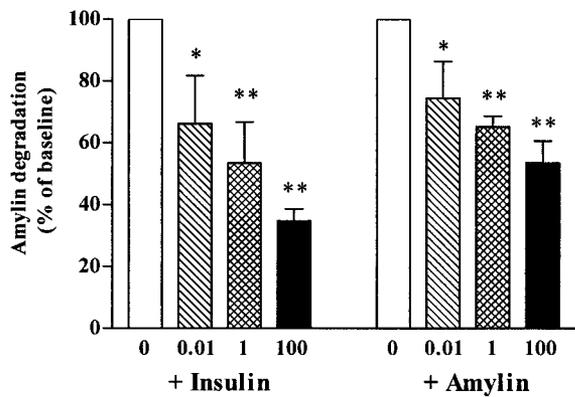
FIG. 3. Amylin degradation in RIN-m5F cells. The degradation of amylin by RIN-m5F cells was measured by the production of acid-soluble products from ^{125}I-amylin in cell culture media. Cells were also treated with the indicated concentrations (nmol/l) of unlabeled amylin or unlabeled insulin. Data are expressed as percent acid-soluble products produced ($n = 6$). *$P < 0.05$, **$P < 0.001$.

lin by the cells. There was no detectable amylin- or insulin-degrading activity in the conditioned medium (data not shown), suggesting that degradation was caused by uptake and intracellular degradation. The presence of IDE was readily detected in the cells by an immunoreactive band at ~110 kDa (Fig. 2). Therefore, RIN-m5F cells possess mechanisms to internalize and degrade both insulin and amylin and to express IDE.

To further characterize amylin and insulin degradation in RIN-m5F cells, the degradation was measured with increasing dosages of unlabeled competitor. The degradation of ^{125}I-amylin was decreased in a dosage-dependent manner by either unlabeled insulin or amylin (Fig. 3). At all concentrations, unlabeled insulin was a more effective inhibitor than amylin. Similarly, the degradation of ^{125}I-insulin was inhibited by both amylin and insulin (Fig. 4), with insulin generally the more effective inhibitor. These observations were consistent with previously determined enzymatic properties of IDE, in which insulin had a three- to sixfold higher affinity for purified IDE than amylin (13). Two possible complications of the cell culture system are the potential for direct interaction between human amylin and insulin (20) and membrane perturbations that may
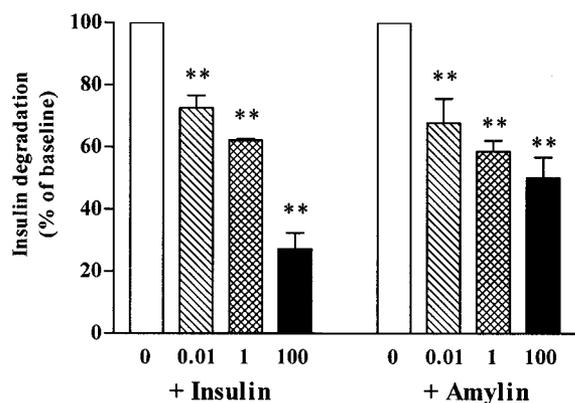

FIG. 4. Insulin degradation in RIN-m5F cells. The degradation of insulin by RIN-m5F cells was measured by the production of acid-soluble products from ^{125}I-amylin in cell culture media. Cells were also treated with the indicated concentrations (nmol/l) of unlabeled amylin or unlabeled insulin. Data are expressed as percent acid-soluble products produced ($n = 6$). **$P < 0.001$.
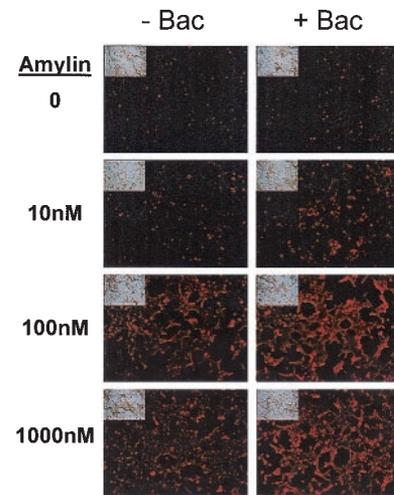

