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Altered β-secretase enzyme kinetics and levels of both BACE1 and BACE2 in the Alzheimer's disease brain

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Abstract **β-Secretase is the rate limiting enzymatic activity in the production of amyloid-β peptide, the primary component of senile plaque pathology in Alzheimer's disease (AD). This study performed the first comparative analysis of β-secretase enzyme kinetics in AD and control brain tissue. Results found V_{max} values for β-secretase to be significantly increased, and K_m values unchanged in AD temporal cortex compared to matched control temporal cortex. The increased V_{max} in AD cases, did not correlate with levels of BACE1, and decreased BACE1 and BACE2 levels correlated with the severity of neurofibrillary pathology (I–VI), and synaptic loss in AD. These results indicate that increased V_{max} for β-secretase is a feature of AD pathogenesis and this increase does not correlate directly with levels of BACE1, the principal β-secretase in brain.**

Keywords: β-Secretase kinetics; BACE1; BACE2; Neurofibrillary tangles; Alzheimer's disease; Amyloid-β-peptide

1. Introduction

The β-secretase enzyme is the subject of significant attention in the understanding and treatment of Alzheimer's disease (AD), as it is the rate limiting activity in the production of the 4 kDa amyloid-β (Aβ) peptide, the primary component of senile plaque pathology in AD [1,2]. β-Secretase cleaves the amyloid precursor protein (APP) at the N terminal Asp-1 position of Aβ, to generate a soluble ectodomain (sAPPβ), and a membrane-bound APP C-terminal fragment of 99 amino acids. The latter can be cleaved subsequently by γ-secretase to produce the 39–43 amino acid peptide, Aβ [2]. Two highly homologous type 1 single transmembrane aspartyl proteases BACE1 (β-site APP cleaving enzyme, also known as, Asp2, Memapsin 2), and BACE2 (Asp1, Memapsin 1) have been identified to have β-secretase activity [3–9], and a number of studies provide evidence indicating that BACE1 is the major β-secretase responsible for Aβ generation in the brain. In particular, mice with targeted homozygous deletion of the BACE1 gene almost completely abolish the production of Aβ peptides in the brain [10–12]. Although BACE2 can cleave at the β-secretase site of APP [4,9], its mRNA tissue distribution [13] and its preference for cleavage within the Aβ region at Phe 19 and Phe 20, is not consistent with requirements for β-secretase activity in the brain [9,14–16].

Previous studies examining β-secretase activity in post-mortem human brain, and in AD, have used a single concentration of substrate, and show variable, but generally increased activity of the enzyme in AD compared to non-AD control brain [17–21]. However, analysis of β-secretase enzyme kinetics is lacking. It is thus unknown whether the reported increased activity in AD brain is reflected by changes in Michaelis constant (K_m) and maximal velocity (V_{max}) values for the protease, which is important for understanding the dynamics of β-secretase activity in AD, and in effective inhibitor design. Moreover, the relationship between the kinetics of β-secretase activity, the severity of AD pathology, and BACE1 protein levels are unknown. Comparative analysis of BACE1 levels in various regions of AD and control brain have detected significant variation, when comparing control and disease groups, with differing molecular weights for BACE1, evident between studies. These are most likely to reflect BACE1 maturation through the secretory pathway from prozymogen (~45–50 kDa) to fully mature *N*-glycosylated forms [22–24]. Thus, BACE1 has been detected at ~70–72 kDa, with a 2.7-fold increase in AD frontal cortex [25], at ~70 kDa, increased by 28% and 32% in AD frontal cortex and hippocampus, respectively [17,19]; bands of ~70 and 50 kDa, showing increases of 14% and an insignificant trend towards an increase of 15% in the AD frontal and temporal cortex, respectively [18], and a ~55 kDa band with unchanged levels in AD parietal cortex, compared to control cases [26].

BACE2 mRNA is expressed at low levels in the brain, when compared to BACE1 mRNA [4,13], however, BACE2 protein has been detected in both control and AD pyramidal neurons [4], and at various molecular weights in human [27,28] and rat [29] brain. No definitive investigation has comparatively quantified BACE2 protein levels in AD and non-AD control brain. This requires investigation, as it is possible that BACE2 levels may be altered in AD. BACE2 activity is predicted for the most part to preclude Aβ generation, and may act antagonistically to BACE1 [30], thus, decreased BACE2 levels may con-

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE, β-site APP-cleaving enzyme; DNP, 2,4-dinitrophenyl; EDTA, ethylene-diamine-tetra-acetic acid; HEK-293, human embryonic kidney 293; K_m, Michaelis constant; MCA, 7-methoxycoumarin-4-acetic acid; NFT, neurofibrillary tangles; PMSF, phenylmethylsulphonyl fluoride; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; V_{max}, maximal velocity; TBS-T, Tris-buffered saline, Tween

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tribute to increased Aβ production and BACE1 biology in AD. Large increases in BACE2 levels in AD brain may contribute to Aβ accumulation, as the BACE2 gene resides on the Down's syndrome obligate region of chromosome 21, and increased levels of the protein have been detected in Trisomy 21 brain which accumulate Aβ [27,28].

