

RESEARCH ARTICLE

Amyloid- β aggregates induce vasculopathy via ferroptosis in brain endothelial cells

Suhyeon Son¹ | Suji Kim¹ | Min-Tae Jeon¹ | Moonseok Choi¹ | Kyu-Sung Kim^{1,2} | Suji Lee^{1,3} | Seung-Yeol Nah⁴ | Do-Geun Kim^{1,2} 

¹Dementia Research Group, Korea Brain Research Institute, Daegu, Republic of Korea

²Daegu Gyeongbuk Institute of Science and Technology, Daegu, Republic of Korea

³Department of Basic and Clinical Neuroscience, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK

⁴Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Republic of Korea

Correspondence

Do-Geun Kim, Dementia Research Group, Korea Brain Research Institute, Daegu, 41062, Republic of Korea.
Email: kimvet0911@kbri.re.kr

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Abstract

Amyloid- β (A β) plaque is the defining pathological feature of Alzheimer's disease (AD) and a target of various therapeutic agents for affected patients. Recent studies have demonstrated the dysfunction of the blood–brain barrier (BBB) in AD; however, how A β plaque induces BBB dysfunction, particularly in brain endothelial cells (ECs), remains elusive. This study investigates the lipid peroxidation-mediated ferroptosis pathway induced by A β via conducting RNA sequencing, phosphorylation analysis, metabolite analysis, western blotting, and immunofluorescent staining both in vitro and in vivo. Here, we demonstrate that A β is associated with lipid metabolic pathways following A β exposure in brain ECs. Additionally, A β aggregates induce the formation and accumulation of peroxidized lipid droplets. Lastly, A β is significantly reduced in brain ECs and 5xFAD mice by the inhibition of the lipid metabolic pathway associated with lipid peroxidation and ROS formation. Our findings from in vitro and in vivo both suggest that A β plays a causative role in the process of lipid peroxidation and might provide a potential target for the development of therapeutic interventions for AD.

KEYWORDS

amyloid- β , blood–brain barrier, ferroptosis, lipid droplets, lipid peroxidation

1 | INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline, memory impairment, and widespread neuronal loss. A hallmark feature of AD is the accumulation of amyloid- β (A β), with its oligomeric and pre-fibrillar forms being the most neurotoxic [1, 2]. Traditionally, A β aggregates have been thought to exert direct toxicity on neuronal cells through mechanisms such as oxidative stress, membrane disruption, and synaptic loss. However, accumulating evidence

demonstrates that A β aggregates can cause pathological changes in non-neuronal cells and subsequently contribute indirectly to neuronal damage. For example, the toxicity of A β aggregates leads to inflammatory responses, including the activation of microglia and astrocytes, which promote the production of pro-inflammatory cytokines and reactive oxygen species (ROS). Although these pathways have been extensively explored as therapeutic targets in AD drug development [3, 4], current treatments targeting these mechanisms have demonstrated limited efficacy, underscoring the urgent need for alternative therapeutic strategies.

Recent studies have shown that pathological protein aggregates, including A β , exert direct detrimental effects

Suhyeon Son, Suji Kim, Min-Tae Jeon, and Moonseok Choi contributed equally to this work.

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on the blood–brain barrier (BBB) [5, 6]. The BBB is the specialized vascular structure that protects the brain from harmful substances in the peripheral system [7]. Brain endothelial cells (ECs) serve as the structural and functional core of this barrier, expressing junctional proteins and transport regulators essential for maintaining vascular integrity, thereby preventing the entry of xenobiotics [8]. The BBB has an integral role in maintaining normal brain physiology, and its dysfunction has been implicated in a wide range of neurodegenerative diseases [9]. Proteotoxicity can lead to pathological changes by promoting the influx of peripheral molecules, such as fibrinogen and complement, that activate pro-inflammatory responses in the brain, emphasizing the importance of BBB integrity in preventing the progression of brain dysfunction [10–12]. However, BBB dysfunction is increasingly recognized as a critical contributor to AD and other neurodegenerative disorders; the molecular mechanisms by which A β impair EC integrity and compromise barrier function remain poorly defined.

Ferroptosis is a relatively new concept of a cell death pathway characterized by iron-dependent lipid peroxidation, which leads to the production of ROS that disrupts normal cellular function and is involved in a variety of pathological conditions, including cancers, neurodegenerative diseases, and kidney diseases [13, 14]. There is accumulating evidence that ferroptosis occurs in patients with Alzheimer's disease (AD). Iron accumulation, a hallmark of ferroptosis, has been detected in the brains of AD patients and correlates with disease progression [15]. In addition, cerebrospinal fluid (CSF) ferritin, which reflects brain iron status, has been reported to associate with cognitive decline and APOE- ϵ 4 genotype, suggesting that elevated CSF ferritin may serve as a predictive biomarker linked to iron dysregulation and ferroptosis in AD [16, 17]. Furthermore, alterations in ferroptosis-related proteins have been observed in the brains of AD patients, particularly in association with A β pathology, supporting the activation of ferroptotic signaling in AD [18]. Although several studies have demonstrated the involvement of ferroptosis in AD pathology, the mechanisms linking ferroptosis to neurovascular dysfunction remain poorly understood. Previous reports have identified ferroptosis as a major contributing factor to atherosclerotic changes in the ECs, suggesting that these changes in the brain ECs could be one of the initial factors inducing neurodegenerative diseases [19]. Moreover, the observed accumulation of iron and lipids within plaques near the vasculature of AD patients suggests that ferroptosis-induced vascular damage is a contributor to the development of AD [20]. However, it remains unclear whether A β can induce pathological alterations in lipid metabolism and lipid-mediated peroxidation in the brain ECs, despite its growing relevance in this field.

In this study, we first examined the mechanisms by which A β compromises brain ECs' integrity, with a

particular focus on lipid metabolic disturbances and ferroptosis signaling. Furthermore, using in vitro and in vivo systems, we confirmed that A β aggregates induce the formation of lipid droplets and activate lipid peroxidation through the induction of ferroptosis. Notably, lipid peroxidation in brain ECs, observed in the early life spans of animal models of AD, was inhibited by treatment with the radical-trapping agent UAMC-3203, which functions as a ferroptosis inhibitor. These observations suggest that A β is a critical driver of lipid peroxidation in brain ECs and highlight ferroptosis-mediated vascular dysfunction as a mechanistically distinct contributor to AD progression.

2 | RESULTS

2.1 | Evidences of vasculopathy and lipid peroxidation in the 5xFAD

BBB disruption in amyloidopathy is closely linked to inflammatory responses that exacerbate neurodegeneration [21]. Consistent with this, we observed prominent extravasation of fibrinogen and immunoglobulin G into the brain parenchyma of 5xFAD mice (Figure S1A,B), indicating a marked increase in BBB permeability. Moreover, A β was co-localized with CD31-positive ECs (Figure S1C), supporting the idea that vascular deposition of A β directly compromises endothelial function.

Lipid peroxidation, the oxidative degradation of polyunsaturated fatty acids in cellular membranes, is a major source of oxidative stress and membrane injury in the brain [22]. Previous studies have shown that A β can promote lipid peroxidation, raising the possibility that vascular lipid peroxidation may be induced in the context of amyloid pathology [23, 24]. Lipid droplets, which store neutral lipids within a phospholipid monolayer, accumulate in response to lipid peroxidation and metabolic stress and are therefore considered indirect indicators of oxidative lipid injury [25]. We examined whether lipid droplets are generated in the brains of 5xFAD mice in vivo. In brain sections from 13-month-old animals, we observed a robust increase in lipid droplet-positive plaques in 5xFAD mice compared with age-matched wild-type (WT) controls (Figure 1A). To determine the onset and distribution of this phenotype, we further analyzed younger 5xFAD cohorts. Lipid droplets were already detectable in the hippocampus at 3 months of age and were more broadly distributed by 6 months (Figure 1B). Given that the hippocampus is the primary site of early A β deposition [26, 27], these findings indicate that lipid droplet formation may initiate in the hippocampus and subsequently expand into wider brain regions as amyloid pathology progresses.

