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Original Contribution

Oxidative modification to LDL receptor-related protein 1 in hippocampus from subjects with Alzheimer disease: Implications for Aβ accumulation in AD brain

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ABSTRACT

Alzheimer disease (AD) is a neurodegenerative disorder characterized histopathologically by the presence of senile plaques (SPs), neurofibrillary tangles, and synapse loss. The main component of SPs is amyloid-β peptide (Aβ), which has been associated with increased oxidative stress, leading to oxidative modification of proteins and consequently to neurotoxicity and neurodegeneration. Low-density lipoprotein receptor-related protein 1 (LRP1) is the primary moiety responsible for the efflux of Aβ from the brain to the blood across the blood–brain barrier. Impaired brain-to-blood transport of Aβ by LRP1 has been hypothesized to contribute to increased levels of Aβ in AD brain. The cause of LRP1 dysfunction is unknown, but we have hypothesized that Aβ oxidizes LRP1, thus damaging its own transporter. Consistent with this notion, we report in this study a significant increase in the levels of the lipid peroxidation product 4-hydroxy-2-nonenal bound to transmembrane LRP1 in AD hippocampus. In contrast, the levels of LRP1-resident 3-nitrotyrosine did not show a significant increase in AD hippocampus compared to age-matched controls. Based on this study, we propose that Aβ impairs its own efflux from the brain by oxidation of its transporter LRP1, leading to increased Aβ deposition in brain, thereby contributing to subsequent cognitive impairment in AD.

Alzheimer disease (AD) is characterized pathologically by the presence of senile plaques (SPs), neurofibrillary tangles (NFTs), and decreased synaptic density [1,2]. The main component of SPs is amyloid-β peptide (Aβ) [3], comprising 40–42 amino acids and generated by proteolytic cleavage of amyloid precursor protein (APP), a transmembrane protein, by β-secretase and γ-secretase. Aβ exists in various soluble and insoluble forms including aggregates, soluble monomers, oligomers, protofibrils, and fibrils [4,5]. Recent studies have suggested that soluble oligomers are the most toxic form of Aβ [6]. Genetic mutations in the *APP* and presenilin 1 (*PS1*) genes in familial AD cases show increased production of Aβ and consequently an early onset of AD, consistent with the notion that Aβ is central to the pathogenesis of AD [7]. Further, elevated levels of Aβ 1–40 and 1–42 have been found in AD hippocampus and cortex and have been associated with high levels of protein oxidation, lipid peroxidation, and DNA and RNA damage [8]. Conversely, brain regions low in Aβ levels, such as the cerebellum, do not have extensive markers of oxidative stress [9–14]. Aβ has been shown to induce oxidative stress in vitro and in AD model systems in vivo, as evidenced by increased levels of protein oxidation (indexed by protein carbonyls and protein resident 3-nitrotyrosine (3NT)) and lipid peroxidation (indexed by protein-bound 4-hydroxy-2-nonenal (HNE)) [15–19]. Studies by Liu et al. show that the addition of HNE to tau protein, the primary component of NFTs, promotes and contributes to conformations conducive to NFT formation, further supporting the role of Aβ in the pathogenesis of AD [20].

The neurovascular hypothesis of AD states that impairment of the efflux of Aβ from the brain to the blood at the blood–brain barrier (BBB) is an important mechanism underlying Aβ accumulation in the brain and contributes to subsequent cognitive impairment in AD patients [21]. The major efflux pump for the clearance of Aβ from the brain to the periphery is the LDL receptor-related protein 1 (LRP1) [22,23]. LRP1 is a membrane-associated protein initially synthesized as a 600-kDa precursor and further processed into two noncovalently linked α- and β-subunits [24]. The 515-kDa α-subunit is extracellular and noncovalently bound to the transmembrane 85-kDa β-subunit. The α-subunit is responsible for ligand binding, and the β-subunit cytoplasmic domain interacts with adapter proteins involved in cell

