Partial Loss-of-Function Mutations in Insulin-Degrading Enzyme that Induce Diabetes also Impair Degradation of Amyloid β -Protein

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The causes of cerebral accumulation of amyloid β -protein ($A\beta$) in most cases of Alzheimer's disease (AD) remain unknown. We recently found that homozygous deletion of the insulin-degrading enzyme (IDE) gene in mice results in an early and marked elevation of cerebral $A\beta$. Both genetic linkage and allelic association in the IDE region of chromosome 10 have been reported in families with late-onset AD. For IDE to remain a valid candidate gene for late-onset AD on functional grounds, it must be shown that partial loss of function of IDE can still alter $A\beta$ degradation, but without causing early, severe elevation of brain $A\beta$. Here, we show that naturally occurring IDE missense mutations in a well-characterized rat model of type 2 diabetes mellitus (DM2) result in decreased catalytic efficiency and a significant ~15 to 30% deficit in the degradation of both insulin and $A\beta$. Endogenously secreted $A\beta_{40}$ and $A\beta_{42}$ are significantly elevated in primary neuronal cultures from animals with the IDE mutations, but there is no increase in steady-state levels of rodent $A\beta$ in the brain up to age 14 months. We conclude that naturally occurring, partial loss-of-function mutations in IDE sufficient to cause DM2 also impair neuronal regulation of $A\beta$ levels, but the brain can apparently compensate for the partial deficit during the life span of the rat. Our findings have relevance for the emerging genetic evidence suggesting that IDE may be a late-onset AD-risk gene, and for the epidemiological relationships among hyperinsulinemia, DM2, and AD. (*Am J Pathol* 2004, 164:1425–1434)

Cerebral accumulation of amyloid β -protein ($A\beta$) is a pathological hallmark of Alzheimer's disease (AD). Although an impressive number of studies support a central role for $A\beta$ in the pathogenesis of this very common neurodegenerative disorder, the underlying causes for gradual accumulation of the peptide are unknown in the vast majority of cases. Overproduction of $A\beta$ has been implicated in only a small portion of cases of early-onset familial AD, representing <2% of the entire AD population.¹ Newly generated $A\beta$ is rapidly cleared from the brain,^{2,3} suggesting that $A\beta$ -degrading proteases could play a critical role in regulating cerebral levels of the peptide. Although much work has focused on the generation of $A\beta$, relatively little is known about $A\beta$ proteolysis, which could be as or more important in the pathogenesis and potential prevention of AD.

Several proteases have been shown capable of degrading $A\beta$, including insulin-degrading enzyme (IDE, insulysin; EC 3.4.24.56),^{4–8} neprilysin (NEP),^{9,10} endothelin-converting enzyme,¹¹ the uPA/tPA/plasminogen system,^{12,13} and angiotensin-converting enzyme.¹⁴ Mice with experimental deletions of the NEP,¹⁵ endothelin-converting enzyme,¹⁶ or IDE¹⁷ genes have elevated levels of endogenous cerebral $A\beta$ of similar magnitude (~60 to 100%). These animal models validate the role of proteolysis in the regulation of cerebral $A\beta$ levels *in vivo*.

IDE, an ~110-kD thiol zinc metalloendopeptidase located in cytosol, peroxisomes, endosomes, and on the cell surface,^{8,18–20} cleaves small proteins of diverse sequence, several of which share a propensity to form β -pleated sheet-rich amyloid fibrils, including insulin, $A\beta$, amylin, atrial natriuretic factor, and calcitonin.^{21,22} An unbiased screen of various cell lines for $A\beta$ -degrading activity revealed that the major such activity was attributable to IDE.^{6,7} Direct comparison of IDE and NEP activity by transfecting their respective cDNAs into stable human APP-expressing cell lines showed that IDE markedly reduced the levels of soluble and insoluble intracellular $A\beta$,

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whereas NEP lowered the levels of only insoluble intracellular $A\beta$.²³ It is not yet fully established which pool of $A\beta$ is more pathogenic, although recent studies suggest that cognitive dysfunction in AD patients correlates more tightly with soluble than insoluble levels of cortical $A\beta$.^{24,25} It has been demonstrated that proteolysis of $A\beta$ by IDE eliminates the neurotoxic effects of the peptide in rat primary neuronal cultures and prevents the deposition of $A\beta$ onto synthetic amyloid aggregates.²⁶ Recently, it has been reported that levels of IDE protein and transcripts are reduced in the hippocampi from AD patients with an apolipoprotein E- ϵ 4 allele compared to either AD patients without this AD-risk allele or normal patients (with or without the allele).²⁷

Consistent with a possible role for $A\beta$ -degrading proteases in human disease, the IDE region of chromosome 10q has been shown to have genetic linkage to late-onset (conventional) AD²⁸ and to age of disease onset in AD families.²⁹ Some studies have also reported allelic association in or near the IDE gene with late-onset AD,^{28,30,31} whereas others have not.^{32,33} In separate reports, the occurrence of AD³⁴ and elevated plasma $A\beta_{42}$ levels³⁵ were each linked to a region of chromosome 10q ~40 Mb centromeric to the linkage peaks over the IDE region. It remains unclear whether these results signify the same underlying locus or two separate loci.