Next, we aimed to deduce the location of lipid droplet accumulation among neurovascular units (NVU) in the brain. The dysfunction of neurovascular units is closely

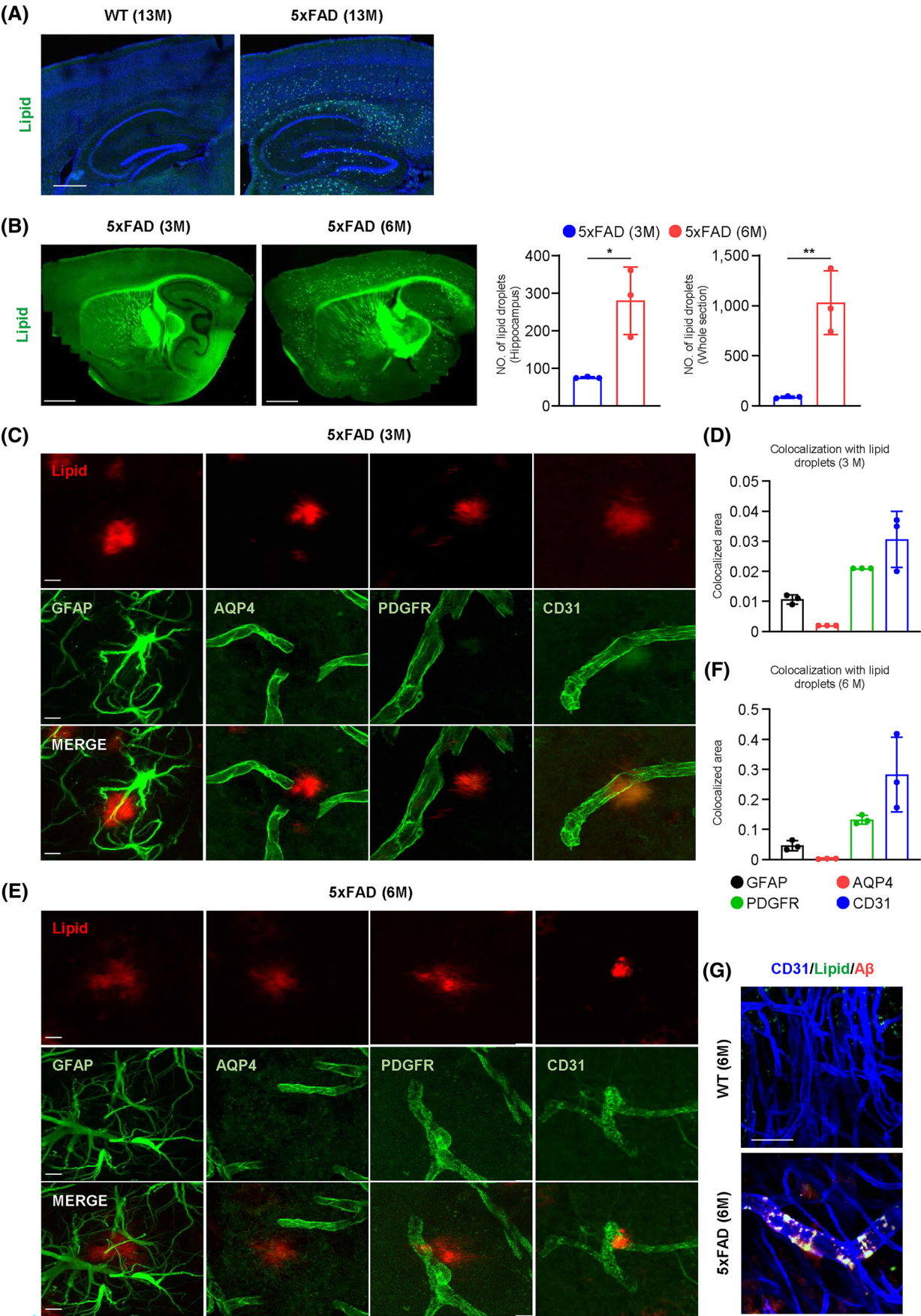


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correlated with A β pathology in AD and A β aggregation can activate the astrocytes and ECs as well as the pericyte degeneration [28]. We found that the lipid droplets were closely related to ECs among the components of NVU, potentially suggesting that they originated from the ECs. However, they were not located in the astrocytes and pericytes (Figure 1C–F). Consistently, intravital imaging demonstrated that lipid signals were predominantly localized around microvessels in the 5xFAD brain, where they colocalized with A β deposits (Figure 1G). These results suggest that lipid droplet accumulation in the 5xFAD brain is primarily associated with ECs.

To assess the clinical relevance of lipid droplet accumulation observed in the NVU in vitro and in vivo models, we examined post-mortem brain tissue from patients with AD. For this, we co-stained the BODIPY to visualize the lipid droplet with CD31 to visualize the brain ECs. We observed that the lipid droplets can be found throughout the brain section of the AD patient (Figure 2A). When we analyzed the localization of lipid droplets with that of the brain ECs, we could successfully find the close spatial association of lipid droplets with brain ECs as we did in the in vivo animal model (Figure 2B). This strongly suggests that lipid droplets are also found in close association with brain ECs and potentially have pathological effects in the dysfunction of the NVU and cause neurological dysfunction as it does in animal models. This strong evidence of lipid droplet formation in various levels of the system has led us to test the pathological mechanisms of the formation of the lipid droplets in order to find therapeutic targets to reverse the formation thereof, with the aim to mitigate damage to the NVU and neurological dysfunctions in AD.

2.2 | Treatment of the A β induces its rapid uptake and aggregate formation mediated by the endosomal trafficking pathway

Brain ECs, which maintain barrier function through tight junctions and regulated vesicular transport, rely on endosomal trafficking pathways to mediate molecular exchange between the circulation and brain parenchyma [29, 30]. In order to test whether exogenous A β can be

internalized and in which endosomal trafficking compartment it is located in the brain ECs, we have treated the brain ECs with A β aggregations for different time points and stained with markers for the endosomal trafficking including early endosome (Rab5), late endosome (Rab7), and lysosome (Lamp1). Upon treatment, we could observe the localization of A β aggregates in the early endosome at the early time point of treatment. However, the puncta-like structure of the A β was gradually observed at the late endosome that was co-stained with Rab7. Twenty-four hours later, most of A β aggregates were observed at the lysosome indicating that the A β has a migratory pattern in the brain ECs through the endo-lysosomal trafficking pathway (Figure 3A,B). Interestingly, the size of the A β aggregate deposits in the brain ECs reached several microns which is observed through the cytoplasm mainly accumulated around the perinuclear area. For further analysis, we have performed a time- and dose-dependent assay. Beginning from 6 h, we could observe the accumulative patterns of A β in the cytoplasm that was highest 24 h after treatment and began to decline 48 h after treatment (Figures 3C and S2). Interestingly, lysosomal activation was still maintained 72 h after treatment even though the level of A β declined at that period of time indicating that the lysosomal activity of brain ECs is constantly activated by A β regardless of the presence of A β (Figure 3C,D).