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signaling [22]. In this study, we tested the hypothesis that LRP1 is oxidized in the hippocampus of subjects with AD. Such oxidative modifications to LRP1 would alter its structure, providing a mechanism by which LRP1's ability to efflux Aβ would be affected. Aβ is hypothesized to lead to lipid peroxidation in AD brain [8,25–29]. We reported elevated HNE bound to the glutamate transporter GLT-1 (EAAT2) [30], which has decreased function in AD [31], and this elevation of HNE could be replicated by addition of Aβ(1–42) to synaptosomes [30]. Based on an analogy to the case of GLT-1, we hypothesize that HNE bound to the β-subunit of LRP1 would lead to increased Aβ accumulation in the brain with subsequent oxidative stress, plaque formation, and AD pathogenesis. Accordingly, in this study, we measured the levels of HNE bound to and 3NT on the β-subunit of LRP1 in AD hippocampus to assess the level of oxidative posttranslational modifications to LRP1. The β-subunit, as described above, contains the membrane-spanning portion of LRP1 and the subunit is rich overall in histidine, lysine, and cysteine residues (UniProt protein database ID Q07954, short name LRP-85), probably providing potential targets in the β-subunit of LRP1 for HNE addition [28].

Materials and methods

Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) with the exception of nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The anti-LRP1 antibody has been described in previously published research [23].

Subjects

Frozen hippocampi from AD patients and age-matched controls were obtained from the University of Kentucky Rapid Autopsy Program of the Alzheimer's Disease Clinical Center (UK ADC). All AD subjects displayed progressive intellectual decline. Control subjects underwent annual mental status testing as a part of the UK ADC normal volunteer longitudinal aging study and did not have a history of dementia or other neurological disorders. Brains from subjects with neurodegeneration were collected after a short postmortem interval (PMI) that averaged less than 5 h. AD brains had Braak stages ranging from 4 to 6. Braak staging indicates the severity of AD pathology (based largely on the number of neurofibrillary tangles and ranges from 1 to 6, with the most severe stage being 6 [32]). All control subjects had test scores for dementia in the normal range and all the control brains had a short PMI averaging less than 3 h and Braak stages of 2 or less (Table 1).

Sample preparation

AD ($n=9$) and age-matched control ($n=9$) hippocampi were minced and homogenized separately in Media-I containing 10 mM Hepes buffer (pH 7.4), 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.1 mM EDTA, and 0.6 mM $MgSO_4$ as well as the protease inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), type II S soybean trypsin inhibitor (0.5 μg/ml), and phenylmethylsulfonyl fluoride (40 μg/Ml). These homogenates were centrifuged at 14,000 *g* for 10 min to remove debris. Protein concentration in the supernatant was determined by the BCA assay using the Pierce kit (Pierce Chemical, Rockford, IL, USA).

Table 1
Demographic characteristics of controls and AD patients

Sample	Age (years)	Gender (M/F)	Postmortem interval (h)	Braak staging
Controls	82 ± 6.2	6/3	2.6 ± 0.8	1–2
AD	85 ± 5.3	5/4	4.8 ± 1.6	4–6

Immunoprecipitation of LRP1

Protein A/G–agarose beads (50 μl per sample, i.e., 900 μl for 18 samples; Amersham Pharmacia Biotech, Piscataway, NJ, USA) were washed with immunoprecipitation (IP) buffer three times for 5 min using a vortex with shaker attachment. IP buffer contained phosphate-buffered saline (PBS) with 0.05% NP-40 and the protease inhibitors leupeptin (4 μg/ml final concentration), pepstatin (4 μg/ml final concentration), and aprotinin (5 μg/ml final concentration), adjusted to pH 8. Hippocampal homogenates from AD and control subjects (300 μg) were first precleared with washed protein A/G–agarose beads (50 μl) for 1 h at 4 °C. Samples were then incubated overnight with anti-LRP1 antibody (5 μg) followed by 1 h incubation with protein A/G–agarose. The antigen–antibody–protein A/G complex was centrifuged at 1000 *g* for 5 min and the resultant pellet was washed five times with IP buffer (500 μl). The final pellet was suspended in deionized water. Proteins were resolved on SDS–PAGE, followed by immunoblotting on a nitrocellulose membrane (Bio-Rad).

