Zinc affects the proteolytic stability of Apolipoprotein E in an isoform-dependent way

He Xu[a], Veer B. Gupta[b], Ian J. Martins[b], Ralph N. Martins[b], Christopher J. Fowler[a], Ashley I. Bush[a], David I. Finkelstein[ac], Paul A. Adlard[ac]

a. The Florey Institute of Neuroscience and Mental Health, Melbourne, Victoria 3010, Australia

b. Centre of Excellence for Alzheimer's disease Research and Care, School of Medical Sciences, Edith Cowan University, 270 Joondalup Drive, Joondalup, 6027 WA, Australia.

c. Contributed equally

Corresponding author:

Paul Adlard

30 Royal Parade, Parkville, 3010, Melbourne, VIC, Australia.

Telephone: +61 3 90356775; +61 3 9035 6680

Email address: paul.adlard@florey.edu.au

Abstract

The pathological role of zinc in Alzheimer's disease (AD) is not yet fully elucidated, but there is strong evidence that zinc homeostasis is impaired in the AD brain and that this contributes to disease pathogenesis. In this study we examined the effects of zinc on the proteolysis of synthetic Apolipoprotein E (ApoE), a protein whose allelic variants differentially contribute to the onset/progression of disease. We have demonstrated that zinc promotes the proteolysis (using plasma kallikrein, thrombin and chymotrypsin) of synthetic ApoE in an isoform-specific way (E4>E2 and E3), resulting in more ApoE fragments, particularly for ApoE4. In the absence of exogenous proteases there was no effect of metal modulation on either lipidated or non-lipidated ApoE isoforms. Thus, increased zinc in the complex milieu of the ageing and AD brain could reduce the level of normal full-length ApoE and increase other forms that are involved in neurodegeneration. We further examined human plasma samples from people with different ApoE genotypes. Consistent with previous studies, plasma ApoE levels varied according to different genotypes, with ApoE2 carriers showing the highest total ApoE levels and ApoE4 carriers the lowest. The levels of plasma ApoE were not affected by either the addition of exogenous metals (copper, zinc or iron) or by chelation. Taken together, our study reveals that zinc may contribute to the pathogenesis of AD by affecting the proteolysis of ApoE, which to some extent explains why APOE4 carriers are more susceptible to AD.

Highlights:

- Zinc destabilizes ApoE in the presence of proteases in an order of ApoE4>ApoE2 and ApoE3.
- Plasma ApoE levels follow an order of ApoE2>ApoE3>ApoE4
- Plasma ApoE levels are not affected by metals

Keywords: Zinc, Apolipoprotein E, Proteolytic stability

Abbreviations: AD, alzheimer's disease; ApoE, apolipoprotein E; CNS, central nervous system; PK, plasma kallikrein; Aβ, amyloid-β; NFT, neurofibrillary tangle.

Introduction

Apolipoprotein E protein (ApoE) is a 34 kDa polymorphic glycoprotein existing in the brain and periphery. Three ApoE isoforms--ApoE2, ApoE3 and ApoE4 are encoded by the same apolipoprotein E gene (APOE) with three alleles (APOE2, APOE3 and APOE4) (1). APOE4, which accounts for 10%-15% of the APOE gene pool in the population, has been identified as the strongest genetic risk factor for late onset Alzheimer's disease (LOAD), increasing the risk level by three times in heterozygous individuals and by twelve times in homozygous individuals (1, 2). The least frequent APOE2 allele (5-10%) appears to protect against AD (1, 3), whilst the most frequent APOE3 allele (70-80%) represents an intermediate risk for disease development (4).

ApoE contains two structural domains by a protease sensitive hinge region: a 22-kDa amino-terminal domain (NT, residue 1-191) that has a four-helix structure and contains the low-density lipoprotein receptor-binding region (residue 136-150) and a 10kDa carboxyl-terminal domain (CT, residue 216-299) existing as an α-helical bundle and containing the major lipid-binding region (residue 244-272) and probably amyloid-β (5). ApoE isoforms differ at two amino acid position, 112 and 158. ApoE2 has cysteine-112 and cysteine-158, whereas ApoE4 has arginine at both positions and ApoE3 has cysteine-112 and arginine-158 (6). Since cysteine is a strong ligand for zinc and arginine is not (7), it is speculated that ApoE2 has the highest affinity for zinc whilst ApoE4 has the lowest. In addition, this cysteine/arginine difference has a dramatic influence on two critical properties of ApoE: protein stability and domain interactions (8). Denaturation studies have demonstrated that ApoE4 is the least stable isoform, whereas ApoE2 is most stable (9, 10). The ApoE4 instability leads to the formation of an ensemble of loosely folded structures referred to as a molten globule state while ApoE2 is most resistant to this, and this increases ApoE4's susceptibility to proteolysis compared to ApoE2 and ApoE3 (10). These effects likely contribute to the interaction of ApoE4 in AD.

Given the strong association between ApoE genotype and the risk of developing AD, numerous studies have investigated whether levels of ApoE are altered in AD patients. Total plasma ApoE levels have been reported to be significantly lower in AD patients, irrespective of APOE genotypes and APOE4 carriers exhibited the largest decrease in total plasma ApoE levels (11, 12). Likewise, several studies have also reported genotype-dependent variability in ApoE levels in the brain with the lowest concentrations found in APOE4 carriers (13-15). Several enzymes, such as thrombin and chymotrysin-like serine protease, have been suggested to mediate ApoE proteolysis to decrease ApoE levels and generate neurotoxic fragments in the brain (16-19). ApoE fragments are found in AD brains associated with amyloid plaques and APOE4 carriers have the most ApoE fragments. Differential proteolysis of ApoE isoforms and toxicity exerted by its fragments is believed to play an important role in AD pathogenesis (20). The potential role/interaction of metal ion homeostasis in this toxicity of ApoE has been recently reviewed (21).

Briefly, alterations in zinc homeostasis have been suggested to be a key factor in the progression to AD (22). Zinc is a potent inducer of Aβ aggregation and is concentrated within the extracellular amyloid plaques (23). Zinc deficiency has been demonstrated in older

healthy individuals, with serum zinc levels even lower in AD patients (24). Of relevance to this study, it has also been reported that zinc is significantly increased in the serum of APOE4 positive AD patients, suggesting the presence of APOE4 alone may increase zinc levels (25). Intriguingly, ApoE has an affinity for zinc (26) and their correlation in the development of AD is gaining increasing attention (21). The metal sequestration properties of ApoE might present metals to Aβ peptides, leading to amyloid deposition or it might account for the antioxidant function of ApoE in AD development (26). In this study we investigated the influence of zinc on the proteolytic stability of ApoE. We found that zinc is able to promote ApoE degradation by proteases in an isoform-dependent way (E4>E2 and E3), which might contribute to AD pathogenesis.

Materials and methods

ApoE preparation

Recombinant ApoE2, ApoE3 and ApoE4 (Escherichia coli, r-ApoE) were purchased from Sigma-Aldrich. The peptides were dissolved in Tris-buffered saline (TBS, 20mM Tris-HCl and 150mM NaCl,pH=7.4) and stored at -20°C until further use. The final concentration of ApoE in our experiment was 40mg/L, which represents the physiological concentration of ApoE in human plasma (12). In addition, lipidated ApoE isoforms were obtained from our collaborators: Prof. Ralph Martins and Ian Martins (Edith Cowan University)

In vitro incubation of r-ApoE isoforms with metals and metal chelators

Different r-ApoE isoforms (r-ApoEs) were incubated with equal volume (5µl) of either metals (zinc chloride or copper chloride; 10 and 100µM) or metal chelators (300µM) at 37°C for 4 hours. Metals are dissolved in TBS (20mM Tris-HCl and 150mM NaCl, pH=7.4). Specifically, the zinc chelator used was N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), the copper chelators were bathocuproine disulphonate (BCS) for Cu(I) and d-penicillamine (DPEN) for Cu(II) and the broad spectrum chelator, ethylenediamine tetra acetic acid (EDTA) was also used.