FIG. 5. Bacitracin increases amyloid deposition in RIN-m5F cells. RIN-m5F cells were treated with 0, 10, 100, or 1,000 nmol/l human amylin, as indicated. Cells were cultured in the absence (*left*) or presence (*right*) of the IDE inhibitor bacitracin (Bac). Deposition of islet amyloid was detected by Congo red fluorescence. Brightfield exposures of the same cells are shown in the insets. Magnification ×100.

impede receptor-mediated uptake of substrates (3). Rat amylin is also an IDE substrate (13), but it does not interact with insulin or cause membrane disruption. Rat amylin (100 nmol/l) inhibited insulin degradation to 49.7 ± 1.2% of baseline insulin degradation, a result similar to that found with human amylin, indicating that reciprocal inhibition of amylin and insulin degradation is attributable to a common pathway of proteolysis.

Because amylin is produced by healthy individuals without the formation of islet amyloid, a mechanism must be in place to prevent amylin aggregation under normal conditions. To examine whether cellular degradation by IDE may contribute to this protective process, a cell culture system to study islet amyloid formation was developed. RIN-m5F cells were cultured on poly-L-lysine-coated glass chamber slides for 3 days of culture, then treated with human amylin (10–1,000 nmol/l) for 48 h. Amyloid was detected by a modified Congo red fluorescence staining method (18), which includes an acidic iron hematoxylin counterstain that minimizes the intense autofluorescence seen using the original method (21). Human amylin induced a dosage-dependent increase in staining, detectable at 100 nmol/l and pronounced at 1 µmol/l (Fig. 5). This finding demonstrated that exogenous human amylin can form amyloid deposits in RIN-m5F cell cultures. When the cells were treated with the IDE inhibitor bacitracin, amyloid staining was markedly increased in intensity at all amylin concentrations compared with cells without bacitracin, including at the lowest amylin concentration (10 nmol/l). These findings suggest impairment of the cells' ability to clear amylin by a protease with characteristics consistent with IDE.

Because amylin has been implicated in β-cell toxicity, the effect of IDE inhibition on RIN-m5F cell viability was determined by MTT reduction (Fig. 6). Treatment with human amylin for 48 h decreased cell viability in a dosage-dependent manner, peaking at 44% with 1,000 nmol/l amylin. In the presence of bacitracin, the cell viability was markedly lower at all amylin concentrations,
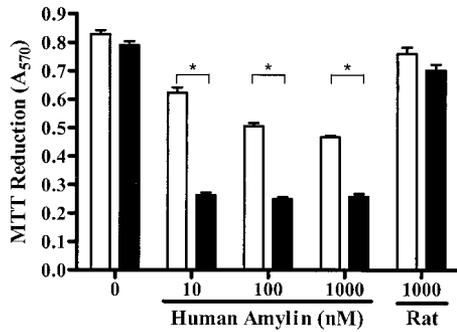
FIG. 6. Bacitracin increases amylin-induced cytotoxicity. RIN-m5F cells were treated with 0, 10, 100, or 1,000 nmol/l human amylin or 1,000 nmol/l rat amylin, as indicated. Cells were cultured in the absence (□) or presence (■) of the IDE inhibitor bacitracin. Cell toxicity was assessed by the degree of MTT reduction by the cells ($n = 6$). *$P < 0.01$.

reaching the maximum reduction (68%) at only 10 nmol/l amylin. Conversely, rat amylin had no effect on viability, whether with or without bacitracin. Therefore, an IDE inhibitor increased the ability of amylin to reduce RIN-m5F cell viability.