The IDE region of chromosome 10q has also been genetically linked to type 2 diabetes mellitus (DM2)^{36–38} and to fasting glucose levels (20-year means).^{38,39} In this regard, it was shown that transferring a ~3.7-cM chromosomal region containing the IDE gene from an inbred rat model of DM2 [Goto-Kakizaki (GK) rat] to a normoglycemic rat recapitulated several features of the diabetic phenotype, including hyperinsulinemia and postprandial hyperglycemia.⁴⁰ The GK allele of IDE in this chromosomal region was found to bear two missense mutations that, when transfected into COS-1 cells, resulted in 31% less insulin degradation by these cells than by cells transfected with the wild-type (WT) allele. Targeted homozygous deletion of the IDE gene in mice, in addition to elevating cerebral levels of $A\beta$, also produced hyperinsulinemia and glucose intolerance in the animals.¹⁷ Thus, although the gene(s) responsible for the linkage and association of AD and DM2 to chromosome 10q has not been identified, the IDE gene is a leading candidate in both diseases.^{37,41} In this regard, there is growing evidence that DM2 and, in particular, hyperinsulinemia are associated with an increased risk of developing AD (see Discussion).

Although it is now clear that experimental deletion of the IDE gene results in acute elevations of cerebral $A\beta$, it is not known whether naturally occurring, partial loss-of-function mutations in IDE can affect $A\beta$ catabolism, and, if they do, whether the effect is subtle enough to be compatible with the genetic evidence that IDE may be a risk-conferring gene for late-onset AD. It is possible that, in the pertinent neuronal compartments, the protease is in sufficient excess relative to its $A\beta$ substrate so that mild hypofunction of IDE will not alter $A\beta$ turnover, or that there is a compensatory increase in the levels of the hypofunctional IDE or another $A\beta$ -degrading protease such that

there is no net effect on $A\beta$ catabolism. For IDE to remain a valid candidate gene for late-onset AD risk, as suggested by the emerging genetic evidence, not only must it be shown that partial loss of IDE function is physiologically relevant, but also that it does not cause an early and severe elevation of $A\beta$ levels. The magnitude of IDE hypofunction in humans would be expected to be significantly less severe than that of the IDE gene-deleted mice, with their complete absence of IDE function and >60% increases in cerebral $A\beta$ by only 12 weeks of age.

In this study, we take advantage of the GK rat to ascertain whether, and to what extent, naturally occurring, partial loss-of-function mutations in IDE that are sufficient to cause features of the DM2 phenotype⁴⁰ can also affect $A\beta$ turnover and alter levels of $A\beta$ in neuronal cultures and brain. Based on our findings, we discuss the potential relationship among mutations in IDE, DM2, and AD.

Materials and Methods

Sequencing of Rat IDE cDNA

GK and Wistar rats were purchased from Taconic M&B (Denmark) and Charles River Labs (Wilmington, MA), respectively. IDE cDNA was amplified from rat liver by reverse transcriptase-polymerase chain reaction with gene-specific primers, ligated into a pcDNA5/FRT (Invitrogen, Carlsbad, CA) vector, and sequenced using an ABI PRISM 377 sequencer.

Preparation and Immunoblotting of Brain Fractions

Fresh-frozen rat cerebral hemispheres were homogenized in 8 vol (w:v) of 250 mmol/L sucrose in 50 mmol/L Tris-HCl (pH 7.4) in a Potter-Elvehjem homogenizer. After pelleting nuclei and unbroken cells, the supernatant was spun again at 100,000 $\times g$ for 1 hour. This resulting supernatant was saved as the soluble fraction, and the membrane pellet was washed in 100 mmol/L Na_2CO_3 (pH 11.3) to linearize microsomes and strip loosely associated proteins.⁴² The membranes were pelleted again, resuspended in 50 mmol/L Tris-HCl (pH 7.4), and sonicated. Protein concentrations were determined using a bicinchoninic acid-based protein assay (Pierce, Rockford, IL). For immunoblotting, membranes were probed with antibodies raised to: IDE (IDE-1),⁸ NEP (56C6; Novacastra, Newcastle, UK); amyloid precursor protein and its proteolytic derivatives C83, C99 (antibody C8),⁴³ and the secreted APP fragment (22C11; Chemicon, Temecula, CA); presenilin N-terminal (Ab14, gift from S. Gandy, Thomas Jefferson University) and C-terminal (4627)⁴⁴ fragments; BACE (Ab-2; Oncogene), and ADAM10 (AB19026, Chemicon).