2.3 | A β aggregates induces the formation of peroxidized lipid droplets in the brain ECs

Since we have hypothesized that the A β aggregates can induce atherogenic conditions in the brain ECs mediated by metabolic shift, we endeavored to assess whether it can indeed induce changes similar to the initial changes in the ECs in the progression of atherosclerosis. For this, we have determined to characterize the aggregation formed in the brain ECs upon A β aggregation treatment. Lipid droplet, which is a form of triglyceride, is known to be involved in various forms of pathological changes [31] and the formation of lipid droplets is widely observed in various types of neurodegenerative diseases and atherosclerotic changes in the blood vessels [32, 33]. Moreover,

FIGURE 1 Endothelial cells in the brain accumulate lipid droplets in 5xFAD. Representative images showing the brain of 5xFAD with lipid droplets staining. (A) Lipid droplet staining revealed plaque-like lipid-rich aggregates in the brains of 5xFAD mice, which were absent in WT controls. Scale bar, 500 μ m. (B) The deposition of lipid droplets began to form in the hippocampus at a young age, and it gradually spread into the whole brain as getting older in 5xFAD mice. Scale bar, 1000 μ m. Bar graphs indicate the number of lipid droplets in the hippocampus (left) and the whole brain (right). All data were statistically analyzed by unpaired two-tailed Student's *t*-test. An asterisk represents the statistical difference compared with the control or treatment. Error bars represent the mean $\pm$ SD (*N* = 3, biological replicates). **p* < 0.05; ***p* < 0.01. (C–F) The astrocyte marker GFAP, astrocytic transmembrane water channel marker AQP4, pericyte marker PDGFR, and endothelial cell marker CD31 (Green) were co-stained with lipid droplets (Red) to observe the location of lipids in the cortex at 3-months (C) and 6-months (E) old of 5xFAD. Representative confocal images suggest that the accumulation of the lipid droplet could induce the activation of astrocytes and pericytes, as well as being internalized in the brain endothelial cell. Scale bar, 100 μ m. Bar graphs indicate the colocalization of lipid with each cell marker in the cortex at 3-months (D) and 6-months (F) old of 5xFAD. (G) Intravital imaging revealed colocalization of lipids (green) and A β with CD31-positive microvessels in the brains of 5xFAD mice. Scale bar, 100 μ m.

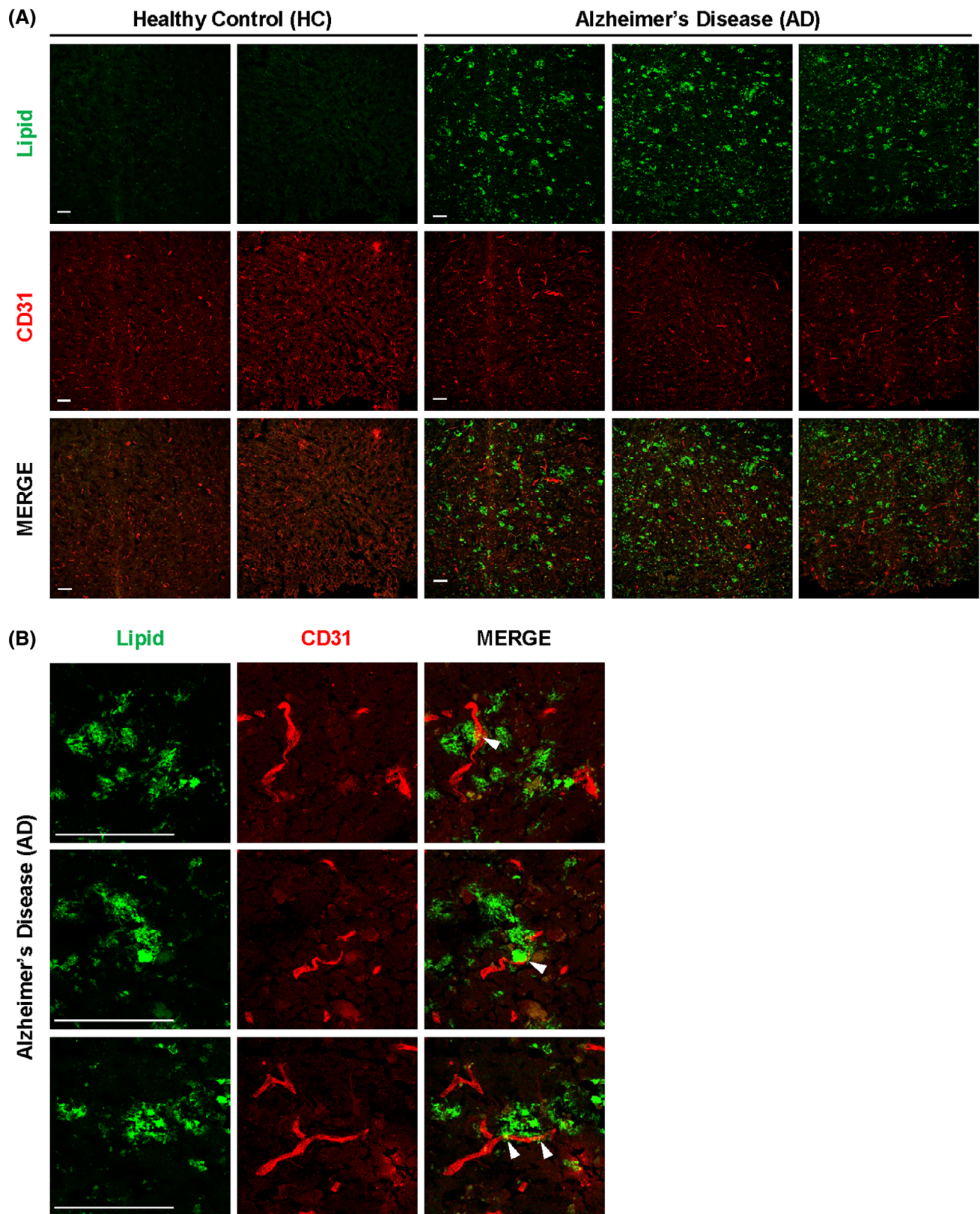


FIGURE 2 Lipid droplet formation in the brain is found in human tissues. (A, B) Representative images showing the lipid droplets staining with CD31 on human brain sections, indicating the lipid droplets were stained only in the brain tissues of AD patient (A). Additionally, lipid droplets existed in various forms and sizes, and they were located near or passing through blood vessels like budding out from endothelial cells (arrow; B). Scale bar, 100 μ m. $N = 1$.