DISCUSSION

A pathologic feature of type 2 diabetes is the presence of islet amyloid deposits composed predominantly of amylin (1). Whether islet amyloid is merely a marker of type 2 diabetes or actually contributes to the disease is a matter of some discussion. Several lines of evidence suggest an association between the presence of islet amyloid and diabetes and reduced β-cell mass. In monkeys, the formation of islet amyloid correlates with early reduced insulin secretion and glucose intolerance, and, with time, correlates with greater deficiency in insulin secretion and glucose tolerance and ultimately hyperglycemia (22). In humans, the degree of amyloid formation correlates with the severity of diabetes, as evidenced by the requirement for insulin replacement therapy (23,24). Other evidence suggests that β-cell death is caused by intermediate-sized fibrils that are precursors of islet amyloid (3,4). In this case, islet amyloid deposits reflect an end-stage effect of amylin aggregation secondary to β-cell death.

The factors that prevent amylin fibril formation, β-cell death, and islet amyloid deposition in the nondisease state are not understood. Because amylin is continually produced in nondiabetic humans with no amyloid formation, the mere presence of amylin is insufficient to cause fibril formation. Indeed, transgenic animals overexpressing human amylin do not form amyloid deposits (5–7,25), confirming that elevated amylin levels alone are insufficient for amyloid formation. However, when diabetic conditions are introduced, such as the *ob* mutation, growth hormone/ steroid treatment, or a high-fat diet, these animals develop islet amyloid, islet dysfunction, reduced insulin secretion, and hyperglycemia (8–10). Therefore, in type 2 diabetes, the balance shifts from amylin clearance to deposition, either by increased amounts of a factor that promotes amylin aggregation or decreased amounts of a factor that clears amylin. Although there have been numerous studies regarding amylin synthesis and secretion, at present there have been few studies of amylin degradation.

The enzyme responsible for the intracellular degrada-

tion of insulin is IDE, a cytosolic metallothioprotease also known as insulysin (11,26). The primary function of IDE in mammalian tissues has traditionally been thought to be the degradation of insulin, but a number of other proteins have been identified as IDE substrates, including proteins structurally related to insulin, such as proinsulin, IGF-II, and relaxin, and seemingly unrelated peptides, such as ANP and glucagon (11). However, insulin, ANP, and glucagon all contain regions that can form β-pleated sheets and are potentially amyloid forming (27). It has been proposed that rather than targeting a primary sequence motif, IDE is specific toward amyloidogenic peptides (28), a concept supported by findings that IDE also degrades amylin and β-amyloid peptide (13,29,30). Recent studies have supported a role for IDE in type 2 diabetes and Alzheimer's disease, two conditions characterized by amyloid. Possible genetic linkages to type 2 diabetes and Alzheimer's disease have been mapped to the chromosomal region of the IDE gene (31–34). In the GK rat, a model of type 2 diabetes, IDE has been identified as a diabetes susceptibility gene; the mutations associated with this model result in lower cellular insulin degradation, hyperglycemia, reduced lipogenesis in fat cells, and reduced glucose uptake in vivo (35). The recently described IDE-null mouse model displays impaired insulin and β-amyloid degradation, as well as hyperinsulinemia, glucose intolerance, and cerebral accumulation of β-amyloid, hallmarks of both type 2 diabetes and Alzheimer's disease (36). Thus, the evidence is mounting that IDE plays a major role in diseases characterized by amyloid formation.