Primary Cell Cultures

For primary fibroblasts, tissue from the abdominal wall and penis of adult rats were minced and partially digested with 0.25% trypsin in Hanks' balanced salt solu-

tion. The dissociated cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. For primary cortical neuronal cultures, neocortices and hippocampi from postnatal day 0 rat litters were partially digested in 0.025% trypsin in Hanks' balanced salt solution, 10 mmol/L HEPES, 10 mmol/L glucose, and 10 mmol/L sucrose, mechanically dissociated, and plated at varying densities onto poly-L-lysine-coated dishes. Cells were maintained in a neuronal-selecting medium (Neurobasal medium with the growth supplement B27; Invitrogen).

Trichloroacetic Acid (TCA) Precipitation Degradation Assays

To measure proteolysis by brain fractions, 100 pmol/L synthetic ^{125}I -A β_{1-40} (human or rodent) (Amersham, Arlington Heights, IL), or 100 pmol/L recombinant human ^{125}I -insulin (Amersham) was incubated at 37°C with 100 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$ of protein, respectively. To determine rates of proteolysis by intact primary fibroblast (22,000 cells/cm²) and neuronal cultures (80,000 and 1,600,000 cells/cm²), cells were washed with serum-free and supplement-free medium (Dulbecco's modified Eagle's medium and Neurobasal, respectively) containing 0.1% bovine serum albumin, then incubated in the same medium with 40 pmol/L of either ^{125}I -insulin or ^{125}I -A β_{1-40} . At each time point, an aliquot of the sample was added to an equal volume of 15% TCA to allow the uncleaved peptides to precipitate. After centrifugation, gamma counts in the TCA-insoluble pellet (undegraded peptide) and TCA supernatant (degraded peptide fragments) were determined, and the percentage of radiolabeled substrate degraded was calculated.

Quantification of Endogenous A β

After primary neurons were allowed to condition Neurobasal media with B27 supplement, the media were removed, 1,10-phenanthroline and a protease inhibitor cocktail (Sigma, St. Louis, MO) were added, and the samples were centrifuged to remove any cellular material. A β was extracted from brain using either the previously described diethylamine protocol^{2,45} or Tris-buffered saline (TBS)/guanidine HCl. In brief, we homogenized cerebral hemispheres in either 9 vol (w/v) 0.2% diethylamine in 50 mmol/L NaCl or 5 vol (w/v) of TBS with the above protease inhibitors using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 100,000 $\times g$ for 1 hour. Supernatants from the diethylamine extractions were neutralized with 1/10 volume 500 mmol/L Tris-HCl (pH 6.8), and the pellets were discarded. Supernatants from the TBS extraction were saved as soluble A β , while the associated pellets were resuspended in a 5-mol/L guanidine HCl and 50-mmol/L Tris-HCl (pH 8.0) buffer and saved as insoluble A β . A β enzyme-linked immunosorbent assays (ELISAs) on neuronal media and DEA brain extracts were performed using the well-characterized BNT-77/BA-27 (A β X-40) and BNT-77/BC-05 (A β X-42) system.⁴⁶ ELISAs on the guanidine HCl and TBS brain extracts and confirmatory ELISAs on the neuronal

media were done with the 2G3/4G8 (A β X-40) and 21F12/4G8 ELISA system (A β X-42).⁴⁷

Transfection of CHO Cells

Equal numbers CHO cells stably expressing human APP₇₅₁ containing the V717F AD-causing missense mutation were transiently transfected with empty vector or vectors containing either the GK or WT IDE allele (as generated above) using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were washed and allowed to condition fresh media for 24 hours (OptiMEM, Invitrogen). The media was then treated identically to the neuronal conditioned media as above, except a different A β ELISA system was used.^{48,49} Thirty-six hours after transfection, cell lysates were made from duplicate dishes of the three transfected constructs, and IDE overexpression was confirmed by immunoblotting.

IDE Immunodepletion

Brain-soluble fractions were prepared as above without protease inhibitors. For each sample, 400 μg of brain-soluble protein were first precleared with protein G-agarose resin (Roche, Indianapolis, IN), then incubated with fresh resin and either no immunoglobulin (sham immunodepletion controls) or the anti-IDE monoclonal antibody 9B12 (titer of 1:100) (gift from R. Roth, Stanford University) at 4°C for 12 hours. The resin was then pelleted by centrifugation, and the IDE-immunodepleted supernatant was saved for A β degradation assays.

Results

We first confirmed that our GK rats, a strain generated by inbreeding Wistar rats having the highest glucose levels on a glucose tolerance test,⁵⁰ carried the three single nucleotide polymorphisms in IDE previously reported⁴⁰ and that our wild-type Wistar rats did not. We cloned and sequenced the complete coding regions of IDE cDNAs from two rats of each strain. As expected, the GK rats had three nucleotide differences from the Wistar controls: CAC→CGC at codon 18 (H18R), GCG→GTC at codon 890 (A890V), and GAT→GAC at codon 934 (silent). The WT Wistar sequence was identical to that in GenBank (accession no. NM 013159). Protein levels of IDE, neprilysin, APP, the APP proteolytic derivatives (C83, C99, secreted APP fragment, and AICD), presenilin NTF and CTF, BACE, and ADAM 10 were indistinguishable by immunoblotting in GK and control rat brain fractions and primary neuronal lysates (not shown).