In vitro protease treatment of ApoE isoforms

In order to understand the effects of zinc on ApoE proteolysis, ApoE samples were pre-incubated with zinc for 4 hours followed by protease treatment at 37°C for 1 hour. Serine protease has been demonstrated to be responsible for the cleavage of ApoE (18, 19, 27), so in our experiment we chose chymotrypsin (Sigma-Aldrich), plasma kallikrein (Molecular Innovations) and thrombin (Sigma-Aldrich) to digest ApoE *in vitro*. The dosages of the three proteases used were optimized for each ApoE isoforms to get a reduction (~ 40-50%) in full-length ApoE after incubation. We normalised the amount of full-length ApoE without protease treatment to 1.0, and within the same experiment, the remaining full-length ApoE after protease treatment was described as the proportion of this value.

Serine protease activity assay

N-Benzoyl-L-tyrosine ethyl ester (BTEE) was used to determine the enzymatic activity of chymotrypsin (Sigma-Aldrich), as previously described (28). A serine protease activity assay kit (Abcam, ab102531) was used to determine the activity of plasma kallikrein (PK) and thrombin, both of which are able to cleave the substrate X-Arg/Lys- p-nitroaniline (pNA) and the product p-NA can be detected at λ=405 nm. Protease activities were tested with and without zinc or copper and performed in triplicates in 96-well plates (pH=7.4, 37°C).

In vitro incubation of human plasma with metals and EDTA

Human plasma samples were obtained from individuals that volunteered for The Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing (AIBL) and were sourced from lithium-heparin tubes. These were incubated with equal volumes of either metals (5µL, 100µM of either zinc chloride, copper chloride or iron chloride) or EDTA (5µL, 300µM) at 37°C for 24 hours. These samples were obtained after they had been approved by the University of Melbourne Human Ethics Committee.

Western blots

Samples were prepared with 20x reducing buffer and 4x sample buffer (Bio-Rad), heated to 90°C for 5 min and separated by SDS-PAGE using a 4-12% Bis-Tris gel and MES running buffer (Bio-Rad) (100V for 90 min). Protein was then transferred onto 0.2µm Polyvinylidene fluoride (PVDF) membrane using a Trans-Blot® Turbo™ Transfer Starter System (Bio-Rad). Membranes were blocked in Tris-buffered saline and Tween-20 (TBST) containing 5% skim milk powder, and then incubated with primary antibody (goat anti-ApoE antibody, 1:3000, Millipore) overnight at 4°C. Blots were rinsed in TBST (10 minutes for 3 times) and incubated in secondary antibody (rabbit anti-goat 1:10000, Dako) (1 hour, room temperature), followed by additional rinsing (10 minutes for 3 times), development with ECL reagent, and imaged using a Fujifilm Luminescent Image Analyser LAS-3000 and Image Reader LAS-3000 software package (Fujifilm).

Assessing the affinity of ApoE for zinc

r-ApoE (ApoE2 and ApoE4, 2µM, 30µl) was dialysed against 30µl of zinc chloride (0-100µM) in a DispoEquilibrium dialyzer (Membrane MWCO 10,000 Daltons). Dialysis was performed at 4°C and samples were recovered after 48 hours. Zinc concentrations were determined using inductively coupled plasma mass spectrometry (ICPMS). We only performed these analyses with ApoE2 and ApoE4, as we confirmed by western blot using an anti-His antibody (data not shown) that they were not His-tagged, unlike ApoE3 (His-tags can bind zinc and affect zinc affinity, thereby confounding any interpretation).

Statistical analysis

Statistical analyses were performed using PRISM 6 Software (GraphPad, La Jolla, CA, USA). Data are presented as mean ± SEM. The difference between the means was determined by one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparisons test. A probability value of $p<0.05$ was taken to be statistically significant. For dialysis data, we used a nonlinear regression specific binding approach for analysis.

Results

Zinc and copper do not affect the stabilityor immunoreactivity of r-ApoE isoforms

Non-lipidated r-ApoEs were incubated with zinc and copper (10 and 100μM) at 37°C for 4 hours (Fig. 1A). The incubation itself did not affect ApoE levels as there was no difference in full-length ApoE (34kDa) levels between the "no incubation" (NI) and "TBS incubation" groups. We also observed that r-ApoEs levels did not change after zinc or copper treatment and 300μM of chelators (TPEN, BCS, DPEN and EDTA) had no influence on the r-ApoEs levels. Lipidated r-ApoE samples were treated similarly and the same results were observed as for the non-lipidated r-ApoE samples (Fig. 1B). Taken together, these suggest that metals do not affect the stability and immunoreactivity of r-ApoE isoforms.

Metal effects on the activity of proteases

Using the colorimetric method of the serine activity assay, we found that 100μM zinc alone had no influence on plasma kallikrein (PK) activity while 1μM and 10μM copper alone led to a significant decrease in PK activity, with 1μM copper causing the loss of approximately 90% of PK activity (Fig. 2A). In contrast, thrombin activity was more susceptible to zinc. 50 and 100μM of zinc inhibited the activity of thrombin (Fig. 2B). Regarding chymotrypsin, we observed that 100μM zinc did not affect chymotrypsin activity(Fig. 2C). In order to exclude the effects of zinc on these proteases, in subsequent experiments we only used zinc concentrations that showed no effect on protease activity. These data of PK and thrombin are consistent with previous studies that showed that ApoE4 is less stable than ApoE2 and ApoE3 (10).

Zinc affects the PK-induced degradation of r-ApoEs (non-lipidated and lipidated) in an isoform-dependent way

After PK treatment (40μg/ml for ApoE2 and ApoE3, 0.35μg/ml for ApoE4), the full-length r-ApoEs decreased to approximately 50-60% of the PK untreated ApoE amount. Pre-incubation of zinc and r-ApoEs did not affect ApoE2 degradation but enhance ApoE4 degradation. The free zinc in the TBS buffer was compared with that in distilled water using Zinquin-ethyl-ester (MELLITECH, #ZQ1). Zinc chloride solutions were made up with TBS or water in the range of 0-100μM, including all the concentrations we used in our study. 10μM of ZQ was added to zinc solutions and incubated at 37°C for 15 minutes, avoiding light. Fluorescence was obtained at excitation 368 nm/ emission 490 nm using a FlexStation 3. We found that there was no significant difference in free zinc immunofluorescence intensity between the two kinds buffer, suggesting that the TBS buffer does not affect the free zinc available (see Supplementary Figure 1).

