BACE2 plays a role in the insulin receptor trafficking in pancreatic β-cells

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BACE2 plays a role in the insulin receptor trafficking in pancreatic β-cells. Am J Physiol Endocrinol Metab 299:E1087–E1095, 2010. First published October 13, 2010; doi:10.1152/ajpendo.00420.2010.—BACE1 (β-site amyloidogenic cleavage of precursor protein-cleaving enzyme 1) is a β-secretase protein that plays a central role in the production of the β-amyloid peptide in the brain and is thought to be involved in the Alzheimer’s pathogenesis. In type 2 diabetes, amyloid deposition within the pancreatic islets is a pathophysiological hallmark, making crucial the study of the pancreas of BACE1 and its homologous BACE2 to understand the pathophysiological mechanisms of this disease. The objectives of the present study were to characterize the localization of BACE proteins in human pancreas and determine their function. High levels of BACE enzymatic activity were detected in human pancreas. In normal human pancreas, BACE1 was observed in endocrine as well as in exocrine pancreas, whereas BACE2 expression was restricted to β-cells. Intra- and intercellular analysis using immunofluorescence showed colocalization of BACE1 with insulin and BACE2 with clathrin-coated vesicles of the plasma membrane in MIN6 cells. When BACE1 and -2 were pharmacologically inhibited, BACE1 localization was not altered, whereas BACE2 content in clathrin-coated vesicles was increased. Insulin internalization rate was reduced, insulin receptor β-subunit (IRβ) expression was decreased at the plasma membrane and increased in the Golgi apparatus, and a significant reduction in insulin gene expression was detected. Similar results were obtained after specific BACE2 silencing in MIN6 cells. All these data point to a role for BACE2 in the IRβ trafficking and insulin signaling. In conclusion, BACE2 is hereby presented as an important enzyme in β-cell function.

β-site amyloidogenic cleavage of precursor protein-cleaving enzyme 1; β-secretase; pancreas; insulin receptor β-subunit trafficking

β-site APP-cleaving enzyme 1 (BACE1) has been identified as the β-secretase that mediates the primary amyloidogenic cleavage of precursor protein (APP) in Alzheimer’s disease (39). BACE2 is a close homologue of BACE1 that belongs to the same family of transmembrane aspartic proteases (34), but its role is still poorly understood. Analysis of the BACE1 and BACE2 gene sequences show high homology in the coding sequence but no similarity in the promoter regions (38); indeed, distinct expression patterns are found. BACE1 mRNA is detected at high levels in the pancreas, at moderate levels in the human brain, and at low levels in most peripheral tissues (36, 39, 43), whereas human BACE2 mRNA is found at very low levels in the brain and is more widely distributed in peripheral tissues (3). Although both BACE1 and BACE2 have been identified in the pancreas, their role in this tissue still remains to be elucidated.

It is worth noting that amyloid deposits have been found in pancreatic islets of type 2 diabetic patients (9). These amyloid deposits are formed by aggregation of the β-cell peptide known as the islet amyloid polypeptide and are related to the loss of β-cell mass that characterizes the disease (4). There is evidence that type 2 diabetes and Alzheimer’s disease share some common mechanisms related to amyloidogenesis; however, the mechanism of islet amyloid polypeptide aggregation is still poorly understood. Therefore, a possible involvement of BACE1 and BACE2 proteins in diabetes-associated amyloidogenesis is suggested, as in Alzheimer’s disease. Although BACE2 has been shown to cleave APP at the β-site in vitro, the cleavage is more efficient at sites within the amyloid-β-peptide (Aβ) region, which argues against its involvement in amyloid production (12).

The present report aims to characterize the localization of BACE proteins in the human pancreas and, by means of pharmacological inhibition, to determine whether modulation of their activity may have potential effects on pancreatic function. We show that the inhibition of β-secretase enzymatic function in pancreatic β-cells affects the intracellular trafficking of the insulin receptor (IR) as well as insulin gene expression and content, suggesting that this secretase plays a significant role in maintaining β-cell function. These studies are physiologically relevant, since secretase inhibitors used for the treatment of Alzheimer’s disease (18, 20) may have adverse effects on pancreatic function.