To initially assess degradative capacity for insulin and A β in intact, living cells of the GK rat, we incubated primary cultured fibroblasts of GK and WT rats with physiological levels (40 pmol/L) of radioiodinated human insulin or human A β_{1-40} in serum-free medium for 0, 3, 6, and 24 hours and then performed TCA precipitation assays at each time point as described in Materials and Methods. We found that the GK fibroblasts degraded

Figure 1. Insulin and A β proteolysis in primary fibroblast cultures of WT control and GK rats. Intact fibroblasts from two GK and two WT rats were incubated in serum-free medium with 40 pmol/L of either human 125 I-insulin (**A**) or human 125 I-A β_{1-40} (**B**). At the indicated times, degradation was quantified by TCA precipitation assays and reported as the percentage of radiolabeled substrate degraded. Graph points represent means, and error bars indicate SEM of 12 determinations (duplicate cultures from each of two rats analyzed in three independent assays; *, $P < 0.01$). The inset tables show the percent decrease in the GK proteolysis compared to the WT control at each time point after normalization to the mean WT percentage degradation in each assay and then combination of the data sets.

insulin $\sim 24\%$ less well than the controls ($P < 0.005$) (Figure 1A), in close agreement with the deficit in insulin degradation ($\sim 21\%$) reported in isolated muscle fibers from rats with the GK IDE allele.⁴⁰ The GK fibroblasts also degraded A β $\sim 20\%$ less than controls ($P < 0.01$) (Figure 1B). Unlabeled insulin (10 μ mol/L), a potent and relatively selective competitive inhibitor of IDE, blocked $\sim 90\%$ of A β degradation in both the GK and control cultures, indicating that virtually all A β proteolysis by these intact cells is attributable to IDE.

To extend this finding to the brain, the principal site of A β production and accumulation, we measured insulin and A β proteolysis in both brain-soluble and Na $_2$ CO $_3$ -washed membrane fractions from GK and WT rats. Brain membranes represent a physiological locus for IDE-mediated A β degradation, because A β , like IDE, is present at the cell surface and in endosomes.⁵¹ For insulin degradation assays, 50 μ g/ml of protein was incubated with 100 pmol/L 125 I-insulin for 0, 1, and 3 hours for membranes or 0, 15, and 30 minutes for the soluble fractions. For A β degradation assays, 100 μ g/ml of protein was

incubated with 100 pmol/L 125 I-A β_{40} for 0, 3, and 8 hours for membranes or 0, 30, and 60 minutes for the soluble fractions. The GK brain membranes showed a highly significant $\sim 30\%$ decrement in the degradation of both substrates at both time points. At 1 and 3 hours, insulin degradation in the WT rats was 37% and 60%, respectively, whereas in the GK rats, it was 24% and 43% ($P < 0.01$ at 1 hour, $P < 0.001$ at 3 hours) (Figure 2A). At 3 and 8 hours, the WT A β proteolysis was 35% and 59%, respectively, while in the GK it was 25% and 40% ($P < 0.00001$ for each time point). The GK brain-soluble fraction also showed degradation deficits. Insulin proteolysis was decreased 26% at 15 minutes ($P < 0.01$) and 19% at 30 minutes ($P < 0.0001$), whereas parallel A β assays showed a 14%, highly significant ($P < 0.00001$) decrease at both 30 and 60 minutes (Figure 2B).

To assess the relative roles of IDE and other proteases in A β degradation and acquire supportive evidence that IDE is responsible for the A β proteolytic dysfunction in GK rats, we examined the protease inhibitor profiles of the brain fractions (Figure 2C). In washed brain membranes, insulin inhibited 77% of control and 71% of GK-mediated degradation, resulting in identical residual proteolysis in the two genotypes, as would be expected if the GK deficit is because of IDE. Glucagon (10 μ mol/L), a less avid competitive inhibitor of IDE than insulin, prevented $\sim 60\%$ of the A β degradation. Thiorphan (1 mmol/L), a potent inhibitor of NEP and angiotensin-converting enzyme, and an inhibitor cocktail containing only serine and cysteine protease inhibitors (Roche) (not shown) each blocked only $\sim 15\%$ of the degradation. In the presence of thiorphan and the serine and cysteine protease inhibitors, the GK membrane fraction continued to show a significant decrease in A β proteolysis, again supporting the attribution of this deficit to IDE. 1,10-Phenanthroline (2 mmol/L), a potent zinc metalloprotease inhibitor that can inhibit both IDE and NEP, prevented $>80\%$ of the A β proteolysis. In the brain-soluble fraction, A β degradation was inhibited $>96\%$ by either insulin or 1,10-phenanthroline, $\sim 75\%$ by glucagon, $<5\%$ by thiorphan, and not at all by the serine and cysteine protease inhibitor cocktail (data not shown). Degradation assays done in brain membranes and soluble fractions using rodent 125 I-A β_{40} iodinated at two different sites than the human peptide used above (ie, either His6 or His14 instead of Tyr10) yielded similar degradation deficits in the GK rats (not shown). These results indicate that the GK A β degradation deficit extends to the rodent peptide, with its three amino acid differences compared with human A β , and that the site of iodination does not affect the results of our A β degradation assays.