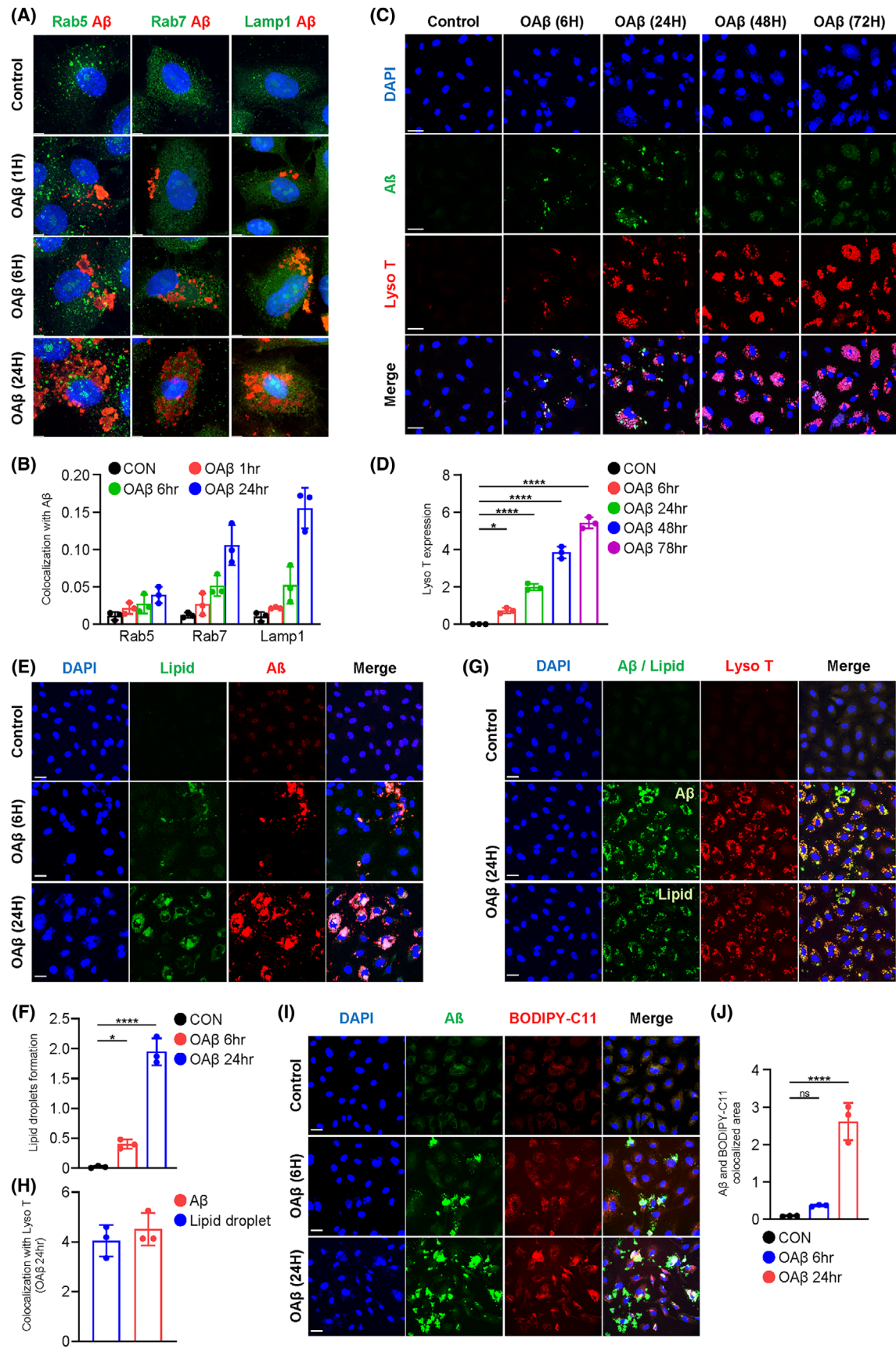


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the alteration of lipid metabolism is found in neurodegenerative diseases, and we hypothesized that the accumulated A β aggregates can induce the alteration of lipid metabolism [34]. To find out whether A β aggregates can indeed increase pathological alteration of lipid metabolism and formation of lipid droplets in the brain ECs, we have aimed to characterize the large vesicle-like structure in the brain ECs. With the hypothesis that the observed vesicular structure which is induced by A β aggregates is indeed the lipid droplet, we treated A β aggregates for different time points and measured the formation of lipid droplets with BODIPY that stains the lipid droplet specifically. With this staining, we found that the A β aggregates could induce the formation of lipid droplets at an early time point and the lipid droplets gradually accumulated after the treatment of A β aggregates up to 24 h in the brain ECs, and it was localized in the perinuclear area (Figures 3E,F and S3A), suggesting the triggering effect of the A β aggregates in the induction of the lipid droplet formation. Moreover, these lipid droplets were co-localized with an upregulated lysosome positive signal (Figure 3G,H), indicating that the A β aggregates could induce the formation of lipid droplets and are promoting increased levels of lysosomal activity.

Pathological lipid droplet formation has shown to be important in disease progression of various diseases, and peroxidation of lipids can cause damage to organelles in cells due to the production of reactive oxygen species [35, 36]. Lipid peroxidation is mediated by various types of factors, and recent studies have provided various evidence demonstrating that the interaction of iron with lipids can cause its peroxidation, leading to ferroptosis [37]. Because lipid droplet accumulation in brain ECs followed A β exposure, we next examined whether these droplets exhibited features of lipid peroxidation. Interestingly, we found that the brain ECs treated with A β aggregates were stained positive strongly with BODIPY-C11 (Figure 3I,J), which is the marker for lipid peroxidation, suggesting the pathological role of the A β in the induction of the lipid droplet formation. Moreover, 4-hydroxynonenal (4-HNE), a well-recognized end product of lipid peroxidation, was increased in A β -treated

ECs in a time-dependent manner and co-localized with lipid droplets (Figure S3B,C). Likewise, ferritin, the major iron-storage protein that buffers redox-active iron, and GPX4, the lipid hydroperoxide-reducing enzyme critical for protecting membranes from peroxidative damage, were both reduced in A β -treated ECs (Figure S3D), indicating impaired iron sequestration and antioxidant defense. This indicates that A β aggregates induce the formation of the peroxidized lipids in the brain ECs, and this led us to focus on the mechanisms behind the lipid peroxidation that could be a potential target of the pathogenesis of vasculopathy in AD.

2.4 | Inhibition of the fatty acid synthesis reduces the formation of lipid droplets induced A β aggregates

Since we have confirmed that the formation of peroxidized lipids upon treatment of the A β aggregates in the brain ECs, we have determined to test the roles of enzymes involved in the formation of lipid droplets using various inhibitors specific for these enzymes involved in fatty acid synthesis. First, we decided to inhibit the activity of long-chain acyl-CoA synthetase (ACSL) by Triacsin-C, the enzyme involved in lipid metabolism that converts fatty acid into Acyl-CoA. We have identified that lipid droplet synthesis has been decreased significantly upon treatment of Triacsin-C suggesting that lipid droplet formation by A β can be mediated by the activation of long-chain fatty acid synthase (Figure 4A,B). Further, to confirm the effect of fatty acid synthesis at the most downstream of the lipid droplet formation pathway, we have inhibited the activity of diacylglycerol acyltransferases (DGAT) 1 and 2, which catalyze the conversion of diacylglycerol to triacylglycerol [38, 39]. Even though we have not seen a decreased level of lipid droplet formation with the separate inhibition of DGAT1 or 2, respectively, we have found that simultaneous inhibition of DGAT1 and 2 caused the decreased level of lipid droplets (Figure 4A,B). Overall, these findings suggest that the inhibition of the fatty acid synthesis pathway results in a

FIGURE 3 A β uptakes and aggregates formation mediated by the endosomal trafficking pathway. (A–D) Representative images showing A β (1 μ M) beginning the localization at the early time point through the endosomal trafficking (A) and the activation of lysosome induced by A β (C) in the HBMVECs. Scale bars, 10 μ m (A) and 100 μ m (C). (B) Bar graph indicates the colocalization of A β with each endosomal marker, Rab5 for early endosome, Rab7 for late endosome, and Lamp1 for lysosome, in the HBMVECs. (D) Bar graph indicates the lysosomal activity revealed by Lyso Tracker labeling in the A β -treated HBMVECs. Statistical data were analyzed by one-way ANOVA with Tukey post hoc test for multiple comparisons. An asterisk represents the statistical difference compared with the control or treatment. Error bars represent the mean $\pm$ SD ($N = 3$, biological replicates). * $p < 0.05$; **** $p < 0.0001$. (E–H) Representative images showing A β induced the formation of lipid droplets (C) as well as the activation of lysosomes (D) in the early time. Scale bar, 100 μ m. (F) The graph indicates lipid droplet formation following A β treatment. Statistical data were analyzed by one-way ANOVA with Tukey post hoc test for multiple comparisons. An asterisk represents the statistical difference compared with the control. Error bars represent the mean $\pm$ SD ($N = 3$, biological replicates). * $p < 0.05$; **** $p < 0.0001$. (H) The graph indicates colocalization of Lyso tracker with A β or lipid droplet. (I, J) Representative images showing BODIPY-C11, a lipid peroxidase marker, are colocalized with A β aggregates, indicating that the lipid droplets induced by A β are peroxidized lipids. Scale bar = 100 μ m. The graph indicates A β and BODIPY-C11 colocalized area in the A β -treated HBMVECs. Statistical data were analyzed by one-way ANOVA with Tukey post hoc test for multiple comparisons. An asterisk represents the statistical difference compared with the control. Error bars represent the mean $\pm$ SD ($N = 3$, biological replicates). ns, not significant; **** $p < 0.0001$.