EXPERIMENTAL PROCEDURES

Human pancreas. Pancreases were obtained from eight nondiabetic human cadaveric organ donors (5 males and 3 females, 47 ± 12 yr old, body mass index, 26 ± 4), following informed family consent and approval by the Hospital Clinic de Barcelona Ethics Committee. Islets were isolated as described previously (7).

Cell culture. Mouse pancreatic β-cell line MIN6 was grown in DMEM at 5.5 mM glucose and supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO2.

Fluorometric BACE activity assay. Human islets and cell lysate aliquots containing 50 μg of protein were resuspended in 100 μl of assay buffer (50 mM Tris, 1.1 mM EDTA, 150 mM NaCl, 10 mM NaH2PO4, 10 mM FNa, and 1% Triton X-100, pH 4.2) and preincubated for 10 min at 37°C with or without 20 μM β-secretase inhibitor II (BI-II) (Calbiochem, San Diego, CA). After 20 μM of β-secretase VII fluorogenic substrate (Calbiochem) was added, substrate hydrolysis was monitored for 3–4 h at 37°C using a microplate spectrophotometer (SpectraMax GeminiXS; Molecular Devices, Sunnyvale, CA). Excitation and emission wavelengths were set at 320 and 420 nm, respectively. BI-II was dissolved in dimethyl sulfoxide (DMSO)
at 1 mM stock solution before use at 10 μM in culture for 24 h. One percent DMSO alone was used as a control.

**Antibodies.** Antibodies directed toward the following proteins were used: insulin (Dako, Glostrup, Denmark), IR β-subunit (IRβ; Santa Cruz Biotechnology, Santa Cruz, CA), glucagon, clathrin, β-actin (Sigma-Aldrich, St. Louis, MO), 130 kDa Golgi matrix protein (Gm130; BD Biosciences, San Jose, CA), Na+/K+-ATPase (Upstate Biotechnology, Lake Placid, NY), transferrin receptor (TFRC; Zymed Laboratories, San Francisco, CA), BACE1Δ58-501, and BACE2Δ496-511 (Calbiochem).

**Western blot.** Cell lyses aliquots of 50 μg of protein were separated by SDS-PAGE, transferred to polyvinylidene difluoride, and immunoblotted with selected antibodies. β-Actin (Sigma-Aldrich) was evaluated after every assay. Changes in protein levels were evaluated by Quantity One software (Bio-Rad Laboratories, Hercules, CA).

**Immunofluorescence and quantitative image analysis.** Human pancreas samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were deparaffinized and rehydrated in ethanol series. Antigen retrieval was performed through trypsin treatment (Sigma-Aldrich), and unspecific binding was blocked by incubation in 3% donkey serum (Jackson ImmunoResearch, Cambridge, UK). Fixed MIN6 cells were permeabilized and blocked with PBS containing 0.1% BSA and 0.1% Triton X-100 and PBS with 1% BSA, respectively. After overnight incubation at 4°C, primary antibodies were revealed with selected secondary antibodies. TO-PRO-3 (1 μg/ml; Invitrogen, Carlsbad, CA) was used for nuclear counterstaining. Images were captured using a fluorescence microscope (DMRB; Leica, Bensheim, Germany) or a Zeiss LSM510 confocal laser microscope equipped with an oil immersion ×63/NA1.3 objective. Micrographs shown are representative optical sections imaged through the center of the cell. Colocalization studies were performed as described previously (27), using ImageJ software (National Institutes of Health) and the Colocalization Highlighter plugin (P. Bourdoncle, Institute Jacques Monod, Service Imagerie, Paris, France).