In the presence of zinc, we observed no significant change in the levels of non-lipidated full-length or fragmented ApoE2 or ApoE3 compared with the control group (PK-treated r-

ApoEs in the absence of zinc). However, there was a significant decrease in non-lipidated full-length ApoE4 and an increase in ApoE4 fragments resulting from increasing concentrations of zinc (Fig 3A). 10μM of zinc caused a decrease in full-length non-lipidated ApoE4 from approximately 0.6 to 0.4 (the amount of PK-untreated full-length ApoE4 was considered as 1.0) and an increase in the ratio of fragmented ApoE4 to total ApoE4 from approximately 0.5 to 0.6. And those zinc's effects became more significant as zinc concentrations increased. The full-length ApoE4 left in the presence of 100μM zinc was approximately 0.3. Similar results were found in lipidated ApoE (Fig 3B). The stability of ApoE2 and E3 in the presence of PK were not altered by zinc except that 100μM zinc caused more degradation of lipidated ApoE3. The levels of lipidated ApoE4 were not affected by low concentrations of zinc (10-50μM), but less full-length lipidated ApoE4 and more lipidated ApoE4 fragments were observed at 75 and 100μM. Taken together, zinc was able to promote ApoE4 (non-lipidated and lipidated) proteolysis by human PK. It's also worthy to note that non-lipidated ApoE4, whose degradation affected by 10μM zinc, is more susceptible to zinc compared with lipidated ApoE4, indicating that non-lipidated ApoE4 is more detrimental than lipidated ApoE4 when zinc dyshomeostasis occurred in ageing and AD brains.

Zinc affects the thrombin-induced degradation of r-ApoEs (non-lipidated and lipidated) in an isoform-dependent way

Thrombin (25μg/ml for ApoE2 and ApoE3, 2.5μg/ml for ApoE4) was used to digest r-ApoEs. Western Blots showed that zinc enhanced non-lipidated and lipidated ApoE4 degradation by thrombin but had no effect on the proteolytic stability of ApoE2 or E3 in the presence of thrombin. Thrombin caused an approximately 50% reduction of the full-length r-ApoEs.

Non-lipidated ApoE2 and E3 levels did not change in the zinc treatment groups compared to the control group (thrombin treatment without zinc), suggesting that zinc had no influence on the degradation of non-lipidated ApoE2 or E3 by thrombin (Fig. 4A). However, non-lipidated ApoE4 degradation was enhanced in the presence of 10 and 20μM zinc (Fig. 4A), resulting in the amount of full-length ApoE4 from approximately 0.5 in the control group to 0.3 (the amount of thrombin-untreated full-length r-ApoEs was considered as 1.0, and the control group was thrombin-treated r-ApoEs in the absence of zinc). As for lipidated ApoE, zinc had no influence on ApoE2 or E3 whereas 20μM zinc promoted ApoE4 degradation, resulting in approximately 0.4 full-length ApoE4 left. And the ratios of fragmented ApoE4 to total ApoE4 rose from approximately 0.3 in the control group to 0.56 in the presence of 20μM zinc (Fig. 4B). Taken together, zinc destabilized r-ApoE4 in the presence of thrombin, resulting in more ApoE4 fragments.

Zinc affects the chymotrypsin-induced degradation of non-lipidated r-ApoEs in an isoform-dependent way

As chymotrypsin has been identified as an important extracellular protease to degrade ApoE in the brain, we investigated whether zinc is able to affect the chymotrypsin-mediated proteolysis of ApoE. 0.1μg/ml chymotrypsin was used for r-ApoEs and it caused a half decrease in full-length r-ApoEs. 100μM of zinc promoted ApoE2 and E3 degradation (Fig. 5), resulting in decreased levels of full-length ApoE from approximately 0.5 in the control group to 0.2 (the amount of full-length r-ApoEs in the chymotrypsin-untreated group was considered as 1.0, the control group was chymotrypsin-degraded r-ApoEs without zinc) and increased ratios of fragmental ApoE to total ApoE from approximately 0.5 to 0.7. ApoE4 appeared to be more sensitive to zinc as in the presence of 1μM zinc there was a significant decrease in full-length ApoE4 from 0.5 in the control group to 0.4 (Fig. 5). ApoE4 degradation was further promoted with higher concentrations of zinc. 100μM zinc has similar effects on ApoE4 with ApoE2 and E3, leading the full-length ApoE4 to be approximately 0.2 and the ApoE4 fragments ratio to be 0.9 (Fig. 5). Taken together, zinc destabilized ApoE isoforms in the presence of chymotrypsin in an order of ApoE4>E2 and E3.

We also investigated the affinity of ApoE2 and ApoE4 for zinc (Supplementary Figure 2). The data demonstrate that the Bmax (maximum specific binding) for ApoE2 and ApoE4 is 4.524 ± 0.7611 mol/mol (mean ± SEM) and 2.503 ± 0.4330 mol/mol (mean ± SEM), respectively, suggesting that ApoE2 can bind more zinc than ApoE4. However, the Kd (equilibrium binding constant) of ApoE2 (8.290μM) and ApoE4 (6.702μM) were similar, suggesting that they might have a similar affinity for zinc.

Plasma ApoE levels in different APOE carriers

Human plasma samples were from both healthy controls (HC) and AD patients with different homozygous APOE genotypes and were divided into five groups: HC E2/E2, HC E3/E3, HC E4/E4, AD E3/E3 and AD E4/E4 (n=5/group). 0.5μl plasma of each group was used to investigate ApoE levels. We found that there was no difference in the amount of plasma ApoE within the same APOE genotype carriers, irrespective of their clinical status. However, consistent with previous studies (11, 12), APOE2 carriers had the highest levels of plasma ApoE while APOE4 carriers have the lowest (Fig. 6A and B). Interestingly, the levels of ApoE fragments showed a similar trend (ApoE2>ApoE3>ApoE4) (Fig. 6A and C) which is in contrast to previous findings showing that more ApoE4 fragments in AD brains compared with ApoE3 and ApoE2 (29). In summary, we found that the amount of both full-length and fragmental ApoE followed an order of ApoE2>ApoE3>ApoE4, which was not affected by disease status.

The level of plasma ApoE is not affected by metals *in vitro*

There was no significant change in plasma ApoE levels in any group after 24-hour incubation with 100μM metals (zinc, copper and iron) or 300μM metal chelator (EDTA) (Fig. 7), indicating that metals alone may not affect the stability of human plasma ApoE.

Discussion

Zinc has been demonstrated to play an important role in the pathogenesis of AD (22), facilitating Aβ aggregation (23, 30) as well as tau phosphorylation and neurofibrillary tangle (NFTs) formation (31). The precise mechanism by which zinc contributes to disease, however, remains unknown. Our study shows that zinc influences the proteolytic degradation of ApoE, which is the most important genetic risk factor for the sporadic form of AD, in an isoform-specific way (ApoE4>ApoE2 and E3). Therefore, zinc dyshomeostasis in ageing and AD brains may affect ApoE4 degradation and its levels, which may partially account for the higher risk of AD in APOE4 carriers.

Brain zinc plays a pivotal role in neurotransmission, enzymatic activity, gene regulation, and structural maintenance and stabilization of proteins (32). Normally, extracellular zinc concentrations are below 1 μM whereas following intense neuronal activity, local zinc concentrations can rise to as high as 300 μM (33, 34). Brain zinc levels are increased with ageing, perhaps resulting from either a reduced ability of zinc re-uptake by neurons and glia or an antioxidant protective response (34, 35). The increased local zinc concentrations in the ageing brain may to contribute to AD pathology in a variety of ways (36). In AD brains, zinc co-exists with ApoE in the amyloid plaques (23, 37, 38). ApoE has been shown to bind zinc, and some studies speculated that ApoE2 has the highest affinity while ApoE4 has the lowest affinity among three ApoE isoforms based on their different structures (26, 38). In our study, we measured the zinc affinity for ApoE directly, showing that ApoE2 has higher maximum binding than ApoE4, suggesting that ApoE2 can bind more zinc than ApoE4. However, the effects of zinc binding on the different ApoE isofroms still need to be clarified in a future study.