Immunodetection

For immunodetection of HNE bound to and 3NT resident on LRP1 the nitrocellulose membranes were blocked with 3% bovine serum albumin (BSA) in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 90 min at room temperature. The membranes were incubated with anti-LRP1 polyclonal antibody diluted 1:5000 in 3% BSA, anti-actin monoclonal antibody (Sigma–Aldrich) diluted 1:5000 in 3% BSA, anti-HNE polyclonal antibody (Alpha Diagnostic, San Antonia, TX, USA) diluted 1:5000 in 3% BSA, or anti-3NT polyclonal antibody (Sigma–Aldrich), diluted 1:2000 in 3% BSA, for 2 h at room temperature with rocking. After completion of the primary antibody incubation, the membranes were washed three times in Wash Blot for 5 min each and incubated with anti-rabbit IgG alkaline phosphatase (ALP)-linked secondary antibody (Sigma), diluted 1:3000 in Wash Blot, for 1 h at room temperature. The membranes were washed in Wash Blot three times for 5 min each and developed using Sigma Fast Tablets (BCIP/NBT substrate; Sigma) The Western blot measuring the levels of the β-subunit of LRP1 (Fig. 1) was incubated with anti-LRP1 antibody (1:5000) as described above, and after completion of the primary antibody incubation, the membranes were washed three times in Wash Blot for 5 min each and incubated with anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody (GE Healthcare, Piscataway, NJ, USA), diluted 1:3000 in Wash Blot, for 1 h at room temperature and visualized using ECL Plus Western blotting detection reagents (GE Healthcare). The blot was subsequently stripped using Reblot Plus Strong antibody stripping solution (Millipore, Billerica, MA, USA) as described by the manufacturer and redeveloped using anti-actin antibody (Sigma) as described above using anti-rabbit–ALP secondary antibody. The Western blot measuring the HNE-bound β-subunit of LRP1 normalized on the same blot with unmodified LRP1 (Fig. 3) was visualized using anti-rabbit–HRP antibody and stripped with prepared Strong stripping solution (62.5 mM Tris–HCl (pH 6.8), SDS (2% wt/vol), and β-mercaptoethanol (10 mM)). The stripped Western blot was washed three times in Wash Blot and blocked with BSA. The Western blot was reprobed with anti-LRP1 antibody and anti-rabbit HRP-linked secondary antibody as described above.

Image analysis

After immunodetection of oxidative modification of LRP1 the membranes were completely dried at room temperature and were then scanned using a Microtek Scanmaker 4900 scanner and a

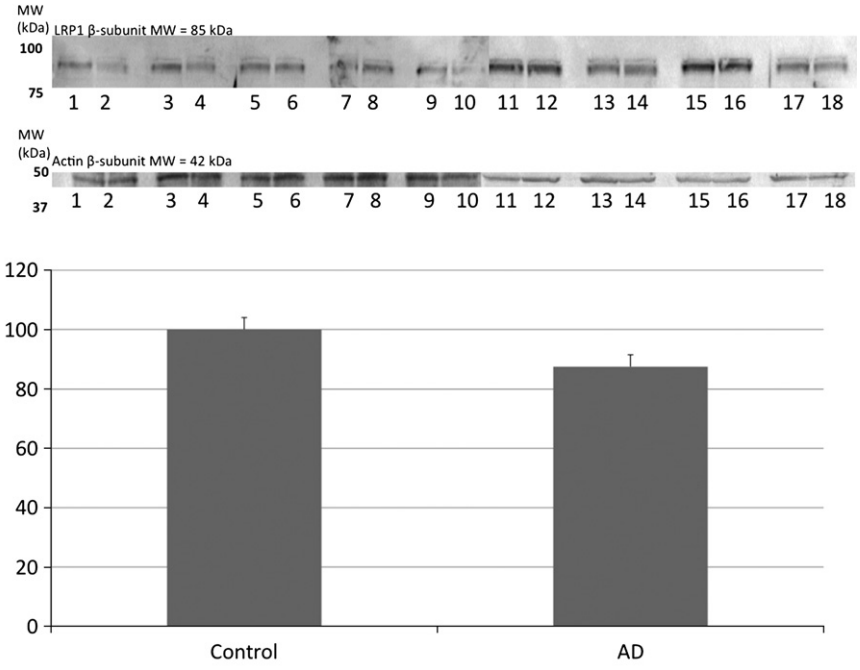

Fig. 1. Levels of LRP1 were determined using Western blotting, and no significant difference in the protein levels of LRP1 was found between AD hippocampus and age-matched controls. Actin was used as a loading control as pictured. Data are shown as percentage control (mean ± SEM). The images shown have odd numbers below the age-matched control hippocampal samples and even numbers below AD hippocampal samples.

Storm860 phosphoimager (GE Healthcare). Images were saved as tiff files in grayscale mode and the intensity of the LRP1 protein modification was quantified using ImageQuant (GE Healthcare) analysis software.

