Neprylisin decreases uniformly in Alzheimer's disease and in normal aging

Roberta Russo[a,*], Roberta Borghi[a], William Markesbery[b], Massimo Tabaton[a], Alessandra Piccini[a]

[a] *Department of Neurosciences, Ophthalmology and Genetics, University of Genoa, Via De Toni 5, 16132 Genoa, Italy*
[b] *Sanders-Brown Center on Aging, Lexington, USA*

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Abstract The proteolysis of β-amyloid (Aβ) requires neprylisin, an enzyme that has been shown as reduced in Alzheimer's disease (AD). We investigated whether a decrease in neprilysin levels contributes to the accumulation of amyloid deposits not only in AD but also in the normal aging. We analyzed neprilysin mRNA and protein levels in cerebral cortex from 10 cognitively normal elderly subjects with amyloid plaques (NA), 10 cases of AD, and 10 control cases free of amyloid plaques. We found a significant decrease in neprilysin mRNA levels in both AD and NA compared to control cases. Thereby, the defect of neprilysin appears to correlate with Aβ deposition but not with degeneration and dementia.

Keywords: Neprilysin; Alzheimer's disease; Amyloid beta-protein; Normal aging

1. Introduction

According to the amyloid cascade hypothesis, accumulation and deposition of β-amyloid (Aβ) in the brain are the major events in Alzheimer's disease (AD) [1–3]. Evidence indicates that Aβ accumulation depends on the balance between Aβ production and Aβ clearance [4]. The physiological metabolite Aβ is constantly produced and removed in the brain, and it has been demonstrated that even small decreases in its removal lead to its deposition [5–7]. Among several proteases involved in the proteolysis of Aβ, neprilysin (NEP) appears to be the most important enzyme. NEP is a 97 kDa plasma membrane glycoprotein that is also known as neutral endopeptidase (EC 3.4.24.11), enkephalinase, CD10, or as common acute lymphoblastic leukemia antigen (CALLA) [8]. In the brain, NEP is expressed on neuronal membranes, both pre- and post-synaptically [9].

Previous studies indicate that NEP mRNA and protein levels are lower in AD compared to non-AD cases free of amyloid plaques, suggesting that low activity of this enzyme may contribute to the pathogenesis of AD [5,10,11]. Abundant amyloid deposits, comprised of Aβ aggregates, are present in the brains of cognitively normal aged people, in spite of little or absent neuronal alterations [12]. Thus, Aβ accumulation and deposition are not exclusive to AD but rather are proportional to aging, and this may depend on a particular neuronal resistance to Aβ due to unknown factors, or more simply put, may derive from the quality of the Aβ accumulated.

In this study, we hypothesize that low levels of NEP may contribute to the accumulation of amyloid deposits in AD. We measured NEP mRNA and protein levels in the cerebral cortex of subjects with sporadic AD and compared them against control cases that were free of amyloid deposits (CTR) and against cognitively normal elderly subjects with amyloid plaques (NA).

2. Materials and methods

2.1. Cases

Frozen cerebral cortex (superior frontal gyrus) samples were used that included: 10 CTR cases free of amyloid plaques, as ascertained by immunocytochemistry using monoclonal antibody 4G8 that recognizes all Aβ species (mean age at death 73 ± 9, post-mortem 7 h); 10 cases with late-onset sporadic AD (mean age at death 86 ± 10, post-mortem 9 h, Braak stage 5) (clinical history of disease; pathological diagnosis according to CERAD criteria; provided by the brain bank of Case Western Reserve University, Cleveland, OH; Dr. Pierluigi Gambetti director); and 10 NA cases (mean age at death 78 ± 10, post-mortem 10 h, Braak stage 2). The latter subjects agreed to be neuropsychologically tested every six months and to be autopsied for research purposes (provided by Alzheimer's Disease Research Center, University of Kentucky, Dr. William Markesbery, director). Analysis of the NA cases showed Aβ deposits and neurofibrillary pathology only in the mesial temporal lobe. The amount of Aβ deposits, semiquantitatively evaluated in frontal cortex sections with anti-Aβ antibody (4G8) immunostaining, was similar in AD and NA cases.

2.2. RT-PCR analysis

Total RNA was extracted from approximately 100 mg frozen brain sample using the TRIzol Reagent (Invitrogen). In all cases, 5 μg of total RNA was reverse-transcribed using random primers and the cDNA was amplified by the polymerase chain reaction (PCR) technique. PCR was performed using master-mix (Fermentas Life Sciences) with 5 min of denaturation at 95 °C, followed by 38 cycles of the following: 30 s at 92 °C, 1.30 min at 58 °C and 1 min at 72 °C. The primers used were: forward 5′-TAAGCAGCCTCAGCCGAACCTACAA-3′ and reverse 5′-GACTACAGCTGCTCCACTTATCCACTCA-3′ (GenBank Accession No. X07166). For mRNA quantification, β-actin was amplified in the same samples using the forward primer 5′-CTCACCCTGAAGTACCCCATCG-3′ and the reverse primer 5′-CTTGCTGATCCACATCTGCTGG-3′.