ApoE, which is produced and secreted primarily by astrocytes, is the most abundant apolipoprotein regulating lipid delivery and redistribution in the brain (39). Several mechanism relating ApoE isoforms to AD pathology have been reported, including both Aβ-dependent and -independent mechanisms (40). In addition, a number of studies have demonstrated genotype-dependent variability in ApoE concentrations, with the lowest levels in APOE4 carriers (14, 15, 29, 41, 42), which account for 65%-80% of all AD cases (43). It is widely believed that not only the different isoforms per se may influence the risk of AD but the different ApoE concentrations may also be an AD risk-modulating factor (39). Some ApoE receptors and the ATP binding cassette A1 (ABCA1) have also been found to influence ApoE levels in the brain (44-48). Since there's no correlation between APOE genotype and gene transcriptional (mRNA) levels (49), we hypothesised that ApoE proteolysis might be one of the mechanisms regulating ApoE protein levels.

ApoE undergoes proteolytic cleavage in the brain to form truncated fragments. Multiple serine enzymes, such as thrombin and chymotrypsin-like protease have been suggested to mediate ApoE proteolysis to generate neurotoxic fragments in the brain (16, 17, 50). The hinge region is the most protease-sensitive region of the protein, and cleavage at this position gives rise to the two major fragments corresponding to the N- (approx. 22kDa) and C-terminal (approx. 10kDa) domains (17). The 22kDa ApoE fragment is neurotoxic (51) and the 10kDa C-terminal portion facilitates amyloid plaque formation, both of which are

increased in AD brains compared to healthy controls (16). Thrombin is thought to be the major protease to generate 22kDa ApoE fragments (52).

Plasma kallikrein (PK), which was thought to be exclusively found in the plasma for a long time, has been found in many regions of brain, such as cerebral cortex and hippocampus, suggesting a role for PK in central nervous system (CNS) (53). Further studies have demonstrated that PK is able to degrade astrocyte-secreted ApoE (54). We found that ApoE4 was degraded to 10, 22 and ~29-30kDa fragments. The latter are proposed to be both due to chymotrypsin-like protease activity and highly enriched in insoluble fractions of AD brains (55). However, we observed its presence after PK digestion of ApoE4 as well, suggesting PK is another important protease related to AD pathology, particularly for APOE4 carriers. A recent study identified a novel way by which ApoE undergoes proteolysis in the extracellular space via chymotrypsin-like serine proteolysis (19). ApoE is cleaved in the human brain by chymotrypsin-like serine protease to form a peptide of ~29-30kDa and the cleavage sites are suggested at Met^{272} and/or Leu^{268} (18, 55). Chymotrypsin-like protease also generates 23-25kDa fragments with the cleavage sites in the hinge region (16). Unlike PK and thrombin, we found that chymotrypsin exhibited equivalent proteolytic efficacy on ApoE degradation which is due to its preferred cut sites on Met, Leu, Phe, Trp and Try, but not cysteine or arginine.

Full-length ApoE is the predominant ApoE species in brain (19) and it plays an important role in lipid transport (39), Aβ clearance (29) and a protective role against neurotoxicity (56). Accordingly, ApoE fragments are observed both in healthy and AD brains. Increased ApoE fragmentation has been observed in AD brains and the increased accumulation of ApoE fragments seems to depend on APOE-e4 dose and causes damage to neurons (19, 29, 57). Several mechanisms have been suggested to account for this, such as increased susceptibility to proteases (10) and less stability and solubility of ApoE4 fragment compared with ApoE2 and E3. In our experiments, more ApoE4 fragments were generated by proteolysis in the presence of zinc compared to ApoE2 and E3. We speculate that increased extracellular zinc in ageing and AD brains can promote ApoE proteolysis, leading to the loss of functional full-length ApoE and increased detrimental ApoE fragments, thereby contributing to neurodegeneration. Furthermore, as ApoE4 proteolysis is more sensitive to zinc, APOE4 carriers might be more susceptible to zinc alterations in terms of ApoE levels in the brain, which may partially account for the higher risk for AD in APOE4 carriers.

Apart from ApoE concentration, the lipid status of ApoE also plays a key role in AD. Lipidated ApoE is more stable and more efficient at binding Aβ and directing Aβ to degradation pathways (58, 59). ApoE4 is less lipidated compared with ApoE2 and ApoE3 under physiological condition, which is a potential mechanism underlying the elevated risk of AD in APOE4 carriers (58-60). We observed that proteases alone had the same efficacy for both non-lipidated and lipidated r-ApoEs, however the degradation of lipidated r-ApoEs was less susceptible to zinc compared with their non-lipidated counterparts, indicating that lipidated r-ApoEs are more resistant to zinc alterations in the brain. Therefore, our results are consistent with the notion that non-lipidated ApoE lacks normal function and could contribute to AD pathology (39) and further, that lipidated r-ApoEs are more resistant to zinc alterations in the brain.

Unlike in the CNS, peripheral ApoE is primarily produced by the liver and macrophages and it does not cross the blood-brain barrier (BBB) (42, 61). Previous studies claimed the lower plasma ApoE levels in AD patients correlated with AD pathology (11). A recent study reported that low plasma levels of ApoE are associated with increased future risk of AD independent of APOE genotype (62). As expected, we found the highest plasma ApoE levels in APOE2 carriers and the lowest levels in APOE4 carriers, consistent with previous reports (11, 42). Anticoagulation of our plasma samples is achieved with the use of heparin, which is an inhibitor of many serine proteases in plasma (including thrombin and PK) (63-65), so the ApoE levels in our heparin-sourced plasma samples are not supposed to be affected by proteases. Additionally, we observed that ApoE4, which is most susceptible to proteolysis, had few fragments in plasma while ApoE2, which is most stable and resistant to proteolysis, exhibited the greatest ApoE fragmentation, suggesting that plasma proteases have a weak influence on ApoE levels and there may be some other factors in the plasma of APOE2 carriers that cause the increased fragmentation. With respect to the effects of metal, plasma ApoE levels did not change after incubation with zinc, copper, iron or EDTA. Those results are consistent with our previous ones that co-incubation of metals and ApoE isoforms in the absence of protease caused no change in r-ApoE levels. Further studies need to be done to identify the functions of ApoE2 fragments in peripheral tissue as well as the factors affecting plasma ApoE levels in the presence of active proteases.

Conclusions

By investigating ApoE proteolytic stability, we present evidence supporting an interaction between zinc and ApoE. We propose that the ability of zinc to promote ApoE proteolysis may contribute to the onset and/or progression of AD in the ageing brain. Furthermore, the isoform-specific effects of zinc may account for the higher risk for AD in APOE4 carriers. Further studies on detailed mechanism of the interactions still need to be clarified.

Conflict of interest

None declared.

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Captions

Figure 1. Metals do not affect the stability and immunoreactivity of r-ApoEs. 10 and 100μM of zinc or copper were incubated with non-lipidated ApoE at 37°C , and we observed no significant changes in full-length ApoE (34kDa) levels after 4 hours (A). In addition, several metal chelators, including TPEN, BCS, DPEN and EDTA, were also added but no changes in ApoE levels was observed. We also investigated zinc's effect on lipidated ApoE using the same method, and we found the same result that zinc (10-100μM), TPEN and EDTA (300μM) did not alter lipidated ApoE levels (B). The ApoE levels in the no incubation (NI) group were normalized to 1.0, and TBS-incubated group is considered as the control group. Data are mean ± SEM, and differences were assessed using one-way ANOVA followed by a Dunnett's post hoc test.