Statistical analysis

Raw values were exported to Microsoft Excel and normalized to percentage control values. The resulting data were analyzed by Student's *t* tests. A value of $p<0.05$ was considered statistically significant.

Results

In this study we measured the levels of LRP1 and the levels HNE-bound LRP1 as well as 3NT modification of LRP1 in AD and age-matched control hippocampus using immunoprecipitation techniques. Fig. 1 is a Western blot showing that the levels of LRP1 β-subunit in AD hippocampus are not significantly different compared to age-

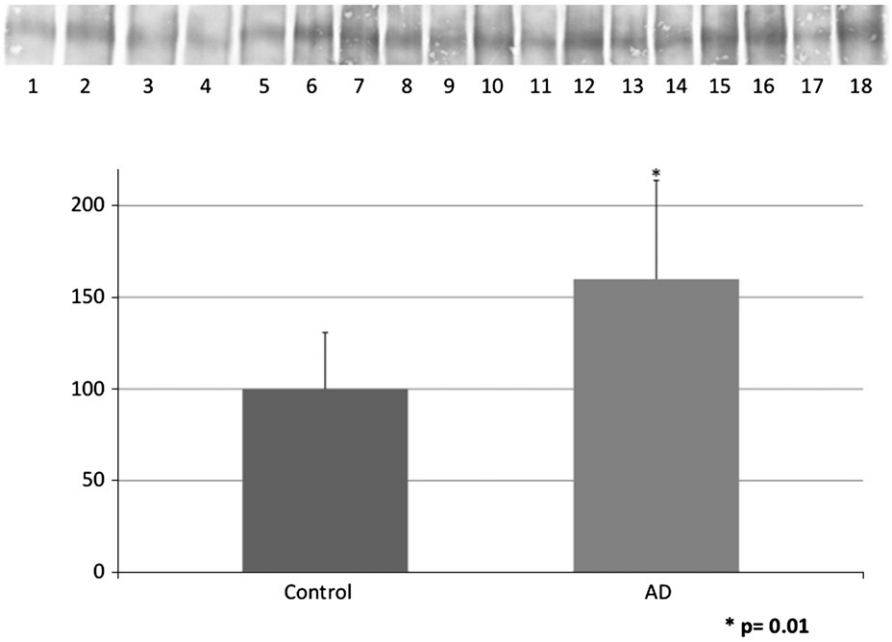

Fig. 2. Levels of HNE-bound LRP1 were determined by immunoprecipitation of LRP1 followed by immunochemical detection with anti-HNE antibody. The level of HNE-bound LRP1 was significantly increased by 60% in AD hippocampus compared to age-matched controls. Data are shown as percentage control (mean ± SEM); $^{*}p = 0.01$. The images shown have odd numbers below age-matched control hippocampal samples and even numbers below AD hippocampal samples.

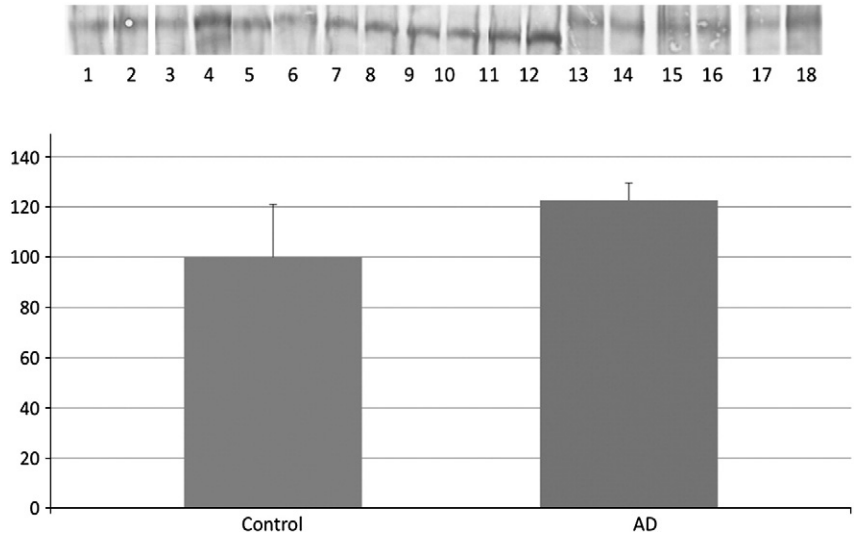

Fig. 3. Levels of LRP1-resident 3NT were determined by immunoprecipitation of LRP1 followed by immunochemical detection using anti-3NT antibody. The levels of LRP1-resident 3NT were not significantly different in AD hippocampus compared to age-matched controls. Data are shown as percentage control (mean ± SEM). The images shown have odd numbers below age-matched control hippocampal samples and even numbers below AD hippocampal samples.