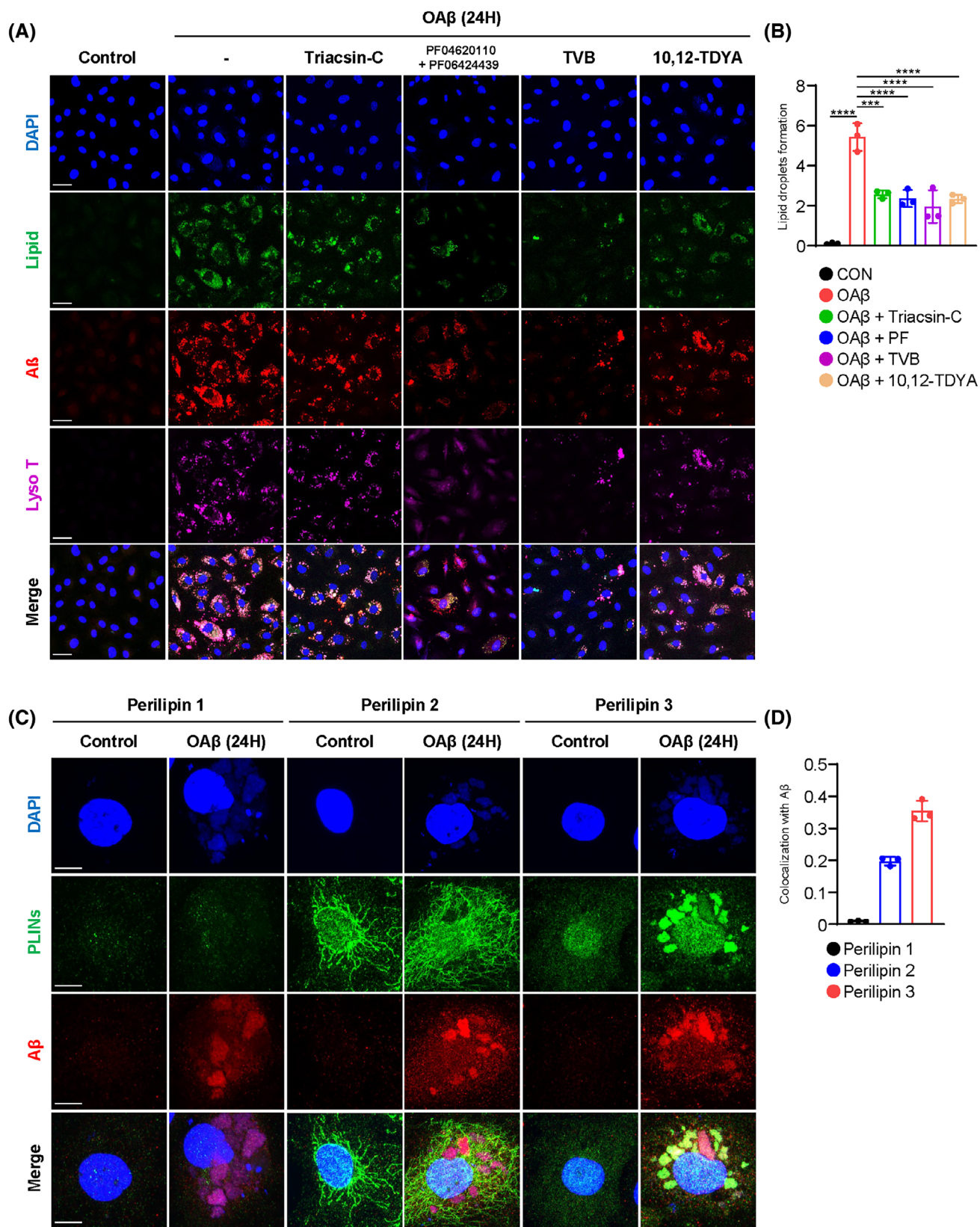


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marked reduction in the accumulation of lipid droplets and A β indicating the importance of the fatty acid synthesis pathway in this pathological alteration in the brain ECs.

Diacylglycerol that was synthesized by DGAT1 and 2 is consequently converted into lipid droplets with the cooperation of perilipins (PLINs), which surround the lipid droplet and regulate the access of the lipases to the lipid droplet content [40–42]. To dissect the subspecies of the PLINs to affect the formation of lipid droplets, we have utilized a co-localization assay to analyze the proximity of PLINs with the signals of lipid droplets. We tested the colocalization of 3 different types of PLINs with that of lipid droplets upon treatment with A β aggregates (Figure 4C,D). We have identified that PLIN1 and PLIN2 were not colocalized with lipid droplets. However, PLIN3 was shown to have prominent colocalization with that of lipid droplets, indicating that PLIN3 potentially has an essential role in regulating the formation of lipid droplets upon treatment of A β aggregates. Overall, we could identify that the lipid metabolic cascade that is involved in the lipid droplet formation is activated by A β aggregates formation, which strongly supports our hypothesis.

2.5 | A β modulates distinct lipid metabolite subspecies acting downstream of fatty acid synthesis in the brain ECs

We aimed to track down the differences in lipid metabolites upon treatment of A β aggregates by utilizing QE/MS-based analysis. We have identified 205 lipid metabolites that were significantly changed, including several acylcarnitines, saturated fatty acids (Palmitic acid), and polyunsaturated fatty acids (Linoleic acid, Stearic acid). Surprisingly, we have observed that the A β aggregates could reduce the levels of linoleic acid and gamma-linolenic acid. Moreover, we have identified that Arachidonic acid (AA) and its downstream product 2-Arachidonoylglycerol (2-AG) have decreased significantly following A β treatment (Figure 5A). It was reported that 2-AG has an essential role in down-modulating the linolenic acid of its separate pathway of fatty acid metabolism [43]. We also observed marked reductions in the omega-3 polyunsaturated fatty acids

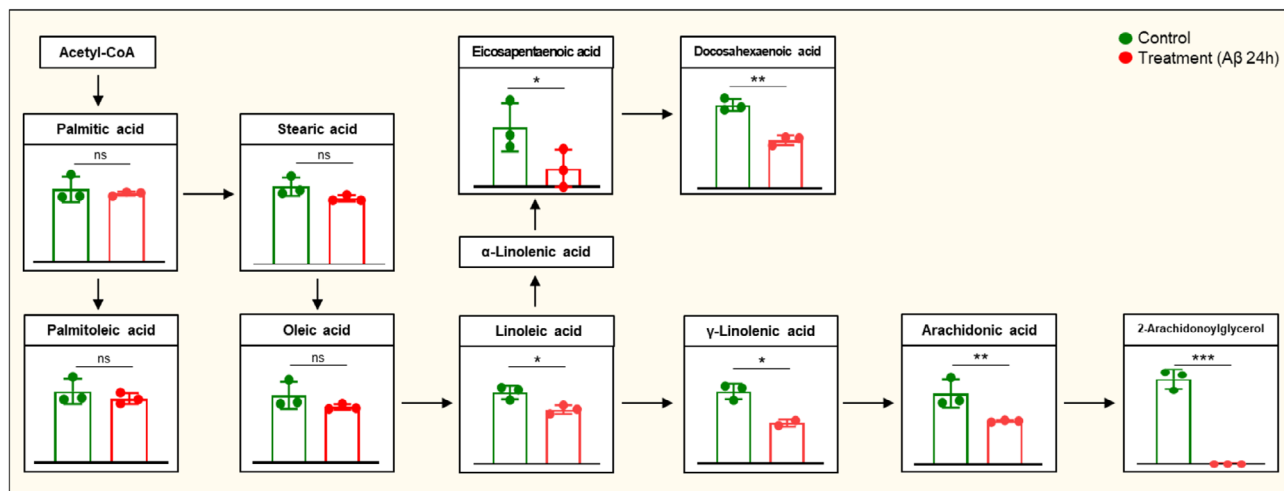
eicosapentaenoic acid and docosahexaenoic acid. These molecules are known to down-modulate the pro-inflammatory pathway in atherosclerosis, suggesting that the A β aggregates can induce the formation of lipid peroxide to induce pathological changes by decreasing this molecule in the brain EC [44].