Next, we quantified insulin and A β proteolysis in intact primary cortical neurons. Neocortical cells from two GK and two WT litters were incubated with 40 pmol/L of 125 I-insulin or 125 I-A β_{40} in serum-free medium for 0, 3, 6, and 24 hours and TCA precipitation assays were performed. Overall, 125 I-insulin proteolysis ranged from $\sim 45\%$ at 3 hours to $\sim 85\%$ at 24 hours, whereas 125 I-A β degradation ranged from $\sim 10\%$ at 3 hours to $\sim 65\%$ at 24 hours. The GK neurons demonstrated less insulin and A β proteolysis than WT neurons at each time point, with the

Figure 2. Insulin and A β proteolysis in brain fractions of WT control and GK rats. Washed brain membranes and soluble fractions were incubated with 100 pmol/L of 125 I-insulin or 125 I-A β_{1-40} , and degradation was determined by the TCA precipitation assay. **A:** Insulin and A β degradation in brain membranes from three WT and four GK rats. Bars represent the mean $\pm$ SEM of 12 control or 16 GK determinations (duplicate samples from each rat analyzed in two independent assays; *, $P < 0.005$; **, $P < 0.001$; ***, $P < 0.00001$). **B:** Insulin and A β degradation in brain-soluble fractions from five WT and four GK rats. Bars represent the means $\pm$ SEM of 20 control or 16 GK determinations for insulin (duplicate samples from each rat analyzed in two independent assays), and 32 controls or 28 GK determinations for A β (duplicate samples from each rat analyzed in two to four independent assays; *, $P < 0.005$; **, $P < 0.0001$; ***, $P < 0.00001$). **C:** Inhibitor profile of A β proteolysis in brain membranes from two WT and three GK rats at 3 hours. The degradation assays were performed in the presence or absence of the indicated protease inhibitor at the concentrations mentioned in Results. Bars represent the means $\pm$ SEM of four to six determinations (duplicate samples from each rat analyzed in two to three independent assays; *, $P < 0.05$ for difference between WT control and GK degradation in a given condition).

Figure 3. Insulin and A β proteolysis in primary neurons of WT control and GK rats. Primary neurons from two GK and two WT postnatal day 0 litters (each litter containing seven to eight pups) were incubated with 40 pmol/L of radiolabeled substrate, and degradation was quantified by the TCA assay. Bars represent the mean degradation for the 6-hour time points. **A:** 125 I-insulin degradation by neurons 17 to 19 days *in vitro*. Bars represent means $\pm$ SEM of seven to eight determinations (160,000 and 80,000 cells/cm 2 cultures from each litter in two independent assays) after normalization to the mean WT percent degradation for each culture density, allowing combination of the two data sets. *, $P = 0.005$. **B:** 125 I-A β_{1-40} degradation and inhibition by insulin by neurons 9 to 11 and 19 to 21 days *in vitro*. Degradation assays were performed in the presence and absence of 10 μ mol/L insulin in the medium. Bars represent means $\pm$ SEM of 9 to 10 determinations (160,000 and 80,000 cells/cm 2 cultures from each litter in two to three independent assays) after normalization to the mean WT percent degradation for each culture density, allowing combination of the data sets; **, $P < 0.001$ for difference between WT and GK degradation).

differences at 6 and 24 hours being statistically significant (6-hour values in Figure 3). Insulin degradation (Figure 3A) in the GK neurons at 3, 6, and 24 hours was 12% ($P = 0.12$), 15% ($P = 0.005$), and 4% ($P = 0.03$) less than WT, respectively. The A β proteolytic deficit (Figure 3B)