matched controls. Fig. 2 shows a 60% increase in the levels of HNE-bound LRP1 β-subunit in AD hippocampus compared to age-matched controls. No significant increase in 3NT-modified LRP1 β-subunit was observed in AD hippocampus compared to age-matched controls (Fig. 3). The raw values for the intensity of the LRP1 β-subunit bands obtained in the immunoprecipitation studies presented in Figs. 2 and 3 were normalized against the LRP1 bands obtained from the Western blot presented in Fig. 1 (e.g., the raw value for the HNE-bound LRP1 β-subunit band in lane 1 of Fig. 2 was divided by the raw value obtained from the LRP1 β-subunit band, corresponding to the same AD subject, from lane 1 in the Western blot of Fig. 1, probing for overall levels of the β-subunit, and multiplied by 100 to obtain percentage control value).

To confirm our results of the increased levels of HNE-bound LRP1 in AD hippocampus, immunoprecipitated LRP1 was probed on a Western blot with anti-HNE antibody, stripped, and reprobed with anti-LRP1 antibody. The HNE-bound LRP1 bands were normalized to the unmodified LRP1 bands obtained from the same blot. The results show a 67% increase in AD hippocampus compared to age-matched controls (Fig. 4).

Discussion

LRP1 is a multifunctional protein that scavenges, serves as a signaling receptor, and transports multiple binding partners, including apoE, α2-

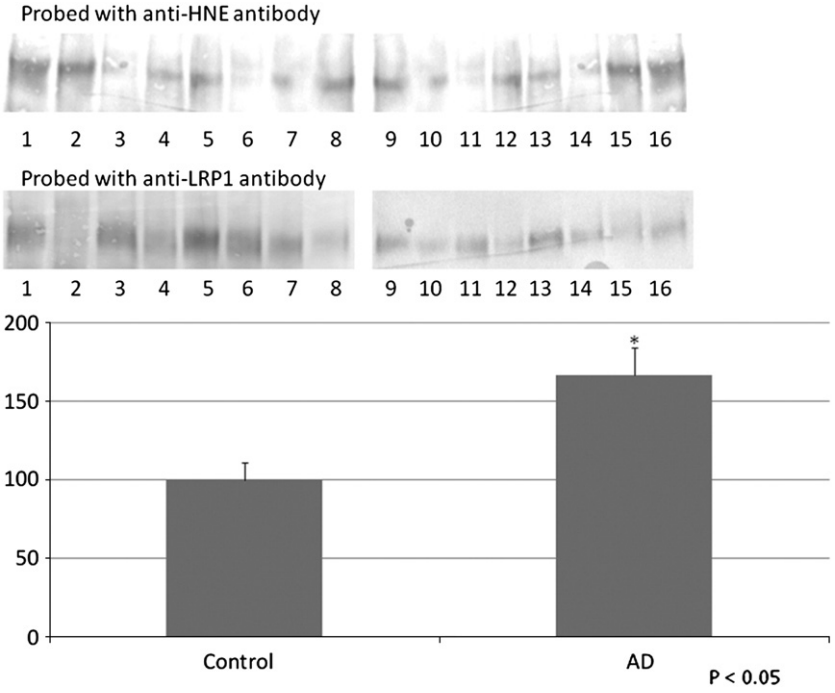

Fig. 4. Confirmation of increased HNE-bound LRP1 levels in AD hippocampus. Immunoprecipitated LRP1 was probed on a Western blot with anti-HNE antibody, stripped, and reprobed with anti-LRP1 antibody. The HNE-bound LRP1 bands were normalized to the unmodified LRP1 bands on the same blot. The results show a 67% increase in AD hippocampus compared to age-matched controls. Data are shown as percentage control (mean ± SEM); $^{*}p<0.05$. The images shown have odd numbers below age-matched control hippocampal samples and even numbers below AD hippocampal samples.

macroglobulin, tissue plasminogen activator, plasminogen activator inhibitor-1, factor VIII, lactoferrin, and Aβ [33–35]. Recent studies show that LRP1 interacts with APP, BACE1, and PS1, proteins involved in Aβ production [36,37], and that LRP1 activity is diminished at the BBB of AD patients [38]. In addition, LRP1 has been shown to mediate both apoE and cholesterol levels in the CNS through APP and to regulate the influence of apoE on microglial inflammation in cell culture systems [23,39,40].