Figure 2. Serine protease activity assay in the presence of metals. 100μM of zinc did not affect the activity of PK whereas copper exhibited significant inhibitory effects on PK activity (A). 50 and 100μM of zinc reduced the activity of thrombin (B) while chymotrypsin is not sensitive to zinc showing 100μM of zinc did not alter the activity of chymotrypsin (C). The control groups are protease enzymatic activity in the absence of metals, and their activities were normalized to 1.0. Data are mean ± SEM, and differences were assessed by one-way ANOVA followed by a Dunnett's post hoc test.

Figure 3. The effect of zinc on the proteolytic stability of r-ApoEs in the presence of PK. Human PK was used to digest r-ApoEs *in vitro*. Non-lipidated ApoE2 and E3 were not influenced by zinc whereas the degradation of non-lipidated ApoE4 was enhanced dramatically by zinc (A). 10μM of zinc promoted the proteolysis of non-lipidated ApoE4, resulting in a decrease in the amount of full-length ApoE4. Higher zinc concentrations further altered the ratio of full-length to fragmented ApoE4 (A). Similar influences of zinc were also present with lipidated ApoE samples (B). Zinc showed no influence on lipidated ApoE2 whereas 75μM and 100μM of zinc enhanced the proteolysis of lipidated ApoE4 by PK. The levels of PK-untreated full-length r-ApoEs were normalized to 1.0 and PK-treated ApoE without zinc is the control group. Data are mean ± SEM, and differences were assessed using one-way ANOVA followed by a Dunnett's post hoc test. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. The effects of zinc on the proteolytic stability of r-ApoEs in the presence of thrombin. Thrombin digested approximately half of the r-ApoEs in the absence of zinc. ApoE2 and E3 degradation were not influenced by zinc whereas the degradation of ApoE4 was promoted dramatically by zinc. 10μM and 20μM of zinc promoted the proteolysis of non-lipidated ApoE4, leading to a change in the ratio of full length to fragmented ApoE (A). Similar influences of zinc were also present with lipidated ApoE4 (B), with 20μM of zinc decreasing full-length lipidated ApoE4 and increasing ApoE4 fragments. The full-length ApoE level in the TBS group (thrombin-untreated) was considered as 1.0. Data are mean ± SEM, and differences were assessed using one-way ANOVA followed by a Dunnett's post hoc test. *P<0.05, **P<0.01.

Figure 5. The effects of zinc on the proteolytic stability of r-ApoEs in the presence of chymotrypsin. 100μM of zinc promoted ApoE2 degradation, resulting in an increase in ApoE fragments and a decrease in full-length ApoE levels. 100μM zinc also enhanced ApoE3 degradation. 1μM of zinc caused a significant decrease in full-length ApoE4 as well as an increase in ApoE4 fragment ratios. ApoE4 proteolysis is further enhanced by increasing the concentration of zinc. The full-length ApoE

levels in TBS group (chymotrypsin-untreated) was considered as 1.0. Data are mean ± SEM, and differences were assessed using one-way ANOVA followed by a Dunnett's post hoc test. *P<0.05, **P<0.01, ***P<0.001.

Figure 6. The levels of human plasma ApoE follow an order of ApoE2>ApoE3>ApoE4. Within HC samples, plasma ApoE levels are highest in APOE2 carriers and lowest in APOE4 carriers (A, B). Similarly, AD patients with an APOE3 genotype have more plasma ApoE than APOE4 carriers (A, B). However, there is no difference in ApoE levels between HC and AD individuals with the same APOE genotype (B). There are high molecular weight (20-25kDa) and low molecular weight (10-15kDa) ApoE fragments in APOE2 and APOE3 carriers whereas APOE4 carriers of HC and AD show few fragments (A). APOE2 carriers have the highest ApoE fragments, which is 34% of total amount of ApoE (C). 24% of ApoE3 are fragments in HC APOE3 carriers and 12% of total ApoE is fragmented in AD APOE3 carriers (C). Full-length ApoE3 levels of HC cases were normalized to 1.0, (HC= healthy control, n=5). Data are mean ± SEM, and differences were assessed using one-way ANOVA followed by a Dunnett's post hoc test. *P<0.05, **P<0.01, ***P<0.001.

Figure 7. Metals do not affect the levels of plasma ApoE isoforms. 5µl of human plasma were incubated with 5µl zinc, copper, iron (100µM) or EDTA (300µM) at 37°C for 24 hours. Neither metals nor EDTA alter plasma ApoE levels after the incubation. The respective TBS group was considered as the control and its full-length ApoE amount was normalized to 1.0 (HC= healthy control, n=5). Data are mean ± SEM, and differences were assessed using one-way ANOVA followed by a Dunnett's post hoc test.

Supplementary Figure 1. Zinc fluorescence in TBS and Water. We used ZQ as an indicator of free zinc. Zinc chloride solutions were made up with TBS or water in the range of 0-100µM, including all the concentrations we used in our study. 10µM of ZQ was added to zinc solutions and incubated at 37°C for 15 minutes, avoiding light. Fluorescence was obtained at excitation 368 nm/ emission 490 nm using a FlexStation 3. We found that there was no significant difference in free zinc between TBS and Water.

Supplementary Figure 2. Zinc affinity for ApoE. ApoE2 and ApoE4 were dialysed against an equal volume (30µl) of zinc solutions at 4°C for 48 hours. ApoE2 and ApoE4 had a similar equilibrium binding constant (Kd) while the maximum binding (Bmax) of ApoE2 (4.524 ± 0.7611 mol/mol) was almost two-fold that of ApoE4 Bmax (2.503 ± 0.4330 mol/mol). Data are mean ± SEM.