was 8% ($P = 0.07$), 15% ($P = 0.0008$), and 7% ($P = 0.0007$) at 3, 6, and 24 hours, respectively. Unlabeled insulin prevented >90% of the neuronal A β degradation ($P < 0.01$), leaving identical amounts of residual activity in the GK and controls (Figure 3B), whereas glucagon blocked >50% of A β proteolysis ($P < 0.05$) (not shown). To determine whether the GK A β -degradation deficit documented in primary neurons occurs for the naturally produced, endogenous peptide, we measured A β levels in the neuronal conditioned media by ELISA. Neocortical cells from two GK and two WT postnatal day 0 litters plated at a density of 40,000 cells/cm² were conditioned for 4 or 7 days. Compared to WT conditioned media, A β X-40 levels in the GK neuronal media were increased 84% after 4 days ($P < 0.0005$) and 162% after 7 days ($P < 0.005$) (Figure 4A). A β X-42 levels were also elevated in the GK neuronal media, increasing by 120% after 4 days ($P < 0.0005$), and 138% by 7 days ($P < 0.05$) (Figure 4B). In confirmatory experiments using a different ELISA system (see Materials and Methods), conditioned media were analyzed from primary neurons plated at 80,000 cells/cm² (twice the density of the first set), and again, the GK media had highly significant elevations of both A β X-40 (60% after 4 days, $P < 0.005$; 57% after 7 days, $P < 0.01$) and A β X-42 (85% at 4 days, $P < 0.001$; 107% at 7 days ($P < 0.05$) (data not shown).

Although the inbred GK line was originally derived from the Wistar rat, the two strains are genetically heterogeneous at more than just the IDE locus. The aforementioned evidence that IDE degrades A β , combined with the demonstrated insulin-degrading abnormality and the proteolytic inhibitor profile, all support the role of IDE in generating the GK A β catabolic deficit. To confirm that the GK allele of IDE is sufficient to explain the above A β -degrading deficit, we performed two types of experiments: overexpression and immunodepletion of the GK and WT IDE isoforms. First, we transiently transfected either the GK or WT alleles of IDE into CHO cell lines stably expressing human APP, which additionally allowed us to determine whether endogenously secreted human A β also accumulates more as a result of the IDE mutations. The two IDE proteins were expressed at equal levels, as assessed by immunoblotting. The conditioned media from cells transfected with the GK and WT IDE alleles had 89.8% and 92.3% lower A β X-40 levels by ELISA than media from an equal number of cells transfected with empty vector (not shown). Compared to WT IDE transfected cells, those expressing the GK allele had 32% higher levels of secreted A β X-40 ($P < 0.005$) (Figure 5A). A β X-42 values were below the level of detection in the media of cells transfected with either IDE allele.

In the second group of experiments, we immunodepleted IDE from the brain-soluble fractions of two GK and two WT rats using the IDE-specific monoclonal antibody 9B12, and performed ¹²⁵I-A β degradation assays on the IDE-depleted supernates. Immunodepletion of IDE from the brain fractions removed ~80% of the enzyme from each sample, as determined by immunoblotting (not shown) and removed ~85% of the A β -degrading activity from the WT samples and ~80% from the GK samples (Figure 5B). Before IDE immunodepletion (pre-ID), WT

Figure 4. Endogenous A β levels in primary neuronal conditioned media of control and GK rats. A β X-40 (**A**) and A β X-42 (**B**) levels were quantified by ELISA in the media of primary neurons (40,000 cells/cm²) from two GK and two WT postnatal day 0 litters. The media were allowed to condition for 4 days (neurons, 6 to 10 days *in vitro*) or 7 days (neurons, 3 to 10 days *in vitro*). Bars represent means $\pm$ SEM of two to six determinations, and each determination is a mean of duplicate ELISA values. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

and GK A β degradation at 30 minutes was significantly different at 33% and 29%, respectively ($P < 0.001$), whereas after IDE removal (post-ID), residual A β degradation was identical at 5.2% ($P = 0.49$). Thus, the consistent difference observed in A β proteolysis between the GK and WT brain samples was eliminated by specific removal of IDE.

To determine whether the IDE-mediated deficit in A β degradation that elevates A β in neuronal cultures also increases steady-state levels of A β in the brain, we

Figure 5. Overexpression and immunodepletion of the GK and WT IDE alleles. **A:** A β in media of cells stably expressing human APP and transfected with the WT or GK IDE allele. A β levels were quantified in the media of CHO cells expressing human APP₇₅₁ containing the V717F AD-causing missense mutation and transiently transfected with either the GK or WT IDE allele. Media were incubated on the cells for 24 hours and submitted for A β X-40 and A β X-42 ELISAs (A β X-42 levels were undetectable in the IDE-transfected cells). Bars represent means $\pm$ SEM of three determinations from two separate transfections (and each determination is a mean of quadruplicate ELISA values) after normalization to the WT A β value in each ELISA, allowing combination of the three data sets (*, $P < 0.005$). For reasons of scale, the normalized A β X-40 value of the vector alone (13.0) is not shown. **B:** A β degradation in brain-soluble fractions from two WT control and two GK rats before (pre-ID) and after (post-ID) IDE immunodepletion. TCA precipitation assays were performed after immunodepletion of IDE with the monoclonal antibody 9B12 (described in Materials and Methods) by incubating 100 pmol/L 125 I-A β_{1-40} with 100 μ g of protein/ml of the pre-ID and post-ID samples for 30 minutes at 37°C. Bars represent the mean degradation $\pm$ SEM of duplicate samples from each rat analyzed in two to three independent assays (*, $P < 0.001$). Sham immunodepletion of the brain-soluble fractions (using protein G-agarose without immunoglobulin) had no effect on A β proteolysis (not shown).