However, it is unclear what causes LRP1 dysfunction in AD. This study shows that HNE-bound LRP1 β-subunit, containing the transmembrane portion of the protein, is significantly increased in AD hippocampus compared to age-matched controls, consistent with the hypothesis that oxidative modification to LRP1 contributes to increased Aβ load in AD brain. LRP1 in other tissues is readily oxidized, with resulting loss of function [41,42]. As noted, previous studies show that oxidative modifications to biomolecules occur in AD brain [8,30,43–45]. Oligomeric Aβ has been shown to induce oxidative stress under in vitro and in vivo conditions, and the Aβ-induced oxidative changes are believed to contribute to neuronal loss and AD pathogenesis [16,19,46]. The numbers of senile plaques are elevated in the hippocampus compared to the cerebellum in AD brain [14]. In addition, histopathological studies show extensive cell loss in the hippocampus from AD subjects [37,47–49]. Previous studies show impaired Aβ efflux at the BBB in transgenic animal models of AD, and as noted there is evidence that LRP1 activity is reduced at the BBB of AD subjects [38,50]. As noted above, studies from peripheral tissues suggest that the oxidation of LRP1 may reduce the activity of this receptor to its other ligands such as α2-macroglobulin [41,42]. Epidemiologic studies propose that oxidation of LRP1 in the blood is one of the risk factors for AD [51,52]. Further, previous studies reported altered levels of LRP1 in AD brain, possibly leading to increased senile plaque formation, cell death, cognitive impairment, and AD pathogenesis [53,54]. Because LRP1 serves as the main efflux pump of Aβ from the brain to the blood, oxidation of LRP1 by its substrate Aβ may be a mechanism of increased accumulation of Aβ in AD brain.

Protein oxidation often leads to loss of function and cell death via necrotic or apoptotic processes [13]. In this study we tested the hypothesis that oxidatively modified LRP1 is increased in AD hippocampus compared to age-matched controls. LRP1 β-subunit was immunoprecipitated and probed for protein-bound HNE and 3NT as indices of lipid peroxidation and protein nitration, respectively, in age-matched control and AD hippocampus. We show, for the first time, that the LRP-1 β-subunit is oxidatively modified by HNE in AD hippocampus, a region of the brain with high levels of Aβ and senile plaques. The observed increase in HNE bound to LRP1 can be explained based on the notion that Aβ, as small oligomers, can insert in the lipid bilayer of brain membranes including brain endothelial cells [27,30,55,56]. The membrane is composed of high levels of polyunsaturated fatty acids, and the incorporation of Aβ into the lipid bilayer alters membrane fluidity and initiates a lipid peroxidation chain reaction, subsequent production of HNE [8,44,57], and, as shown in this study, a resulting Michael addition-mediated binding of HNE to LRP1. As presented in the introduction, we previously demonstrated Aβ-induced lipid peroxidation to another membrane-bound protein, the excitatory amino acid transporter 2 (EAAT2) in rat synaptosomes, and we found elevated HNE bound to EAAT2 in AD brain [30]. This transporter has decreased activity in AD [31] and we speculate that LRP1 activity will decrease with HNE modification. However, further studies are needed to confirm this hypothesis.

Aβ is a neurotoxic peptide that contributes to oxidative stress in AD brain [15–19], and this neurotoxic peptide is removed from the brain by LRP1 [33–35]. Our results from this study support the notion that Aβ-induced lipid peroxidation inhibits its own efflux mechanism from the brain by increasing the levels of HNE-bound LRP1. The results of this study are consistent with the concept that oxidative modification of LRP1 and not the reduction in levels of LRP1 may be responsible for the increased level of Aβ accumulation in the hippocampus of subjects with AD. Further research is in progress in our laboratories to understand the role of LRP1 oxidation in AD pathogenesis and progression.

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