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Age-dependent decline of neprilysin in Alzheimer's disease and normal brain: Inverse correlation with Aβ levels

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Abstract

Brain deposition of amyloid-β (Aβ) is a pathological hallmark of Alzheimer disease (AD) but Aβ is also detected in non-demented elderly individuals. Neprilysin has been shown to be an important enzyme to degrade Aβ in brain. We investigated whether decreased neprilysin levels contributes to the accumulation of Aβ in AD and in normal aging. No difference in neprilysin protein and mRNA levels were found between AD subjects and age-matched controls. Protein levels of neprilysin were reduced with age in the temporal and frontal cortex of AD and normal brain. A significant positive correlation between insoluble Aβ 40 and Aβ 42 with age was found in cortex of normal brain whereas in AD brain the correlation between age and Aβ was weaker. Our findings of an inverse correlation between neprilysin and insoluble Aβ levels in both groups suggest that neprilysin is involved in the clearance of Aβ. The observed age-dependent decline in neprilysin may be related to the increased Aβ levels during normal aging. The similar rate of decline in neprilysin with age may not be the major cause of the high levels of Aβ associated with AD but is likely to be a trigger of AD pathology.
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1. Introduction

Neuropathologically, Alzheimer disease (AD) is characterized by the accumulation of extracellular plaques, mainly comprised of amyloid-β (Aβ), and intracellular neurofibrillar tangles, consisting of aggregates of hyperphosphorylated tau protein (Selkoe, 2001). Aβ is generated from amyloid precursor protein (APP) by enzymatic cleavage involving β-secretase (BACE) and γ-secretase activities (Evin and Weidemann, 2002; Selkoe and Schenk, 2003). In familial AD, Aβ accumulation is known to occur as a result from mutations in APP or presenilin 1 and 2 genes. For the remaining sporadic cases, which account for the vast majority of AD cases, the underlying pathogenic mechanism is currently unclear. The steady-state level of Aβ is determined by the metabolic balance between its rate of synthesis and its rate of clearance, and it has been suggested that the onset of sporadic AD may in many cases be attributed to an impaired clearance of Aβ (Higuchi et al., 2005; Leissring et al., 2003).

Several proteases have recently been shown to play a role in regulating steady-state levels of Aβ in brain. To date, most attention in this area has focused on the proteases neprilysin and insulin-degrading enzyme (IDE), although other proteases have been implicated in Aβ degradation (Carson and Turner, 2001; Eckman and Eckman, 2005; Vardy et al., 2005). Both neprilysin and IDE may contribute to overall Aβ degradation in the brain, but each may play a role in distinct subcellular loci and with different forms of Aβ. Neprilysin (also known as neutral endopeptidase, EC3.4.24.11, enkephalinase, and CD10) is a 97 kDa type II membrane-associated protein enzyme that cleaves various peptides (Roques et al., 1993; Turner et al., 2001). Neprilysin is primarily localized at the presynaptic terminals and on axons with its ectodomain

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facing the extracellular space (Fukami et al., 2002). The presence of neprilysin at the presynaptic sites is of particular importance, because neprilysin is capable of degrading both monomeric and oligomeric forms of Aβ (Kanemitsu et al., 2003). Recent studies have indicated that particular forms of soluble Aβ such as oligomeric forms and protofibrills cause dysfunction or modification of synaptic transmission in vivo, and to disrupt cognitive function (Cleary et al., 2005; Walsh et al., 2002).

Considerable data has emerged indicating that neprilysin plays a key role in decreasing the levels of cerebral Aβ deposition. Neprilysin-deficient mice show a significant dose-dependent increase in cerebral Aβ levels (Iwata et al., 2001; Marr et al., 2004). Conversely, over-expression of neprilysin leads to a reduction in Aβ levels in a dose-dependent manner (Hama et al., 2001; Iwata et al., 2004; Marr et al., 2004). Furthermore, infusion of neprilysin inhibitors into brains of APP transgenic mice elevated Aβ levels in brain (Marr et al., 2004). In post mortem human brain the levels of neprilysin mRNA has been found to be particularly low in regions vulnerable to senile plaque development, such as hippocampus and midtemporal gyrus (Yasojima et al., 2001a,b). In addition the neprilysin mRNA levels were lower in AD compared to age-matched controls, suggesting that low activity of this enzyme may contribute to the pathogenesis of AD (Yasojima et al., 2001b). Neprilysin is expressed in GABAergic and metabotropic glutamate receptor 2/3-positive neurons, but not in catecholaminergic or cholinergic neurons in mouse hippocampal and neocortical areas (Fukami et al., 2002). If similar cell type-specific expression of neprilysin exists also in human brain, it may help to explain the particular vulnerability of cholinergic neurons against Aβ in AD brain (Kar et al., 2004).