Since we have observed altered pathways in lipid metabolism in the brain ECs by A β aggregates, we have aimed to find the molecular pathways governing the pathological changes induced by A β aggregates. Oxidized lipid formation is highly associated with inflammatory responses, and it is reported to be triggered by a well-characterized signaling pathway [45, 46]. To further characterize this, we profiled whether there is a potential effect on gene transcript levels and protein phosphorylation during A β exposure in the brain ECs using RNA sequencing and protein phosphorylation array. First, we performed RNA sequencing of brain ECs treated with A β for 1 h, 4 h, 12 h as well as untreated controls (Figure 5B,C,F). With a differentially expressed genes (DEGs) threshold of |fold change| ≥ 2 and p value < 0.05 , we performed differential expression analysis across all pairwise comparisons among control, A β 1 h, A β 6 h, and A β 12 h (Table S1). These contrasts together yielded approximately 1300 unique DEGs. KEGG pathway analysis of this DEG set exhibited enrichment of lipid metabolism-related pathways, and 31 DEGs were annotated to lipid metabolism-associated KEGG terms (Figure 5C). To better characterize the functional context of these lipid-related changes, we then focused on this subset of 31 lipid-associated DEGs and examined their KEGG pathway annotations, which highlighted alterations in lipid synthesis pathways (LPIN3, PNPLA3, DGKG) as well as immune and inflammatory response pathways (AKR1B10, MAP2K6, CXCL2) in A β -treated brain ECs (Figure 5F). Next, we performed phosphoarray analysis of brain ECs treated with A β for 1 h, 6 h, 24 h (Figure 5D,E,G). We observed 433 phosphorylated proteins were significantly regulated by A β and these proteins were also dominantly correlated with lipid metabolic pathways (KDR, CRK, LIPE, PLCG1) (Figure 5E,G).

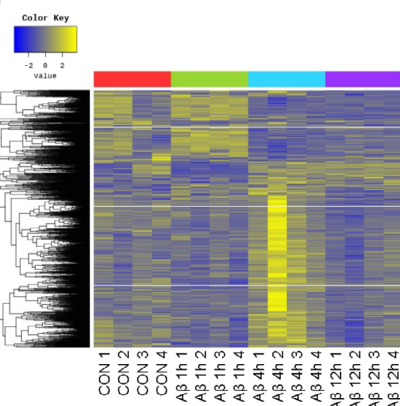
With this, we could conclude that A β aggregates primarily regulate the lipid species associated with anti-inflammation and atherosclerosis related genes. These results indicated that they have crucial roles in the

FIGURE 4 Inhibition of the fatty acid synthesis reduces the formation of lipid droplets induced by A β . (A) Representative images showing most of the fatty acid metabolic inhibitors can reduce the accumulation of lipid droplets and A β aggregates as well as lysosome activity in the HBMVECs. Triacsin-C, ACSL inhibitor (5 μ M); PF-04620110, DGAT1 inhibitor (10 μ M); PF-06424439, DGAT2 inhibitor (10 μ M); TVB-3664 (TVB), FASN inhibitor (10 nM); 10,12-Tricosadiynoic acid (10,12-TDYA), ACOX inhibitor (3 μ M). Scale bar, 100 μ m. (B) The graph indicates that A β -induced lipid droplet formation was inhibited by such inhibitors. Statistical data were analyzed by one-way ANOVA with Tukey post hoc test for multiple comparisons. An asterisk represents the statistical difference compared with the control or OA β treatment. Error bars represent the mean $\pm$ SD ($N = 3$, biological replicates). *** $p < 0.001$; **** $p < 0.0001$. (C) Representative images showing perilipin 3, one of the proteins of the surface of the lipid droplets in the brain endothelial cells, is prominent for colocalization with A β aggregates, indicating that the lipid metabolic cascade that is involved in the lipid droplet formation is activated by A β aggregates. Scale bar, 10 μ m. (D) The graph indicates colocalization of A β with perilipins in the A β -treated HBMVECs.

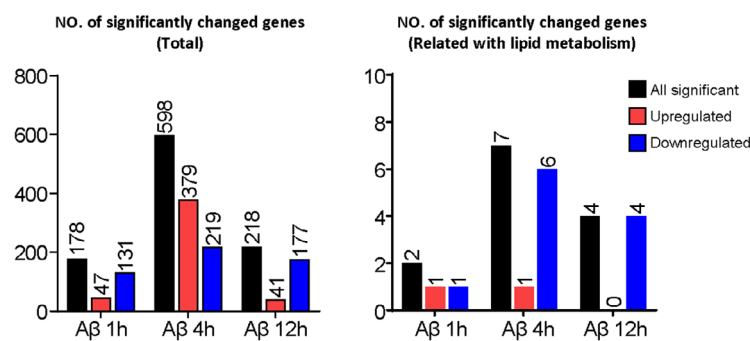
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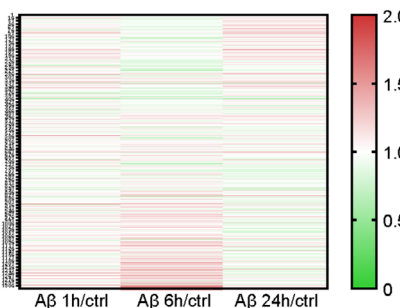
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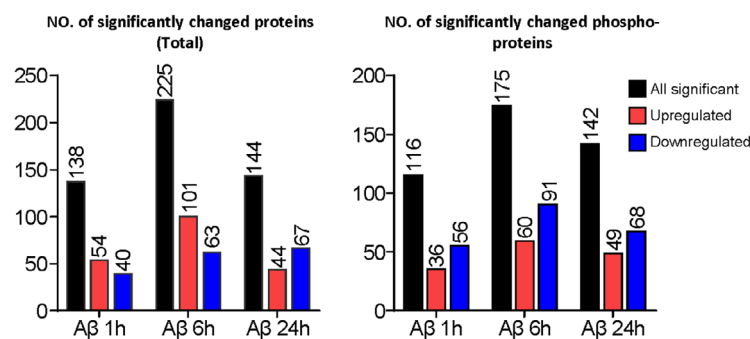
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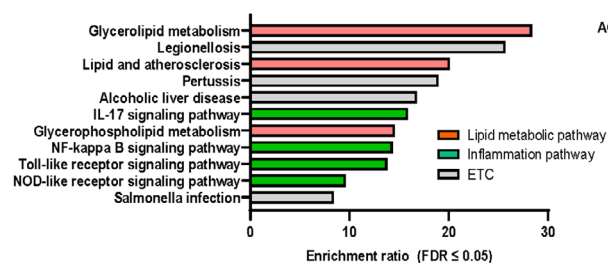
(D)



(E)



(F)



(G)

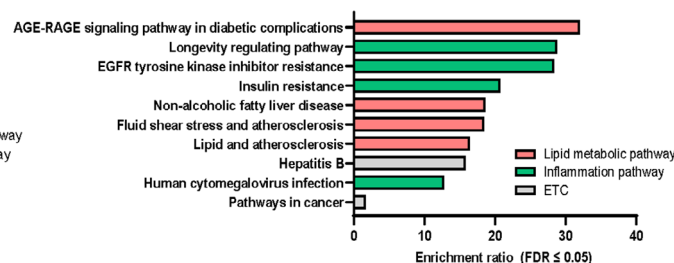


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modulation of atherosclerotic changes in the ECs, suggesting that A β can induce a metabolic shift of lipids, causing the environment favorable for inducing atherogenic changes in the brain ECs.