quantified levels of the peptide in GK and WT brains by ELISA. Cerebral A β was extracted using two separate protocols, which yielded three distinct pools of A β . The diethylamine protocol, which extracts the aqueous soluble and a portion of the aqueous insoluble A β , was performed on one cerebral hemisphere from each of 34 rats divided into two age groups. The other hemisphere from a subset of these animals (19 rats) was first extracted in TBS to acquire the soluble A β , and then the TBS-insoluble material was extracted in guanidine HCl to obtain insoluble A β . There were no statistically significant differences between GK and WT brain A β X-40 and A β X-42 levels in any of the three types of extracts at either 5 to 7 months or 10 to 14 months of age (Table 1).

To better understand the nature of the IDE proteolytic deficit conferred by the GK missense mutations, we performed quantitative kinetic analyses using a fluorescence polarization-based A β degradation assay.⁵² We used GK and WT brain-soluble fractions because, as shown above, >96% of the A β -degrading activity in these fractions is attributable to IDE. In each individual assay, A β degradation from 50 μ g/ml of protein from one of three GK or three WT samples was analyzed. After completing eight assays per rat strain, the mean $V_{max} \pm$ SEM and $K_m \pm$ SEM were calculated for the GK and WT samples. The mean V_{max} of A β degradation for the GK soluble fraction was significantly less relative to WT (30.0 ± 3.7 versus 47.6 ± 3.3 nmol/L/minute; $P < 0.005$), whereas the K_m values did not differ significantly (1.5 ± 0.3 versus 2.1 ± 0.3 μ mol/L, respectively). The catalytic efficiency (V_{max}/K_m) of the GK brain-soluble fraction was reduced by

~15% relative to WT, which is in good agreement with the data presented above.

Discussion

Our findings demonstrate that naturally occurring, partial loss-of-function mutations in IDE, which are sufficient to cause a diabetic phenotype, result in significant impairment of A β degradation in brain membrane fractions and intact neurons, and this causes substantially elevated levels of neuronally secreted A β . Thus, there does not seem to be an increase in the levels of IDE or other A β -degrading proteases sufficient to compensate for the enzyme's decreased catalytic efficiency in neurons. Furthermore, the relationship between IDE activity and available A β substrate is such that even mild IDE hypofunction alters turnover of the peptide and results in a rise in neuronally generated A β . The realization that IDE activity is rate-limiting in the neuronal proteolysis of A β recommends activation or disinhibition of IDE as a viable therapeutic target in patients with AD.

Although the relatively small (~15 to 30%) decrement in A β proteolysis led to considerable increases in endogenous A β levels in neuronal cultures, we detected no quantifiable rise in steady-state levels of the peptide in brains from rats up to age 14 months. In the intact brain, microglia,^{6,7} astrocytes,⁵³ and transport into the circulation could each play roles in A β clearance,⁵⁴ and these may compensate for the relatively small decrease in IDE function. This is in sharp contrast to full loss of IDE function by homozygous deletion of the gene in mice, which was found to lead to a ~65% elevation of endogenous cerebral A β_{40} levels by 3 months of age.¹⁷ Combining the results from these two different animal models strengthens the hypothesis, based on the recent human genetic data that IDE mutations could serve as a risk factor for late-onset AD. Whereas the gene-deleted mice provide proof-of-principle that IDE dysfunction can significantly elevate cerebral A β levels *in vivo*, the GK rats demonstrate that naturally occurring, subtle loss-of-function mutations in IDE are physiologically relevant (decreasing the catabolism of A β in intact neurons), but not so severe as to cause significant elevations of rodent A β in the brain during the 14-month life span of our rats. In late-onset AD patients, the imbalance between rates of A β production and clearance is such that the presymptomatic accumulation of cerebral A β occurs very slowly throughout decades, and the symptomatic phase of the disease is measured in years. If IDE turns out to be a genetic risk factor for late-onset AD, the magnitude of IDE hypofunction in humans would be expected to resemble that of the GK rat more than that of the IDE gene-deleted mice. It is possible that a modest decrease in neuronal A β clearance would only lead to detectably elevated cerebral A β levels after many years. We speculate that a subtle decrease in IDE function resulting in elevated levels of monomeric A β could lead to more accumulation of A β in humans than rodents, because the human peptide is far more prone to form stable aggregates that resist degradation by proteases than is the rodent form.