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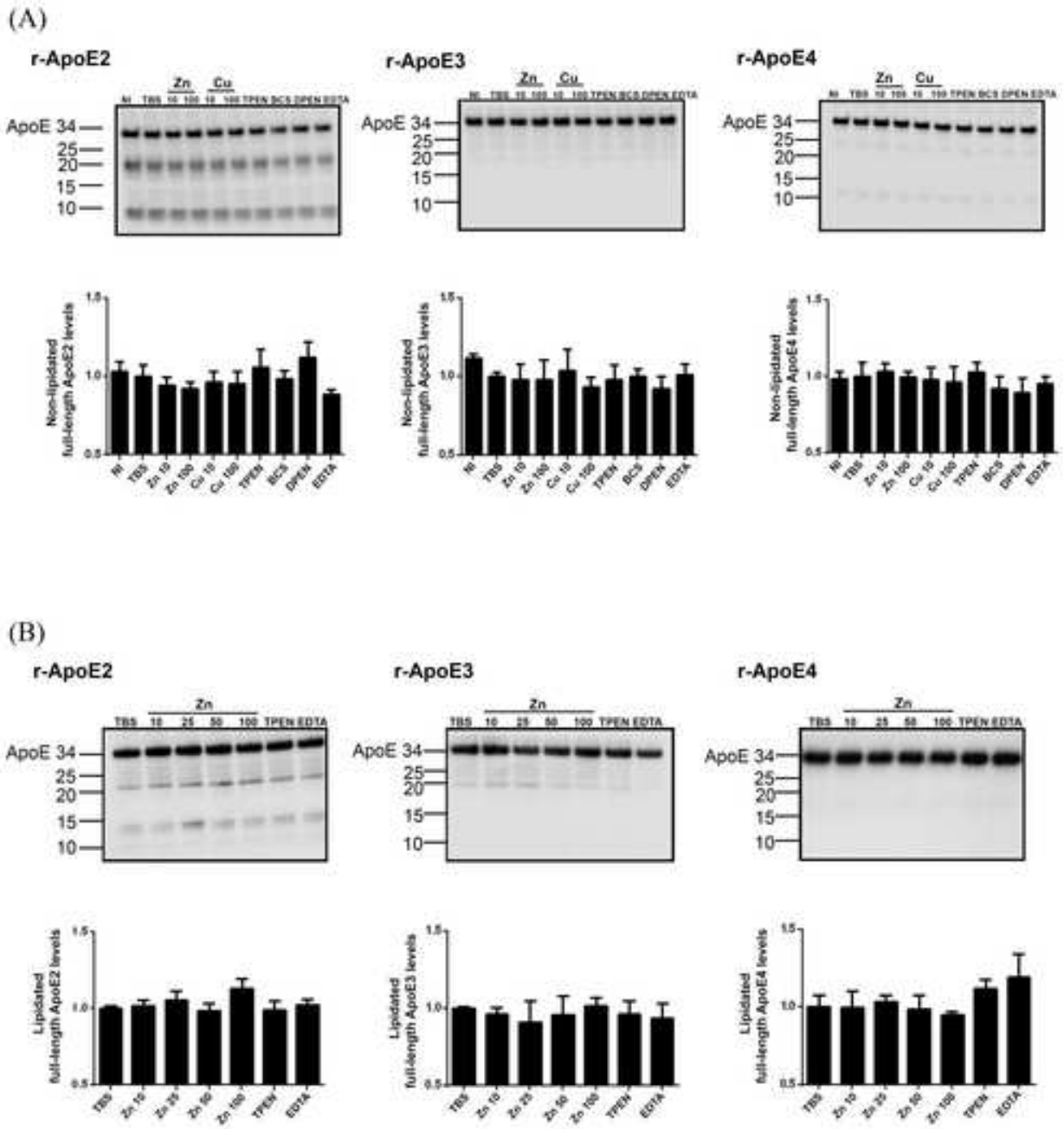

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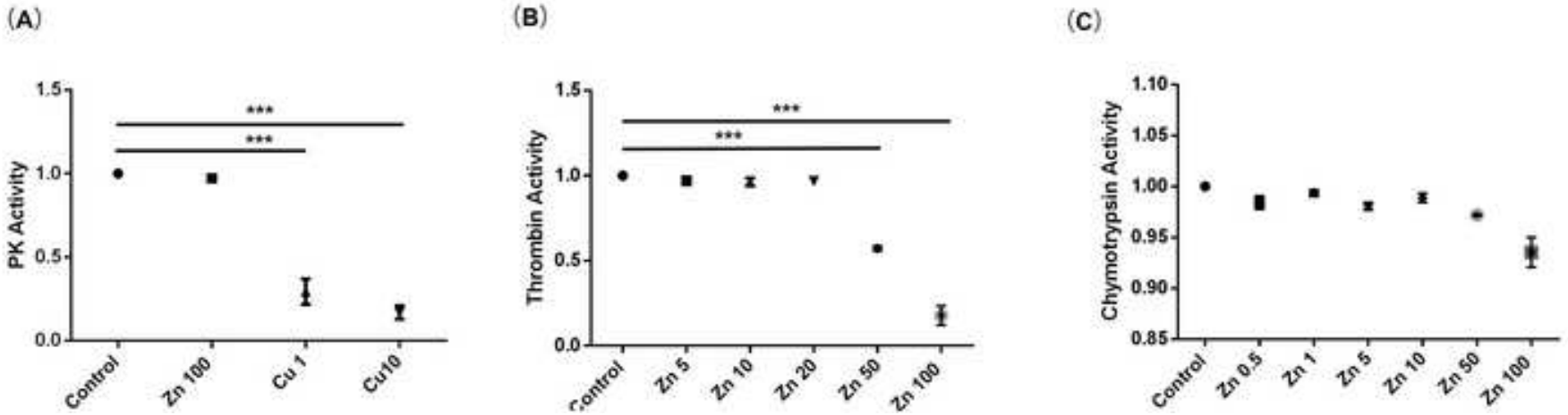

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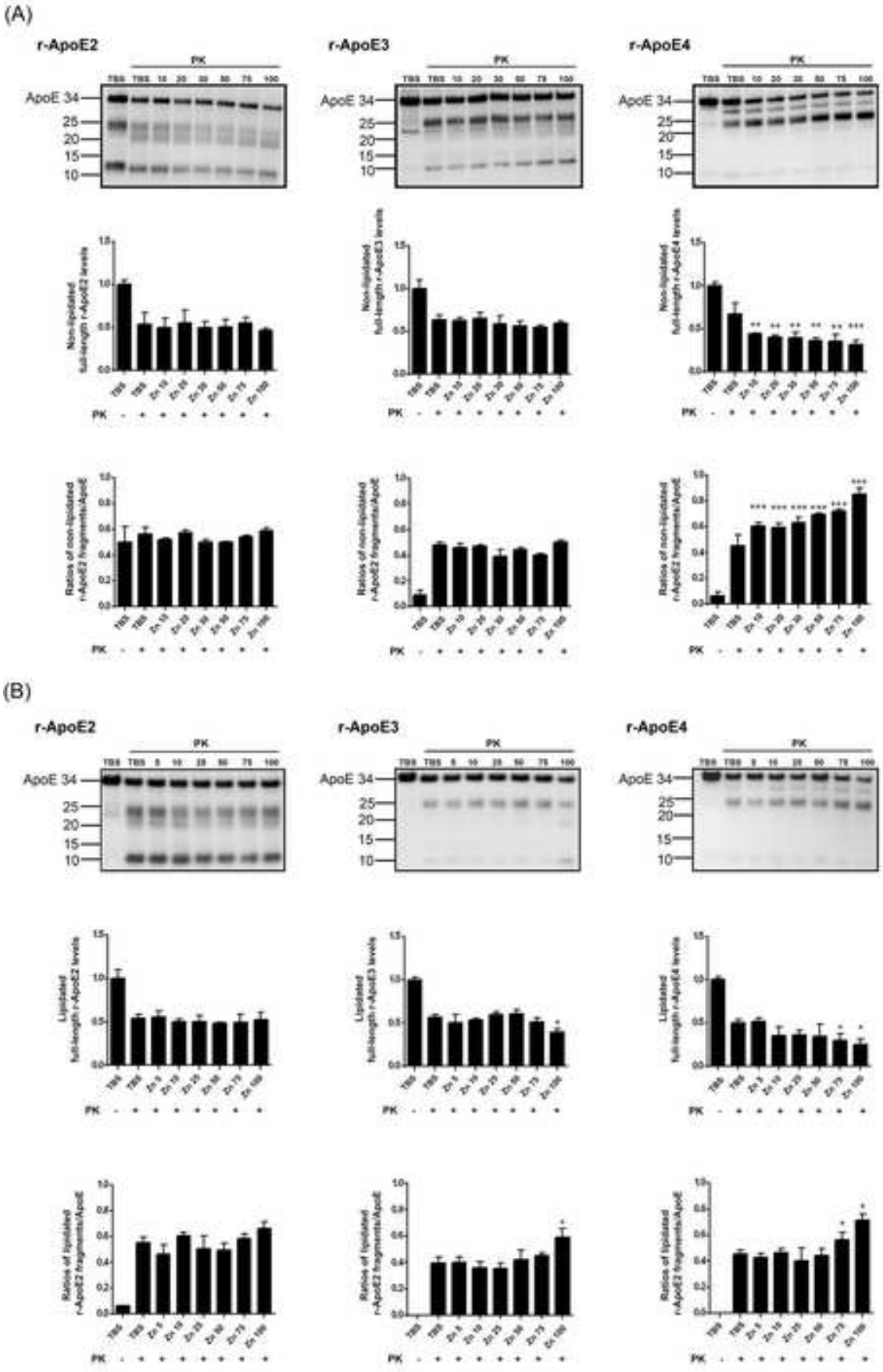