2.6 | Ferroptosis is the major pathological process inducing the formation of aggregation in the brain ECs

Since we have observed that the A β aggregates can induce the peroxidation of lipids that can be potential pathological inducers in the brain ECs and the changes in the lipid species upon A β treatment, we aimed to find the underlying mechanisms. It is known that the brain ECs constantly take up transferrin, leading to ferroptosis, a new mechanism of non-apoptotic cell death (Figure 6A) [47]. Therefore, we decided to test whether ferroptosis is involved in the lipid droplet peroxidation following A β treatment. To investigate whether inhibition of ferroptosis attenuates lipid droplet formation, cells were treated with the radical-trapping agent UAMC-3203 (C₂₅H₃₇N₅O₂S), which functions as a radical scavenger to suppress ROS generation and lipid peroxidation [48, 49]. As a result, we found that lipid droplet formation is decreased, indicating that lipid droplet formation induced by A β aggregates treatment in the brain ECs is caused by ferroptosis-induced ROS synthesis (Figure 6B,C). Further, the level of lysosomal activity measured with lysotracker in the samples was decreased, suggesting that the autophagic effect shown in the aforementioned experiments was mediated by ROS formation. This indicates that ferroptosis contributes to A β -induced lipid peroxidation and lipid droplet formation in brain ECs.

In addition, GPX4 is the main co-regulator of ferroptosis and it reduces peroxide accumulation and ROS production, and the GPX4 inhibitor accelerated the ferroptosis for the development of death of brain ECs [50, 51]. For this, we have treated RSL3 (RAS-selective lethal), which is the activator of ferroptosis and inactivates GPX4, to test if ferroptosis induction can upregulate the formation of lipid droplets. Our results show that

the RSL3 inhibitor caused the prominent aggregation of lipid droplets and A β aggregates compared to that of the non-treated control in the brain ECs (Figure 6D,E). This indicates that the peroxidation of lipid droplet formation by A β is exacerbated by RSL3, GPX4 inhibitor, and ferroptosis is the novel process for regulating the lipid metabolism in ECs.

2.7 | Radical-trapping agent significantly reduces the accumulation of the lipid droplets and A β plaques at 6-months old in 5xFAD mice

Having observed that inhibition of ferroptosis can decrease the formation of lipid droplets and A β through human brain ECs in vitro, we tested whether the inhibition of ferroptosis can reduce the accumulation of lipid and A β in the mouse brain in vivo. For this, 6-month-old 5xFAD mice were injected with 10 mg/kg of UAMC-3203 (Figure 7A), a radical-trapping agent that functions as a ferroptosis inhibitor, or PBS as a vehicle control once a day, and then all mice were sacrificed after 1 week (Figure 7B). Surprisingly, the number of lipid droplets and A β -positive signals were significantly reduced by approximately 50% in UAMC-3203-injected mice compared to PBS controls, both in the cortex (Figure 7C–J) and in the hippocampus (Figure S4A–D), indicating that the inhibition of ferroptosis may induce decreased accumulation of lipid droplets and A β plaques in correlation with the in vitro model. Moreover, we observed that the activation of astrocytes was significantly downregulated (Figures 7C,D and S4A) and the deficiency of astrocytic endfeet appeared restored (Figures 7E,F and S4B) by UAMC-3203 in the 5xFAD mice. This suggests that the inhibition of ferroptosis can reverse the formation of lipid droplets and it subsequently inhibits the pathological alteration of astrocytes. Consistent with these cellular changes, IgG leakage into the brain parenchyma, a marker of BBB permeability, was markedly reduced in UAMC-3203-treated 5xFAD mice compared with PBS controls (Figure 7K,L), indicating that ferroptosis inhibition preserves BBB integrity in A β -induced vasculopathy. In addition, we analyzed the localization of lipid droplets

FIGURE 5 A β regulates biological processing and several types of genes that are linked to lipid and atherosclerosis pathways in the brain ECs. (A) Bar graphs showing the synthesis fluxes of fatty acid subspecies regulated by A β aggregates, analyzed by LC/MS in HBMVECs, indicating that A β significantly decreased the lipid species known to reduce toxicity and inflammation of nerve cells like AA and 2-AG. These data were statistically analyzed by one-way ANOVA with Tukey post hoc test for multiple comparisons. An asterisk represents the statistical difference compared with the control or treatment. Error bars represent the mean $\pm$ SD ($N = 3$, biological replicates). Ns, not significant; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$. (B–F) Overview of the dataset of RNA-sequencing (B, C, F) and phospho-array (D, E, G) analysis in HBMVECs. (B) The heat map of RNA-seq transcriptome analysis between the A β group and the control group in HBMVECs. (C) Significant gene expression changes over time in the entire gene list and genes related to lipid metabolism ($|FC| < 0.5$). Black bars indicate the total number of regulated genes, while red bars and blue bars indicate the number of upregulated and downregulated genes, respectively. The numbers at the edge of the graph indicate the number of genes that changed. (D) The heat map of phospho-array analysis between the A β group and the control group in HBMVECs. (E) The significant lipid-related protein expression changes over time in the entire proteins and phosphorylated proteins. Functional enrichment analysis of GO terms in regulated genes of RNA-seq (F) and proteins of phospho-array (G). The results indicate that A β highly regulates the changes of genes related to lipid metabolism and immune responses pathway.