**Insulin internalization.** Trypsinized cells were maintained for 90 min in suspension with assay binding buffer, and measurement of insulin internalization was performed with 125I-insulin (Millipore, St. Charles, MO), as reported previously (25). Cell-associated radioactivity represented insulin bound to the plasma membrane receptor and internalized radioactivity. After the cells had been preincubated at 37°C for 4 h in the presence or absence of 10 μM BI-II, as reported previously (1, 17, 31), they were rapidly cooled to 4°C through five different washings with PBS at 4°C to inhibit all intracellular processing and release of intracellular radioactivity. The binding buffer, titrated to pH 3.5, was added to the dishes and vigorously shaken at 4°C for 10 min. The cells were washed three more times with acidic binding buffer PBS at 4°C and three times with PBS at 4°C. Using this method, the non-acid-extractable radioactivity indicated internalized insulin. Control experiments to determine the efficiency of the method, the non-acid-extractable radioactivity indicated internalized radioactivity. The binding buffer, titrated to pH 3.5, was added to the dishes and vigorously shaken at 4°C for 10 min. The cells were washed three more times with acidic binding buffer PBS at 4°C and three times with PBS at 4°C. Using this method, the non-acid-extractable radioactivity indicated internalized radioactivity. Control experiments to determine the efficiency of the method, the non-acid-extractable radioactivity indicated internalized insulin. Control experiments to determine the efficiency of the method, the non-acid-extractable radioactivity indicated internalized insulin.

**Luciferase assay.** The vector pGL-LUC200 containing a 200-bp human insulin gene promoter fragment was kindly provided by Dr. Kevin Docherty, University of Aberdeen, Aberdeen, UK, and the vector pRL-CMV encoding Renilla reniformis luciferase under cytomegalovirus immediate early gene promoter control was obtained from Promega (Madison, WI). pGL2Luc200 and pRL-CMV at 1:400 were transfected into MIN6 cells using Lipofectamine 2000 (Invitrogen). Renilla and luciferase activities were measured in a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany). Luciferase activity values were normalized to those of renilla.

**Insulin content.** Cells were disintegrated by sonication at 4°C in acid ethanol solution (75% ethanol, 0.15 N HCl). Cell lysates were centrifuged for 30 min at 3,500 rpm, and supernatants were kept at −80°C until use. Insulin content was assayed by Ultrasensitive Insulin ELISA (Merdocia, Uppsala, Sweden).

**Real-time PCR.** cDNA was synthesized from 1 μg RNA using the First-Strand cDNA Synthesis Kit for RT-PCR (avian myeloblastosis virus; Roche Diagnostics, Mannheim, Germany). Mouse insulin, **BACE1**, and **BACE2** genes were amplified using SYBR Green PCR Core Reagents (Applied Biosystems) with primers 5′-CTGCCATACCTGAGTCCGACA-3′ and 5′-TCATAATACCATCCTCCCGC-3′, 5′-TGGACGGCGGAGTGAAT-3′ and 5′-AGCTTTGATGGCCTGCTG-3′, 5′-GCAAAACTGGACTGGT-3′, 5′-ATGCTGGTGCCACGACTGTC-3′, and 5′-CCTCTGAGAAAGTGCGATGT-3′, respectively. PCR was run with 1 ng of cDNA using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. Expression levels were normalized to 18S rRNA (Applied Biosystems) and expressed in arbitrary units.

**RNA interference.** siRNA experiments were performed with short-hairpin RNA lentiviral transduction particles obtained from Sigma-Aldrich for mouse **BACE1** and **BACE2**. Infections were carried out according to the manufacturer’s instructions. Cells were processed and analyzed after 48 h of infection. Cultures infected with lentiviral transduction particles for nontargeting siRNA were used as a control, whereas those for TurboGFP were used to calculate infection efficiency.

**Statistical analysis.** Data are presented as means ± SE. Comparisons were made by t-test, except when variables were nonnormally distributed, in which case the U-test was used.