2.3. Immunoprecipitation of NEP

From each brain, 400 mg of tissue was homogenized in 5 vol (w/v) of ice-cold 10 mM Tris–HCl buffer (pH 8.0) containing 0.25 M sucrose and a protease inhibitor cocktail. The homogenates were centrifuged at 10 000 rpm at 4 °C for 15 min and the supernatants were further centrifuged at 45 000 rpm at 4 °C for 20 min. The pellets were solubilized

*Corresponding author. Fax: +39 10 506938.
E-mail address: robertaruss@tiscali.it (R. Russo).

Abbreviations: CTR, control cases free of amyloid deposits; NA, cognitively normal elderly subjects with amyloid plaques; AD, Alzheimer's disease; NEP, neprilysin

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in Tris–HCl buffer containing 1% Triton X-100 (v/v) for 1 h on ice. The solubilized membranes were again centrifuged at 45 000 rpm at 4 °C for 20 min [9]. The supernatants, used as the membrane fraction, were immunoprecipitated overnight at 4 °C with 15 μl of monoclonal anti-human CD-10 antibody (Santa-Cruz Biotechnology, Inc.).

2.4. Western blot and protein quantification

Immunoprecipitated proteins were resolved on 8% SDS–PAGE gels, transferred to PVDF membranes (Amersham Biosciences), and probed with anti-NEP antibody (Santa-Cruz Biotechnology, Inc.) and with anti-HNE antibodies HNEJ-2 (Japan Institute for the Control of Aging), HNE11-S (Alpha Diagnostic) and HNE-pyrrole [13]. Signals were detected with ECL (Amersham Biosciences). The specific 97 kDa band was measured with densitometric analysis using Quantity One software system (Biorad). Absolute values for NEP were calculated for each immunoprecipitated sample with the Lowry protein assay kit (BioRad) and normalized to mg of proteins.

2.5. Statistical analysis

All data were analyzed using the one-way ANOVA with Bonferroni's Multiple Comparison Test as the post-test with GraphPad Prism software. Data are presented as means ± S.E. of the three groups of cases (CTR, NA, AD).

3. Results

Expression of *NEP* was analyzed using RT-PCR in cerebral cortex tissue samples taken from 10 CTR, 10 NA, and 10 AD cases. The *NEP* mRNA level was normalized to the corresponding amount of *actin* mRNA. Relative RT-PCR was performed in triplicate and the data of the three experiments was statistically analyzed. The levels of mRNA were significantly decreased ($^{**}P < 0.01$) in the NA cases (0.48 ± 0.07) and the AD cases (0.50 ± 0.1) compared to CTR cases (0.86 ± 0.2) (Fig. 1).

In the same tissues, NEP was immunoprecipitated with anti-NEP antibody and Western blot analysis was performed using anti-NEP and anti-HNE antibodies. The anti-NEP antibody detected a band migrating at 97 kDa, as predicted for NEP. The quantification analysis revealed a lower level of NEP protein in the NA (0.043 ± 0.03) and AD (0.045 ± 0.01) cases in comparison to the CTR cases (0.050 ± 0.05), but without revealing a significant difference among the three groups (Fig. 2). The 97 kDa band did not show any HNE reactivity with the three different anti-HNE antibodies in all cases examined (data not shown).

4. Discussion

The overproduction of Aβ, or the failure to remove it, is a complex process which leads to the formation of amyloid plaques and to the development of Alzheimer's disease [2–4]. Recent findings indicate that NEP plays a key role in decreasing the levels of cerebral Aβ deposition. Several studies have shown that in vitro overexpression of NEP significantly decreases Aβ levels [7,14]. Moreover, downregulation of NEP promoted Aβ deposition in the brain of transgenic mice [15–17] and the role of NEP in reducing Aβ accumulation was also demonstrated in transgenic mice overexpressing human neprilysin [18]. In agreement with these findings, Yasojima et al. [10] reported that NEP mRNA and protein levels were reduced by 50% and 70% in AD patients, respectively, compared to aged-matched controls.

The present study aimed to extend these findings by including cognitively normal elderly cases with abundant Aβ deposits and scarce neurofibrillary pathology (NA individuals). The normal elderly subjects that we considered differed from AD not in the amount but in the quality of Aβ species, arguing that AD is pathogenetically a distinct disease and not an accelerated form of aging [19].

We observed that *NEP* mRNA levels in cerebral cortex were significantly lower in both NA and AD groups compared to subjects free of amyloid deposits. NEP protein levels were also lower in NA and AD cases compared to the amyloid-free cases, although without significant difference. This discrepancy could be due to reduced protein degradation or to an increased efficiency of mRNA transcription and might be compensated by analyzing larger groups of cases.