Aging is the most significant risk factor for the development of AD. Aβ accumulation and deposition are not exclusive to AD but during aging, most humans start to accumulate Aβ in brain. A predominance of Aβ 42 levels and Aβ 42-immunoreactive diffuse plaques have been found in subjects in brains of elderly subjects, in absence of signs of neuronal degeneration or dementia (Funato et al., 1998; Hellström-Lindahl et al., 2004b; Morishima-Kawashima et al., 2000; Price and Morris, 1999; Thai et al., 2002; Walker et al., 2000). The reasons for the absence of pathogenic effect exerted by Aβ in normal aging and in cognitive normal elderly subjects with abundant Aβ deposits are unknown. Several studies have shown a significant reduction in neprilysin expression in the hippocampus and cortex of aged mice compared to young mice (Apelt et al., 2003; Caccamo et al., 2005; Iwata et al., 2002). If an aging-dependent decrease of neprilysin also occurs in the human brain, as in aging mice, the down-regulation of neprilysin is likely to be related to the Aβ deposition associated with normal aging and AD pathology. The aims of this study were: (1) to investigate if there were any difference in neprilysin protein and mRNA between AD subjects and elderly non-demented subjects, (2) to investigate if there were an age-dependent decrease of neprilysin in normal and AD brain, (3) to measure aged-related increase in soluble and insoluble Aβ levels and look for possible correlations between Aβ and neprilysin, and (4) to investigate if the level of neprilysin is influenced by nicotine since we recently have found reduced Aβ levels in smoking individuals (Hellström-Lindahl et al., 2004b). We here report for the first time an age-dependent decrease in neprilysin protein levels in the brains of normal individuals and AD subjects, as well as an inverse correlation between neprilysin and insoluble Aβ levels.

2. Methods

2.1. Human post mortem brain tissue

Frontal cortex (medial frontal gyrus) and temporal cortex (medial temporalis gyrus) from 15 AD patients and 23 normal healthy individuals were obtained from the Netherlands Brain Bank. Autopsies were performed on donors from whom written informed consent has been obtained either from the donor or direct next of kin. The clinical diagnosis of demented patients was performed according to the NINCDS-ADRDA criteria (Mc Khann et al., 1984) and the severity of the dementia was estimated according to the Global Deterioration Scale (Reisberg et al., 1982). The control subjects had no known history or symptoms of neurological or psychiatric disorders. All cases were neuropathologically confirmed using conventional histopathological stains on formalin-fixed specimens. The diagnosis was based on the presence and distribution of the classical hallmarks for AD. The Netherlands Brain Bank uses a scoring system in which the density of senile plaques, neurofibrillary tangles (NFT), disrupted interneuronal-network (dINN), neuropil threads, congophylic plaques and vessels are estimated in Bodian and Congo stains in four neocortical areas. For the staging of the various pathological hallmarks a combination of a quantitative grading system and the Braak staging was applied to all specimens (Braak and Braak, 1991; Ravid et al., 1998). The individual case histories of the subjects are listed in Table 1. Initially, a few AD subjects around 60 years were included in the study, but were later excluded since some of these AD patients had relatives with dementia and possibly suffered from familial AD. Samples from both temporal and frontal cortex were not obtained from all cases and also neprilysin, synaptophysin and Aβ were not analyzed all together in every sample, which makes the number of observations between different parameters analyzed somewhat misleading in comparison with the total number of cases in Table 1.

2.2. Brain tissue preparation for Western blot

Frozen tissues were homogenized in 10 volumes of cold 10 mM Tris buffer–HCl, pH 8, with 0.25 M sucrose and protease inhibitor cocktail (Complete, Roche Diagnostics). After centrifugation for 20 min at 35,000 × *g* at 4 °C, pel-

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