Together, the evidence above led us to examine the kinetics of β-secretase enzyme activity, as well as BACE1 and BACE2 protein levels in AD and matched control mid-temporal cortex, in which the brains were staged for disease severity, as measured by Braak staging for neurofibrillary changes [31]. We report a significant increase in the V_{max} for β-secretase activity in AD cases compared to control cases which did not correlate with the severity of AD, or with levels of BACE1, the principal β-secretase in the brain. Together results indicate a complex control of β-secretase enzyme kinetics and its major associated enzyme BACE1 in AD.

2. Materials and methods

2.1. Human brain tissue

Human brain tissue was provided by the Netherlands Brain Bank (see Table 1 for case details). Ethical approval and written informed consent from the donors or the next of kin was obtained in all cases. AD cases ($n = 9$) were diagnosed according to the NINCDS-ADRDA criteria, and severity of dementia rated by the Global Deterioration Scale. Non-disease controls ($n = 7$) had no history or symptoms of neurological or psychiatric disorders. All cases were neuropathologically confirmed, using conventional histopathological techniques, and diagnosis performed using the CERAD criteria. Neuropathological staging of neurofibrillary changes (I–VI) and amyloid plaques (O–C) was performed according to Braak and Braak [31]. The nine AD and seven non-demented control cases were matched for post-mortem delay (control ± S.E.M., 6.0 ± 0.4 h; AD ± S.E.M., 5.7 ± 0.6 h; $P = 0.66$); age (control ± S.E.M., 72 ± 4.6 years; AD ± S.E.M., 77.9 ± 3.6 years; $P = 0.29$); brain-weight (control ± S.E.M., 1230 ± 60 g; AD ± S.E.M., 1112 ± 54 g; $P = 0.14$) as well as tissue pH (control ± S.E.M., 6.9 ± 0.2; AD ± S.E.M., 6.7 ± 0.1; $P = 0.41$), the latter, as an index of agonal status and tissue quality. P-values indicate no significant difference between post-mortem delay, age, brain weight or tissue pH when comparing AD and control cases.

2.2. Preparation of tissue fractions

Human brain tissue fractions from the mid-temporal cortex were prepared as previously described [32], using conditions that were compatible with measuring both β-secretase activity, and detection of BACE1/BACE2 by western immunoblot analysis. Briefly, frozen brain tissue was thawed rapidly at 37 °C in homogenisation buffer [20 mM PIPES–KOH (pH 7.4), 0.32 M sucrose, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)]. Tissue was homogenised using 10 volumes (w/v) of ice-cold homogenisation buffer, and centrifuged in a Beckman ultracentrifuge at $100000 \times g_{max}$ for 60 min at 4 °C (Beckman Type 42.1 rotor). Particulate fractions ($100000 \times g_{max}$ pellet), enriched in membranes, were separated from cytosolic fractions ($100000 \times g_{max}$ supernatant) and suspended in 5 volumes (w/v) of homogenisation buffer, prior to storage of fractions at −70 °C. Rat brain cortical tissue from female Sprague-Dawley rats (age 12–16 weeks) was homogenised in ice-cold homogenisation buffer as above, aliquoted and stored at −70 °C. Protein concentrations were determined using a modified Lowry procedure with bovine serum albumen as standard [33]. Brain homogenates from BACE1 knock-out (KO) and wild-type litter mate controls were prepared as previously described [10].