In a previous study using cell-free extracts (13), IDE readily degraded amylin, but with a lower affinity than for insulin. Radiolabeled amylin covalently cross-linked to IDE, and a monoclonal antibody directed against IDE immunoprecipitated both insulin- and amylin-degrading activities, confirming that IDE was the amylin-degrading protease. We have extended those findings to a rat β-cell–derived line. Both amylin and insulin were degraded by RIN-m5F cells, but insulin was degraded much more slowly. Possible reasons for this observation include differences in binding and internalization through the different receptors or differences in receptor availability (e.g., by desensitization or cell toxicity). The differences may also be attributed to the nature of the assay. Unlike amylin, multiple cleavages are required to produce TCA-soluble fragments of insulin, and therefore, this assay tends to underestimate the level of insulin degradation. Amylin degradation began quickly, then leveled off with time. The probable explanation for this observation is based on insulin secretion by the cells. The cells were changed into fresh media containing labeled substrates, so initially there was no endogenously produced insulin in the media. RIN cells rapidly secreted insulin (reaching 5–10 nmol/l in 1 h), which would act as a competitive inhibitor of amylin degradation. Because the amylin was present in tracer amounts, sufficient insulin to competitively inhibit amylin degradation may well have been produced within 30 min. Both insulin and amylin were capable of competitive inhibition of either substrate, with insulin in general a better competitive inhibitor than amylin. As discussed above, this is likely attributable to the greater affinity of

insulin for IDE and possibly also to the different internalization properties for insulin and amylin. The efficiency of unlabeled insulin inhibition of insulin degradation was consistent with that in previous reports showing a 50% inhibition of 50–100 nmol/l insulin using RIN-m5F cells (37). Taken together, the data suggest that insulin and amylin share a common pathway of degradation.

The specificity of amylin- and insulin-degrading activity was investigated using the IDE inhibitor bacitracin. Although bacitracin is not a specific inhibitor of IDE, other IDE inhibitors (e.g., N-ethylmaleimide and 1,10-phenanthroline) were highly toxic to RIN-m5F cells (R.G.B., unpublished observations), whereas bacitracin was well tolerated by the cells. Amylin and insulin degradation by RIN-m5F cells was markedly inhibited by bacitracin. Bacitracin also accelerated islet amyloid formation at all amylin concentrations, suggesting that inhibition of amylin degradation accelerates amylin aggregation and amyloid deposition. Because amylin has been implicated in β-cell death, cell viability was examined under the same conditions. Cytotoxicity induced by human amylin was markedly increased with bacitracin treatment, whereas rat amylin had no effect, suggesting that amylin degradation is responsible for reducing cell toxicity caused by amylin. Amylin-induced cytotoxicity in β-cells has been shown to be caused by intermediate amylin precursors rather than mature amyloid (3). If IDE degrades amylin monomers, then the formation of both intermediate precursors and amyloid fibrils would be prevented. The experiments performed here (48-h treatment of freshly prepared amylin) cannot determine which amylin species caused toxicity in RIN cells. However, the degree of cell death seen with bacitracin treatment was increased to the maximum attained, even at the lowest amylin concentration, which could mean that the toxicity preceded amyloid deposition. Although the amylin concentration required for detection of amyloid and cell toxicity (10–100 nmol/l) is high relative to circulating levels, the local amylin concentration around β-cells is unknown, but may well be higher, especially under conditions that stimulate insulin and amylin secretion.

In summary, RIN-m5F cells clear amylin through a bacitracin-sensitive mechanism with properties consistent with those of IDE. Inhibition of this mechanism increased amyloid formation and cell toxicity. These data are consistent with the emerging concept of a role for IDE in diseases characterized by amyloid. In this regard, it is interesting to note again that transgenic animals expressing human amylin in general do not develop islet amyloid unless diabetic conditions are introduced. One of these conditions is a high-fat diet (9). Interestingly, it has been shown that increased lipid levels result in impaired insulin degradation (38) and free fatty acids directly inhibit IDE activity (39). The combination of increased expression of amyloidogenic amylin and impaired insulin-degrading activity may result in the formation of islet amyloid in these models. Therefore, it is possible that conditions leading to impaired IDE activity (e.g., elevated free fatty acids) contribute to amylin accumulation and aggregation, β-cell toxicity, islet amyloid formation, and the progression of type 2 diabetes.

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