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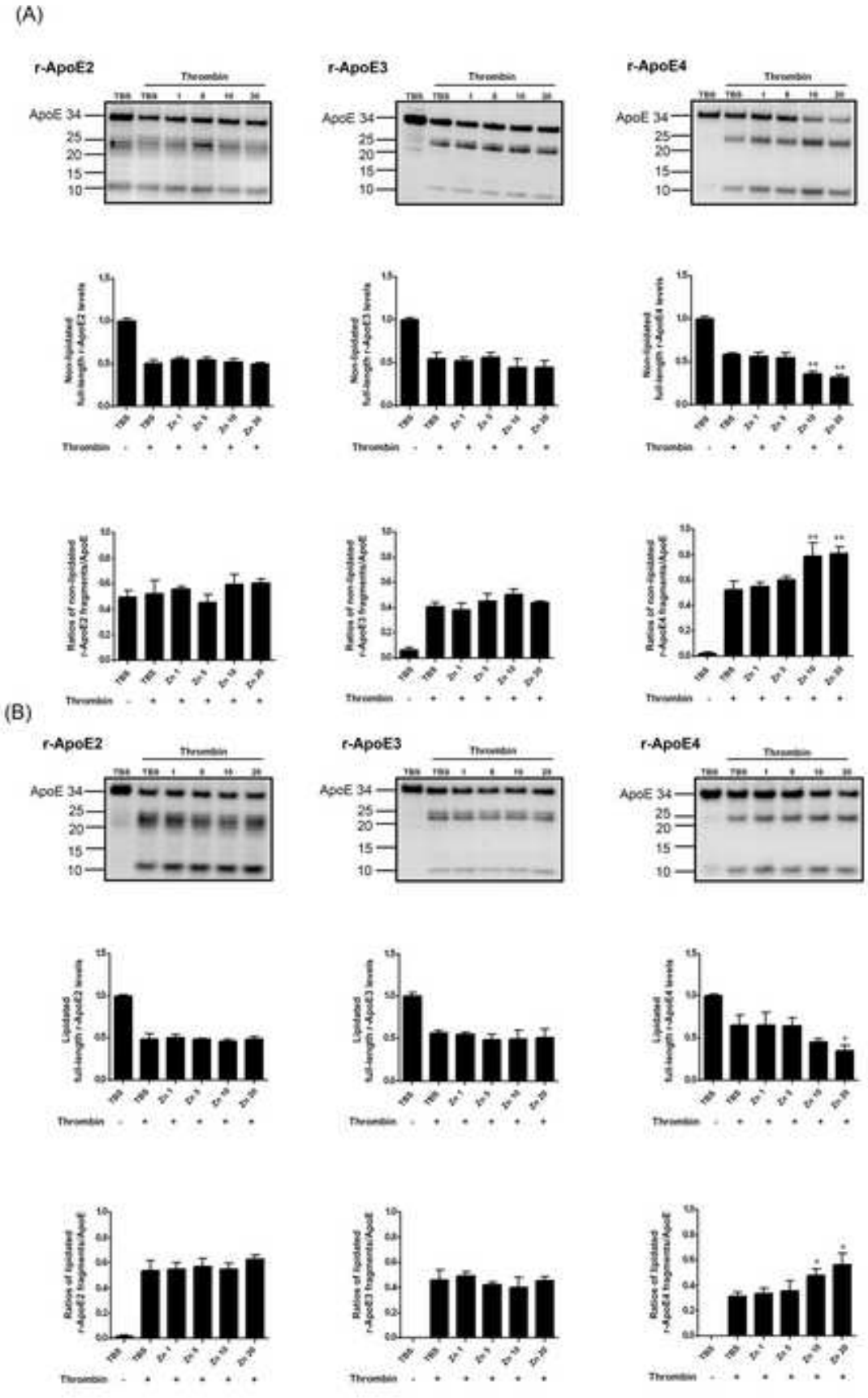

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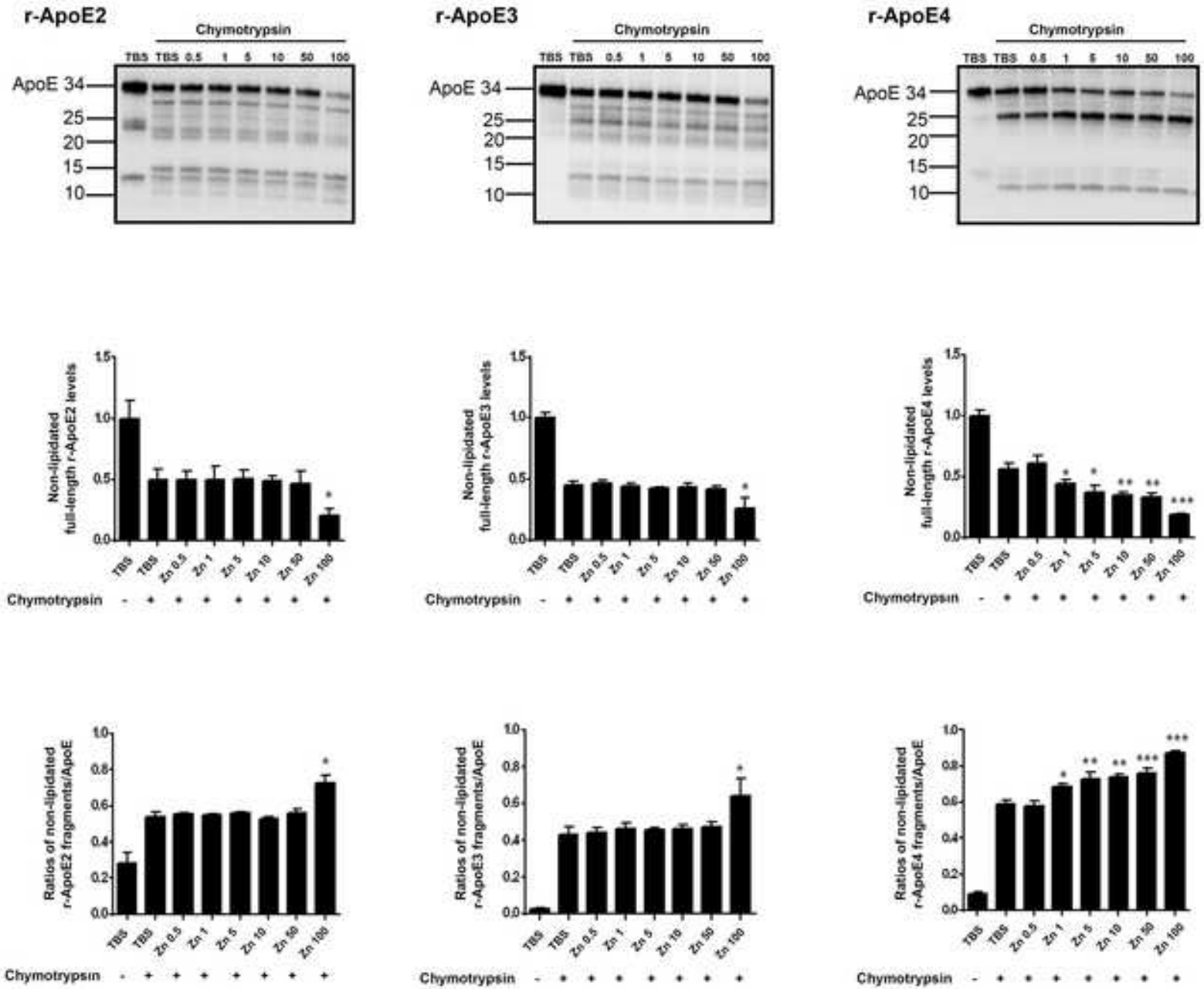