2.3. β-Secretase enzyme activity assay

β-Secretase activity and kinetic analysis was measured using a quenched fluorescence assay, with a synthetic peptide substrate based on the β-secretase cleavage site of wild-type APP (MCA)Glu-Val-Lys-Met-↓-Asp-Ala-Glu-Phe-Lys(DNP) (Calbiochem, UK), and a modification of a previously described assay system [34], employing the principal of fluorescence resonance energy transfer (FRET). The substrate peptide flanks the β-secretase cleavage site, separating a fluorophore donor (MCA), from an acceptor quenching group (DNP). Hydrolysis of the peptide separates the fluorophore from the quenching group, resulting in an increase in fluorescence proportional to the amount of peptide hydrolysed. Enzyme reactions were carried out at 37 °C, in brown microfuge tubes (Sarstedt, DE), in a final volume of 100 μl of reaction buffer (50 mM sodium acetate, pH 4.5), containing varying concentrations of substrate, and 20 μl of diluted brain fraction. Unless otherwise stated, 1 μg of protein from control and AD particulate fractions was used in all reactions, with an incubation time of 1 h, after linearity between protein concentration (0.3–12 μg), time (examined up to 120 min, data not shown) and fluorescence intensity was determined in control human brain fractions ($n = 3$). For inhibitor studies 10 μl of β-secretase inhibitor P10–P4′ StatVal (H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Stat-Val-Ala-Glu-Phe-OH [Stat = (3S,4S)-Statine]) (Calbiochem), at concentrations of 0.1 nM to 6.5 μM, was incubated with 20 μl of protein for 30 min at 37 °C, prior to addition of reaction buffer with 15 μM substrate, and incubation at 37 °C for 1 h. For kinetic analysis 1 μg of protein was incubated with increasing concentrations of substrate (1–50 μM) for 1 h at 37 °C with reaction buffer containing a maximum of 1.4% dimethyl sulfoxide. All reactions were stopped by boiling samples at 100 °C for 10 min, followed by centrifugation at $9500 \times g_{max}$ for 10 min, 80 μl of reaction sample was removed and diluted in 400 μl of deionised water and flourescent 7-methoxycoumarin-4-acetic acid (MCA) cleavage product measured. An excitation wavelength of 320 nm and an emission wavelength of 393 nm were used to measure the hydrolysis of substrate on a Perkin-Elmer LS50 B fluorescence spectrofluorometer, and readings compared against a standard curve of MCA (Sigma-Aldrich, UK) dissolved in 50 mM sodium acetate pH 4.5.

2.4. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown to confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% L-glutamine. All reagents were obtained from Sigma-Aldrich, unless otherwise stated. Cells were maintained in a humidified 37 °C incubator with 5% CO_2. Cells at 70% confluency were transfected, using the calcium phosphate DNA precipitation technique. Briefly, 4 μg of pcDNA3.1 vector encoding C-terminal Myc-His tagged BACE1, C-terminal Myc-His BACE2 (generous gifts from Dr. J.V. McCarthy), or empty vector were diluted in 62 μl of 2 M $CaCl_2$, brought to 500 μl with deionised water and mixed, before drop wise addition to 500 μl of 2× Hank's balanced saline solution solution, and addition of the mixture to cells for 8 h. Media was refreshed, and cells left to grow for 16 h before harvesting. Cells were lysed in 0.1% NP-40 (v/v), 150 mM NaCl, 50 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], 1 mM EDTA, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 0.5 mM PMSF, for 20 min on ice and centrifuged at $20000 \times g_{max}$ for 10 min at 4 °C. Supernatants were retained and protein concentration determined using Bio-Rad reagent (Bio-Rad, UK) with bovine serum albumen as standard. For deglycosylation experiments, cell lysates (100 μg protein) were denatured by boiling at 100 °C for 10 min in 0.1% SDS, 0.5 mM 2-mercaptoethanol and 50 mM sodium phosphate buffer (final volume of 100 μl). NP-40 was added to a final concentration of 0.75% (v/v) before treatment with or without 1 U of PNGase F (Sigma-Aldrich) for 17 h at 37 °C. Enzymatic digestion of samples was examined by immunoblotting.

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

20 μg of protein was resolved on 10% SDS–PAGE for all Western blot analyses. Proteins were transferred electrophoretically to nitrocellulose membranes (0.45 μM, Schleicher and Schuell, DE) using a wet transfer blotting apparatus (75 V, 75 min). The transfer buffer for immunoblots consisted of 48 mM Tris, 39 mM glycine and 20% ethanol. Nitrocellulose membranes were blocked for 1 h in 150 mM NaCl, 10 mM Tris–HCl, pH 8.0, 0.1% Tween 20 (TBS-T, Tris-buffered saline, Tween) and 5% w/v non-fat dry milk. Immunoblots were incubated overnight at 4 °C with the various primary antibody solutions diluted in TBS-T with 5% non-fat dry milk. Antisera dilutions and sources were as follows: BACE1 485-501 (1:2000 dilution, Calbiochem);