**RESULTS**

**BACE enzymatic activity and expression in the human pancreas.** Significant levels of BACE enzymatic activity were detected in protein extracts of human pancreatic islets and exocrine tissues when tested in a fluorometric β-secretase proteolytic activity assay (Fig. 1A). Therefore, we sought to determine the cell types expressing **BACE** in the human pancreas. Western blots with anti-BACE antibodies detected **BACE1** expression indistinctly in human pancreatic islets and exocrine tissue extracts, whereas **BACE2** expression was observed only in extracts from human islets (Fig. 1B). The Western blot analysis was confirmed by the immunohistochemical results, revealing **BACE1** in human islets and exocrine tissue and **BACE2** in the islets (Fig. 1C). Furthermore, in human islets, **BACE1** was localized in both α- and β-cells, as shown by the costaining with glucagon and insulin (Fig. 1D), whereas **BACE2** only colocalized with insulin, indicating that it was present in β-cells, and was almost undetected in α-cells (Fig. 1E).

**BACE1 and BACE2 are located in different compartments of β-cells.** To analyze the intracellular localization of **BACE**, we used MIN6 cells. This murine pancreatic β-cell line showed β-secretase enzymatic activity and expression of both **BACE1** and **BACE2** proteins (Fig. 2, A and B). Immunofluorescence in MIN6 cells revealed that BACE proteins were distributed in different intracellular compartments. **BACE1** expression was largely colocalized with insulin, whereas no coexpression of **BACE2** with this marker was detected (Fig. 2C). Moreover, **BACE2** showed random spotting colocalization with the plasma membrane marker Na+/K+-ATPase and a large colocalization with clathrin (Fig. 2D), which indicates that it is expressed specifically in endocytic vesicles. No staining of **BACE1** at the plasma membrane level (Na+/K+-ATPase marker) was highlighted (Fig. 2D).

**E1088 THE ROLE OF BACE2 IN THE IR TRAFFICKING**
Inhibition of β-secretase activity affects BACE2 intracellular localization. To study the role of BACE on β-cells, we used a selective and fast cell-permeable substrate-based inhibitor designed from the β-secretase cleavage sites named BI-II (1, 15). Localization of BACE proteins in MIN6 cells treated for 24 h with 10 μM BI-II was studied by immunofluorescence. BACE2 content in plasma membrane (Fig. 3A) and clathrin-coated vesicles (Fig. 3B) was increased significantly.
compared with the control (from 4.8 ± 0.8 to 9.7 ± 2.6%, \( P < 0.05 \), and from 14.0 ± 2.6 to 26.2 ± 1.9%, \( P < 0.001 \), respectively; Fig. 3E). In contrast, no significant changes in BACE1 intracellular colocalization with insulin were observed between treated and nontreated MIN6 cells (14.6 ± 4.6 and 12.7 ± 3.2%, respectively; Fig. 3, C and E). MIN6 protein extracts immunoblotted for anti-BACE1 and anti-BACE2 showed no protein content alteration in BI-II-treated cells with
respect to untreated cells (Fig. 3D). These results indicate that the inhibition of β-secretase activity affects BACE2 intracellular localization but not that of BACE1.

**β-Secretase activity is required to maintain β-cell function.** Studies involving different in vitro model systems show that, in addition to transducing insulin signal, the IR mediates the internalization of the hormone by receptor-mediated endocytosis, a multistep process that also requires the redistribution of insulin receptor complexes at the level of the clathrin-coated pits (6). The coexpression of BACE2 with clathrin suggests a possible involvement of this secretase in the IR-mediated insulin internalization and/or recycling by clathrin-coated vesicles. For this reason, we focused our next analysis on the study of BACE2 control over the β-subunit of the IRβ trafficking.

We first observed that the 125I-insulin internalization rate after glucose stimulation is reduced in BI-II-treated MIN6 cells compared with the control (from 0.30 ± 0.02 to 0.23 ± 0.02%, P < 0.05; Fig. 4A). Despite a decreased insulin internalization rate, the evaluation of IRβ expression revealed a twofold increase in protein extracts (Fig. 4B). Then, the localization of IRβ was studied by immunofluorescence. Treatment of MIN6 cells with BI-II induced a significant decrease in receptor expression at the plasma membrane compared with the control (from 4.9 ± 1.9 to 0.3 ± 0.01%, P < 0.05), with the receptor pool being retained in the pericentrosomal region (Fig. 4C). The same pattern of intracellularly retained IRβ was observed in MIN6 cells treated with BACE2-siRNA (Fig. 4D). Colocalization studies revealed that the intracellularly retained IRβ was located within the Golgi apparatus, as indicated by the expression of the trans-Golgi apparatus marker Gm130 (Fig. 4E). No evidence of colocalization was detected by the recycling endosome marker TFRC (Fig. 4F).