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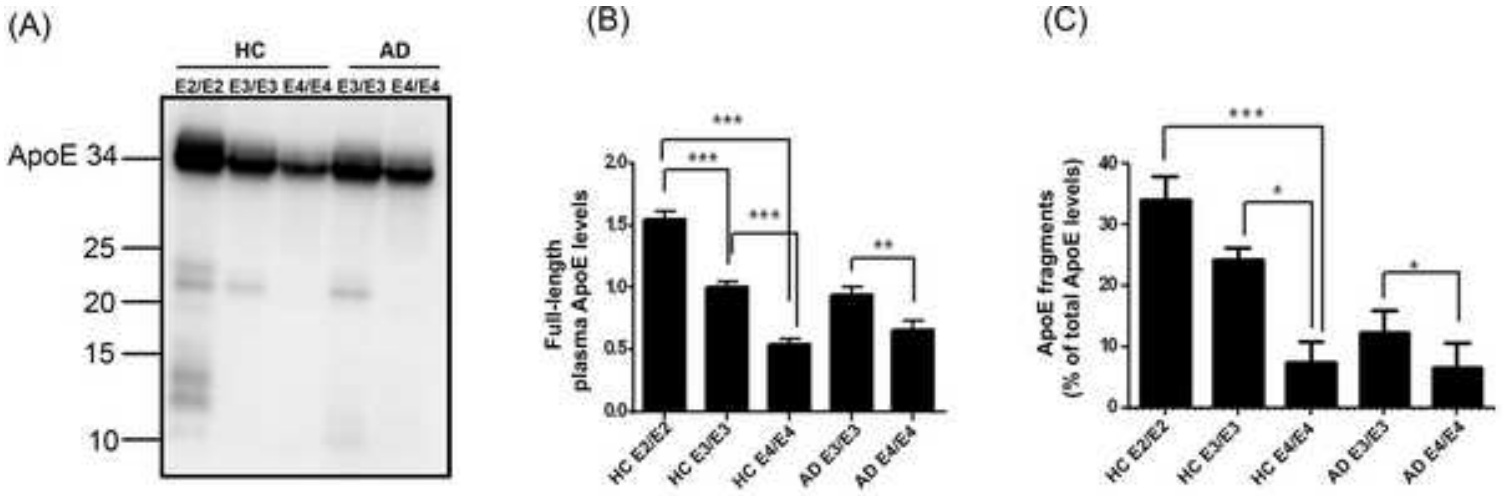

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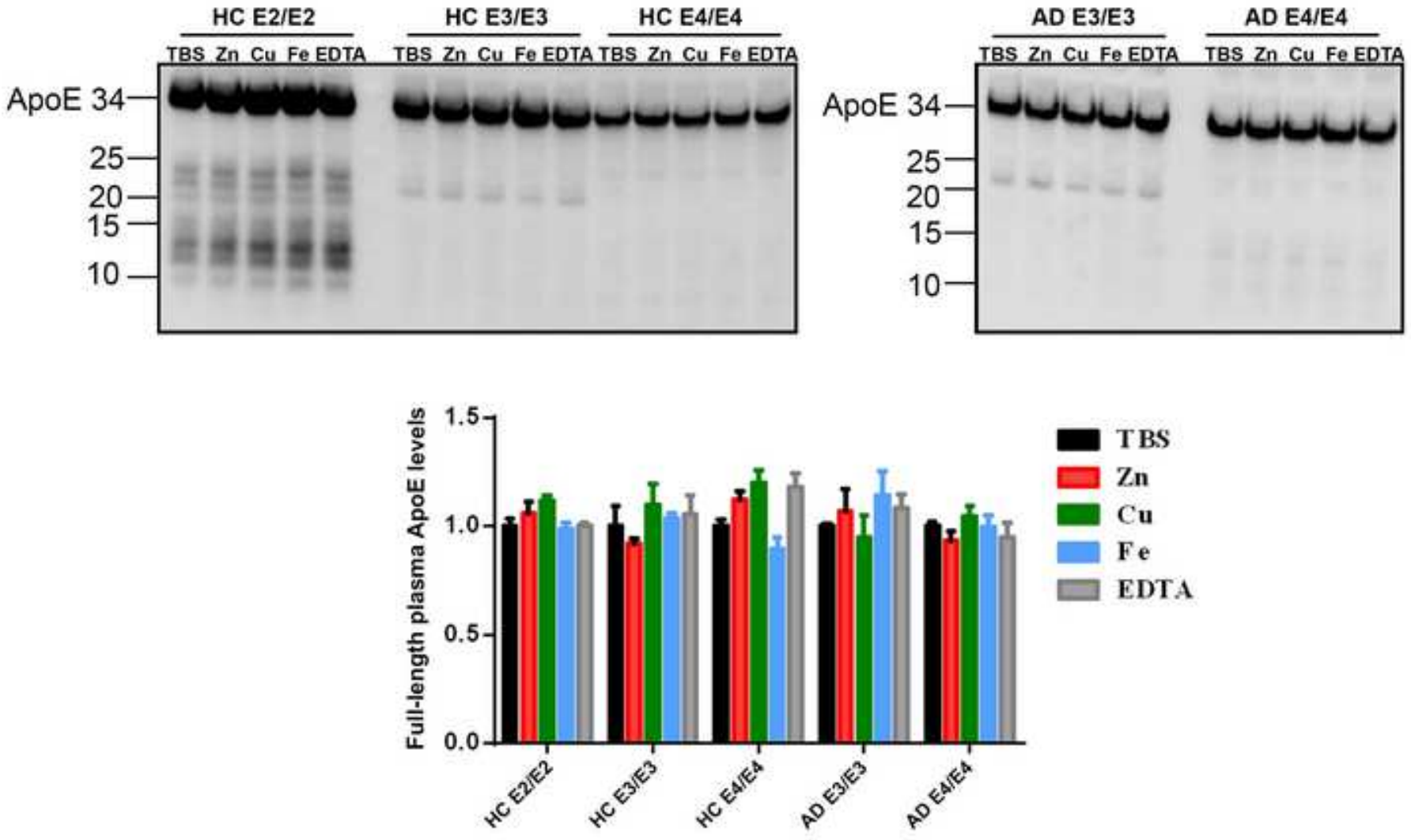

Supplementary Figure 1

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