Table 1. A β Levels in GK and WT Rat Brains (Means $\pm$ SEM, in pmol/g of Brain)

Age	5 to 7 months		10 to 14 months		7 months	
Extraction	Diethylamine		Diethylamine		Guanidine HCl	
Rat Strain	GK	WT	GK	WT	GK	WT
Number	11	12	7	4	6	7
A β X-40	0.55 $\pm$ 0.06	0.53 $\pm$ 0.05	0.54 $\pm$ 0.08	0.53 $\pm$ 0.12	0.91 $\pm$ 0.07	0.97 $\pm$ 0.08
	$P = 0.39$		$P = 0.47$		$P = 0.31$	
A β X-42	0.41 $\pm$ 0.03	0.42 $\pm$ 0.02	0.41 $\pm$ 0.03	0.42 $\pm$ 0.06	0.42 $\pm$ 0.06	0.05 $\pm$ 0.00
	$P = 0.34$		$P = 0.43$		$P = 0.13$	

We have extended the finding that muscle fibers from rats with the GK allele degrade insulin less well than controls⁴⁰ by showing that these diabetic rats also have a deficit in insulin proteolysis in primary fibroblasts, brain fractions, and intact neurons. The consequences of having chronic insulin-degrading deficits in the brain are unclear at present, but there is mounting evidence that insulin plays numerous roles in the central nervous system.⁵⁵ When Fakhrai-Rad and colleagues⁴⁰ transfected COS cells with the GK and IDE alleles, they were able to detect the GK insulin-degrading defect in intact cells, but not in lysates of the same cells. They hypothesized that the IDE defect is coupled to receptor-mediated internalization of insulin or other activities that require intact cell structures. In contrast, we clearly detected the GK IDE defect not only in untransfected intact fibroblasts and neurons, but also in tissue fractions, providing the simpler explanation that the IDE missense mutations directly decrease the ability of the protease to cleave insulin, independent of other processes. Furthermore, we have begun to characterize the kinetic effects of these insulin- and A β -elevating mutations, showing that they lower the $V_{\max}$ without changing the K_m of the protease, resulting in a modest but significant decrease in catalytic efficiency.

By demonstrating faulty A β degradation in a well-characterized animal model of DM2, our findings are directly relevant to emerging evidence that diabetes and, in particular, hyperinsulinemia, are associated with a heightened likelihood of developing AD. It is well known that DM2 is a risk factor for vascular diseases, including vascular dementia, but the relationship between the occurrence of DM2 and AD has only recently been addressed in large-scale epidemiological studies. Two of these studies in which other causes of dementia were carefully excluded showed that individuals with DM2 were approximately twice as likely to develop a clinical diagnosis of AD compared to normals, independent of vascular factors,^{56,57} whereas a third group found that diabetic patients had a 60% increased risk of developing either frank AD or mild cognitive impairment (a clinical state frequently progressing to AD).⁵⁸ Another population-based study of Japanese-American men reported no association between AD and midlife diabetes,⁵⁹ but a more recent report based on the same cohort concluded that diabetic patients' relative risk of AD was 1.8 and that this increased risk was not related to vascular disease.⁶⁰ The latter report hypothesized that the small number of diabetic patients included in first analysis limited its ability to find an association between DM2 and AD, but as the

study population aged, the prevalence of DM2 more than doubled, giving the second study more power. Although it is not clear from the above epidemiological studies which particular aspect of the DM2 phenotype (eg, hyperglycemia, hyperinsulinemia) is most associated with an increased risk of AD, elevated plasma insulin levels in nondiabetic patients have been linked to dementia⁶¹ even after adjustment for vascular disease, and specifically AD.^{62,63} Supporting a role for IDE in these findings is evidence that the exaggerated insulin response to administered glucose seen in AD patients is secondary to an insulin clearance defect, rather than to overproduction of the hormone.⁶⁴

Thus, although the mechanisms underlying the associations of DM2 and hyperinsulinemia with AD are not yet clear, the demonstration that naturally occurring partial loss-of-function mutations in IDE affect the catabolism of both insulin and A β , combined with the genetic linkage of both DM2 and AD to the IDE region of chromosome 10q, recommend IDE hypofunction as an intriguing explanation. Our results have attendant therapeutic implications in that compounds that subtly increase IDE activity, either directly or via blocking a natural IDE inhibitor, could chronically decrease A β levels in the human brain.

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Table 1. *Continued*

10 to 14 months		7 months		10 to 14 months	
Guanidine HCl		TBS		TBS	
GK	WT	GK	WT	GK	WT
4	2	6	6	4	2
1.02 $\pm$ 0.09	1.07 $\pm$ 0.08	0.31 $\pm$ 0.04	0.37 $\pm$ 0.03	0.32 $\pm$ 0.04	0.36 $\pm$ 0.01
$P = 0.35$		$P = 0.17$		$P = 0.24$	
0.05 $\pm$ 0.00	0.05 $\pm$ 0.01	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.00 $\pm$ 0.00
$P = 0.48$		$P = 0.39$		$P = 0.08$	

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