It is known that IR signaling acts as an autocrine β-cell regulator, influencing insulin gene expression and protein control (16). For this reason, since we have observed changes in the IR trafficking, we studied the BACE role in insulin expression and content. MIN6 cells and human islets treated with BI-II showed a decrease in the insulin protein content (Fig. 5A). Insulin gene promoter activity was then analyzed by transfecting MIN6 cells with the pGL-Luc200 luciferase report construct. Transcriptional activity of the insulin promoter in cells treated with β-secretase inhibitor was decreased significantly.
compared with controls (Fig. 5B). Then, a time course studying insulin content (Fig. 5C) and transcription (Fig. 5D) was performed, observing a 1.4-fold reduction in insulin content after 4 h of treatment and a 1.3- to 4.0-fold decrease in insulin mRNA after 2–24 h of treatment. Notably, the effects observed by the BI-II inhibitor are mimicked after MIN6 treatment with siBACE2 but not with siBACE1, detecting a twofold decrease in insulin expression after BACE2 silencing (Fig. 5E).

siRNA’s efficiency was assessed by analyzing both gene and protein downregulations (Fig. 5, F and G). These results indicate that inhibition of BACE enzymatic activity in H9292-cells decreases insulin gene expression by affecting the insulin-signaling pathway, and they suggest that BACE2 was the enzyme responsible for the observed effects.

DISCUSSION

BACE1 and BACE2 have been reported to be expressed in murine and human pancreas, but until now their potential role in pancreatic physiology has not been investigated thoroughly. Moreover, the investigations on BACE1 and BACE2 knockout mice have been focused on central nervous system and behavioral alterations, except for a general histological evaluation of nonneural tissues that did not unveil any abnormalities at the pancreatic level (10, 28, 30, 32). In the present study, we localized both proteins in different β-cell compartments and demonstrated that the inhibition of β-secretase enzymatic activity in pancreatic β-cells affects the IR intracellular trafficking and leads to downregulation of insulin gene expression.

Our colocalization studies highlighted widespread BACE1 expression in human pancreatic exocrine and endocrine tissues, whereas BACE2 was restricted to islet β-cells. The specificity of BACE2 in β-cells had not been detected in a previous study that evaluated the human pancreatic expression of BACE1 and BACE2 mRNAs using in situ hybridizations and Northern blot analysis (3, 13). However, in our study, we determined that different spliced forms of both BACE1 and BACE2 coexist within human islets (data not shown), as shown previously for BACE1 (11), which may explain these discrepancies. Our data are in accordance with more recent reports (14) that have found specific BACE2 protein expression in rodent β-cells. Nevertheless, although we detected that BACE1 colocalizes with insulin in the human pancreas and BACE2 colocalizes with clathrin-coated vesicles, Finzi et al. (14) have not examined BACE1 localization and have detected BACE2 in secretory granules of rodent pancreases. In this respect, the different experimental models and epitope specificity of the antibodies used may account for these discrepancies.
BACE proteins are synthesized in the endoplasmic reticulum as precursors, and proBACE proteins are short-lived and undergo rapid maturation into ∼70-KDa forms in the Golgi apparatus (5). Moreover, BACE proteins were previously described as cycling between different compartments of the secretory pathway in varying proportions in the trans-Golgi, plasma membrane, and endosomes (5, 19, 44). In agreement with this finding, our colocalization studies, which were performed on a pancreatic cell line, clearly showed a differentiated expression pattern, with BACE1 being located mostly in the secretory pathway and BACE2 in the endocytic pathway. This distinct cell distribution of BACE1 and BACE2 suggests different functions of these proteins within the human pancreas and provides valuable data to help understand the role of BACE proteins in pancreatic ß-cells.