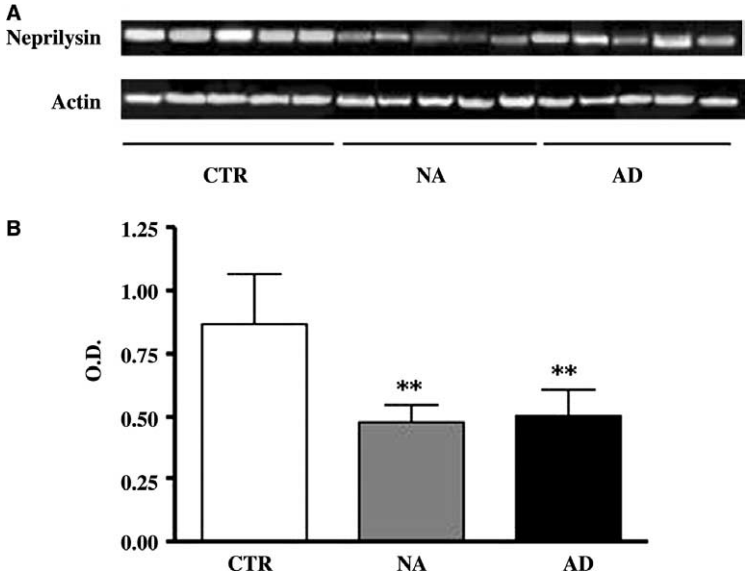

Fig. 1. Total mRNA levels for neprilysin in frontal cortex of CTR, NA and AD cases. (A) RT-PCR data for neprilysin and corresponding actin in five representative cases for each group. (B) Histograms represent neprilysin mRNA levels for all 30 cases, normalized to the corresponding amount of actin mRNA. Data are expressed as mean values ± SEM of three different experiments for each group ($^{**}P < 0.01$).

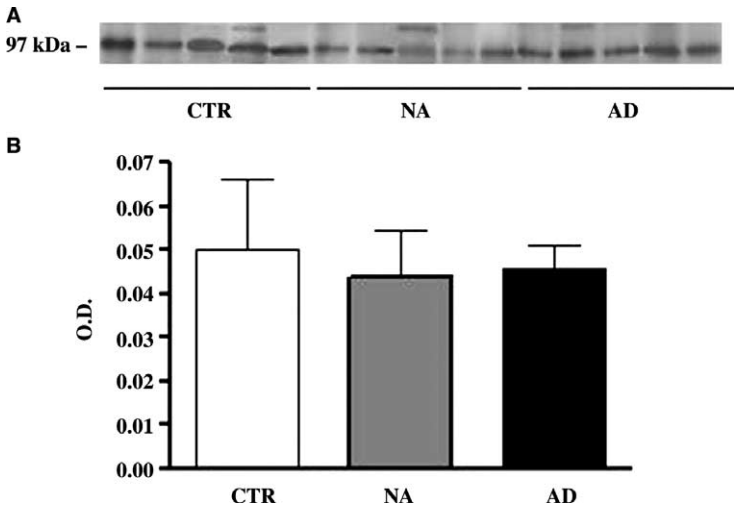

Fig. 2. Total neprilysin protein levels from CTR, NA and AD cases. (A) A specific protein band (97 kDa) was revealed with anti-NEP antibody in five representative cases for each group. (B) Histograms indicate the densitometric analysis of neprilysin western blot bands normalized to the corresponding total protein for all 30 cases.

Our data show that NEP expression is correlated with the presence of amyloid plaques and not with cognitive dysfunction. Therefore, the decrease in NEP levels, observed also in NA subjects, indicates that this event is not a peculiarity of AD, and that different mechanisms are responsible for the neuronal dysfunction and degeneration observed in AD. For example, the harmless amyloid deposition seen in NA could depend on a particular neuronal resistance to Aβ due to unknown factors, or more simply, may derive from the quality of the accumulated Aβ [19,20]. Furthermore, several other mechanisms besides the toxic effect of Aβ are putatively involved in the pathogenesis of AD [21,22]. Wang et al. [23] suggest that NEP activity is impaired in AD because the protein is partially altered by oxidation. In our cases presented here, we did not find oxidized NEP in either the AD or normal brains, as demonstrated by the absence of HNE reactivity in the extracted NEP.

In contrast from our findings, Wang et al. [24] reported a decrease in NEP immunoreactivity in their AD cases compared to ''pathological aging''. The subjects that were selected for this study were cognitively normal, as demonstrated by neuropsychological tests, and their neocortex was absolutely free of neurofibrillary pathology. It is possible, then, that the pathological aging cases analyzed by Wang et al. and our NA cases substantially differ in the extent of neurodegeneration, thus accounting for the contrasting results.

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