The transcriptional regulation of BACE genes has been shown to differ significantly, and, compared with that of BACE1, the BACE2 promoter is partially suppressed in normal neural cells and is likely to be highly regulated and expressed in a tissue type-specific manner (24, 38). Moreover, several putative transcription factor sites, particularly hepatocyte nuclear factor-1α (HNF1α; TCF1), HNF3β, epidermal growth factor receptor, peroxisome proliferator-activated receptor, and HES1, were predicted to be present within the BACE2 promoter (29), and interestingly, most of these are widely recognized as required factors for pancreatic ß-cell function (35), which could explain the enriched BACE2 expression in ß-cells.

It has been demonstrated the proamyloidogenic effect of BACE1 secretase activity in human brain tissue, where it is responsible for the proteolytic processing of the APP in cytotoxic Aβ in Alzheimer’s disease (8). However, BACE2 in neuronal cells seem to cleave at the level of nonamyloidogenic APP pathways, suggesting that BACE2 plays a protective role in neural amyloid deposition (37). Besides their amyloidogenic role, BACE proteins have been implicated in the proteolytic processing of several type I transmembrane proteins with a receptor-like structure, suggesting that they could participate in the process of receptor ectodomain shedding (21, 22, 26, 33, 40, 42). Moreover, it has been reported that, unlike BACE1, BACE2 propeptides require autocatalytic prodomain processing of several type I transmembrane proteins but might also constitute a way for their enzymatic activation (19). Furthermore, interference in the proteolytic shedding of transmembrane proteins by way of BACE enzymatic inhibition may not just block the secretion of protein fragments but might also constitute a way of deregulating transmembrane protein function.

The coexpression of BACE2 with clathrin suggests a possible involvement of these secretases in the IR-mediated insulin internalization and/or recycling by clathrin-coated vesicles. Studies involving different in vitro model systems have shown that, in addition to insulin signal transduction, IR-mediated internalization of the hormone by a receptor-mediated endocytosis takes place, a multistep process that also requires the redistribution of IR complexes at the level of the clathrin-coated pits (6, 16). Endocytosis of the IR complex leads to insulin degradation, whereas most of the unoccupied receptors...
are recycled to the plasma membrane. After prolonged insulin stimulation, the receptor itself is degraded, resulting in receptor downregulation and attenuation of the insulin signaling. Internalized insulin receptors are catalytically active kinases, suggesting that insulin-stimulated internalization is important for signal transduction (41). Employing a pharmacological strategy, we showed that, by inhibiting β-secretase activity, insulin internalization is reduced and that IRβ accumulates in the Golgi apparatus. Moreover, besides impairing IRβ trafficking to the plasma membrane, the inhibition of proteolytic activity seriously affects insulin gene expression and consequently insulin content, probably due to an interruption of the insulin-signaling cascade. Since BACE2 localization, but not that of BACE1, is affected when β-secretase activity is inhibited, BACE2 is most likely the enzyme involved in the IR trafficking alterations observed. To confirm the specific involvement of BACE2, we used a lentiviral construct to silence BACE2, which showed the same effects on IRβ trafficking and insulin gene expression as the pharmacological inhibition strategy.

The data presented here suggest a role of BACE2 in the insulin receptor trafficking, which is a critical step in the insulin signaling and sensitivity, since it has been reported that this might function as a sensor in the plasma membrane to signal mobilization of the IR. Correct intracellular trafficking of the IR is critical for insulin signaling and sensitivity, since it has been reported that impaired IR transport across the plasma membrane leads to diabetes in humans (2, 23). On the whole, our data indicate that BACE2 is possibly an enzyme essential to β-cell function. Future studies aimed to identify BACE1 and BACE2 intracellular pathways will help to elucidate the role of these two proteases and provide further knowledge about the regulation of β-cell function. Such studies are needed since the secretase inhibitors under development for the treatment of Alzheimer’s disease, particularly those that target BACE proteins (18, 20), may have adverse effects on pancreatic function and, therefore, on glycemic control.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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