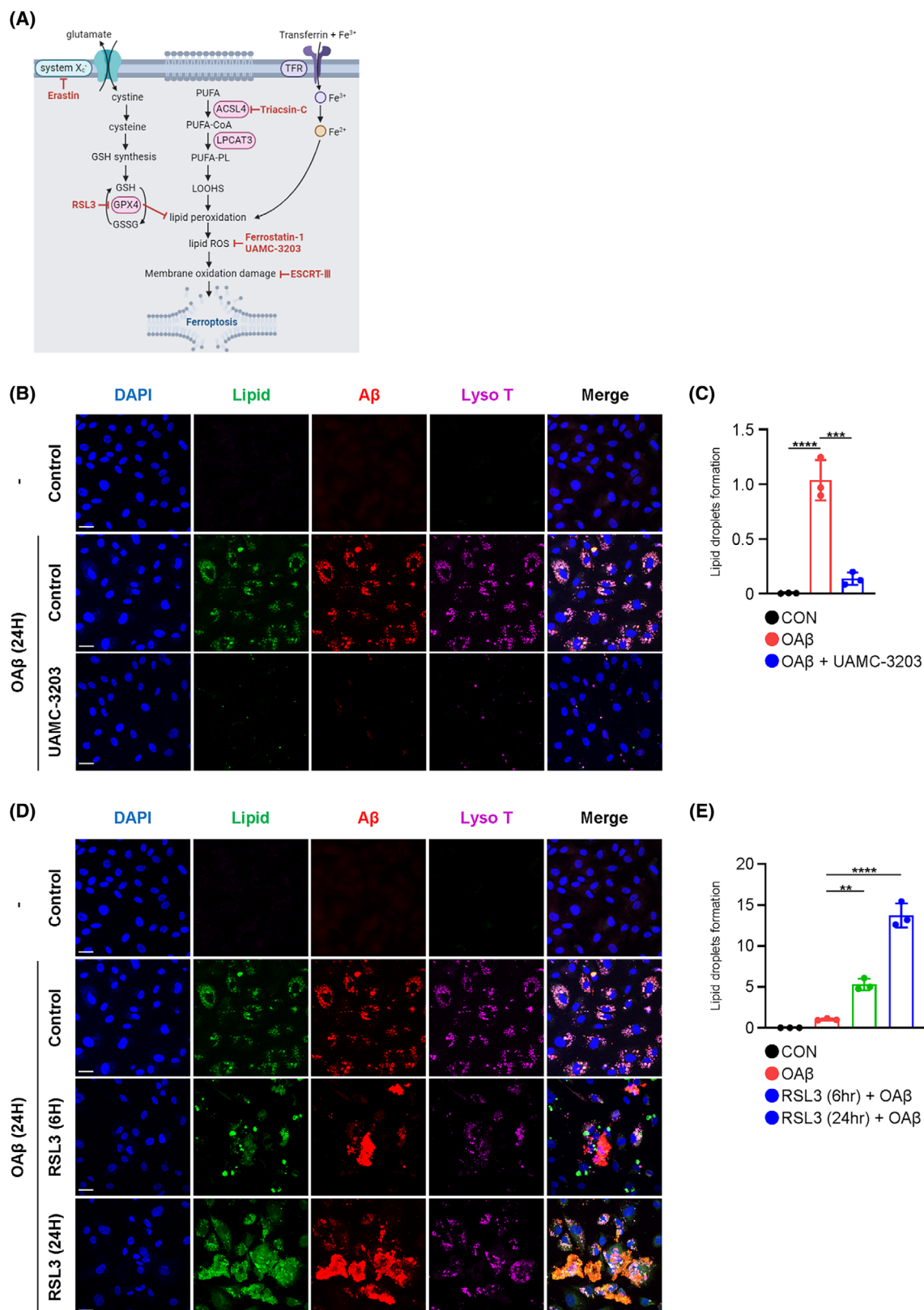


FIGURE 6 Legend on next page.

with blood vessel staining by PDGFR and CD31 in the 5xFAD mice. We observed that the lipid droplets within vascular ECs were observed twice more frequently than they were in pericytes, suggesting that the origination of these lipid plaques would be more likely to occur in brain ECs (Figures 7G–J and S4C–F). In contrast, when we performed the same treatment in 10-month-old 5xFAD mice, UAMC-3203 injection did not lead to significant changes in lipid droplet or A β accumulation compared with PBS controls (Figure S5). Overall, our results indicate that A β pathology regulates lipogenesis and changes in the neurovascular unit, and UAMC-3203 significantly reduces the effects induced by A β deposits. These findings strongly suggest that ferroptosis inhibition reduces lipid droplet and A β accumulation in early-stage but not late-stage 5xFAD mice, and it could be a potential target of the pathogenesis of vasculopathy in AD. Notably, this therapeutic effect was not evident in 10-month-old 5xFAD mice, suggesting that ferroptosis inhibition is more effective at earlier disease stages and underscoring the importance of early intervention.

3 | DISCUSSION

The brain is the principal component for cognitive function, and its dysfunction is closely linked to neurodegenerative diseases. Accumulating evidence demonstrates that damage to the brain ECs can cause the dysfunction of various brain disorders [52]. Among the causal factors inducing the changes of neurodegenerative diseases, aggregate-prone proteins such as A β have long been considered major contributors to AD. Recently, the developments and clinical approval for the therapeutic effect of A β targeting antibodies have supported this long-standing hypothesis [53, 54], however, their therapeutic effects remain limited, primarily benefiting only patients with mild cognitive impairment [55] and clinical use is further constrained by the substantial risk of amyloid-related imaging abnormalities (ARIA), which necessitates stringent patient selection and intensive safety monitoring to prevent severe adverse events. This raises the concern that A β toxicity may not be the sole factor in AD but rather one of several contributing factors. A major limitation in understanding the pathological mechanism lies in the lack of insight into A β -induced changes in non-neuronal cells [56, 57]. Identifying the right targets and exact mechanisms contributing to the development of AD

is therefore essential for developing effective therapeutics for neurodegenerative diseases. Recent studies have shifted attention toward non-neuronal cells as potential contributors to neurodegenerative diseases. In particular, dysfunction of NVU has been relatively neglected.

In this study, we investigated novel A β -induced pathological changes, focusing on altered lipid-related pathways in brain ECs. Although A β pathology has been primarily studied in neurons and glial cells, increasing evidence suggests that A β can also act directly on ECs. This is particularly relevant in the context of cerebral amyloid angiopathy (CAA), where A β deposits accumulate along cerebral vessel walls. This is particularly relevant in the context of cerebral amyloid angiopathy (CAA), where A β deposits accumulate along cerebral vessel walls. Consistent with this, our *in vivo* observations in 5xFAD mice revealed prominent A β deposition around cerebral microvessels, indicating that A β may directly target brain ECs and induce vascular pathology in AD. Upon treatment with A β aggregates, we observed their rapid internalization into brain ECs, where they co-localized with cargo vesicles such as early endosomes and accumulated in lysosomes. This process is consistent with previously reported findings in microglia, astrocytes, and neurons, where lysosomal degradation was shown to involve autophagy activation [58, 59]. We also found that exposure of brain ECs to A β aggregates led to the accumulation of lipid droplet-like structures. Furthermore, the internalization of A β appears to induce lipid droplets formation by activating enzymes such as Lipin, which is known to promote lipid droplet formation in other cell types. These structures were suppressed by Triacsin-C and DGAT1/2, suggesting that their formation depends on specific enzymatic pathways involved in lipid processing. Notably, inhibition of DGAT1 and DGAT2 decreased A β aggregation in brain ECs.

In our studies, we found that the treatment with A β aggregates, even without co-treatment with oleic acid, induced the formation of lipid droplets. Recent studies have linked lipid droplet formation in the liver and other organs to lipid-induced toxicity [60–62]. However, since the importance of lipid droplet formation and its pathological effect in the ECs have been scarcely reported, this observation warrants further investigation into the specific signaling mechanisms by which A β triggers such vesicle-like structures in ECs.

Finally, to confirm whether similar changes occur *in vivo*, we examined 5xFAD transgenic mutant mice

FIGURE 6 Ferroptosis is the major process inducing the formation of aggregation in the brain endothelial cells. (A) Summary of the ferroptosis pathway. Ferroptosis inhibitors are indicated in red and the main regulator enzymes are indicated in red circles. (B–E) Representative images showing the lipid droplets staining with A β in the HBMVECs treated with the ferroptosis inhibitor (UAMC-3203) and inducer (RSL3, GPX4 inhibitor). The lipid droplets induced by A β aggregates are downregulated by UAMC-3203 (B); however, lipid droplets are more peroxidized by RSL3, GPX4 inhibitor (D). These results suggest that A β is involved in ferroptosis and is the causative agent for lipid peroxidation. Scale bar, 100 μ m. (C, E) Bar graphs indicate lipid droplet formation in the A β -treated HBMVECs with the ferroptosis inhibitor (C) or inducer (E). Statistical data were analyzed by one-way ANOVA with Tukey post hoc test for multiple comparisons. An asterisk represents the statistical difference compared with the control or OA β treatment. Error bars represent the mean $\pm$ SD ($N = 3$, biological replicates). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

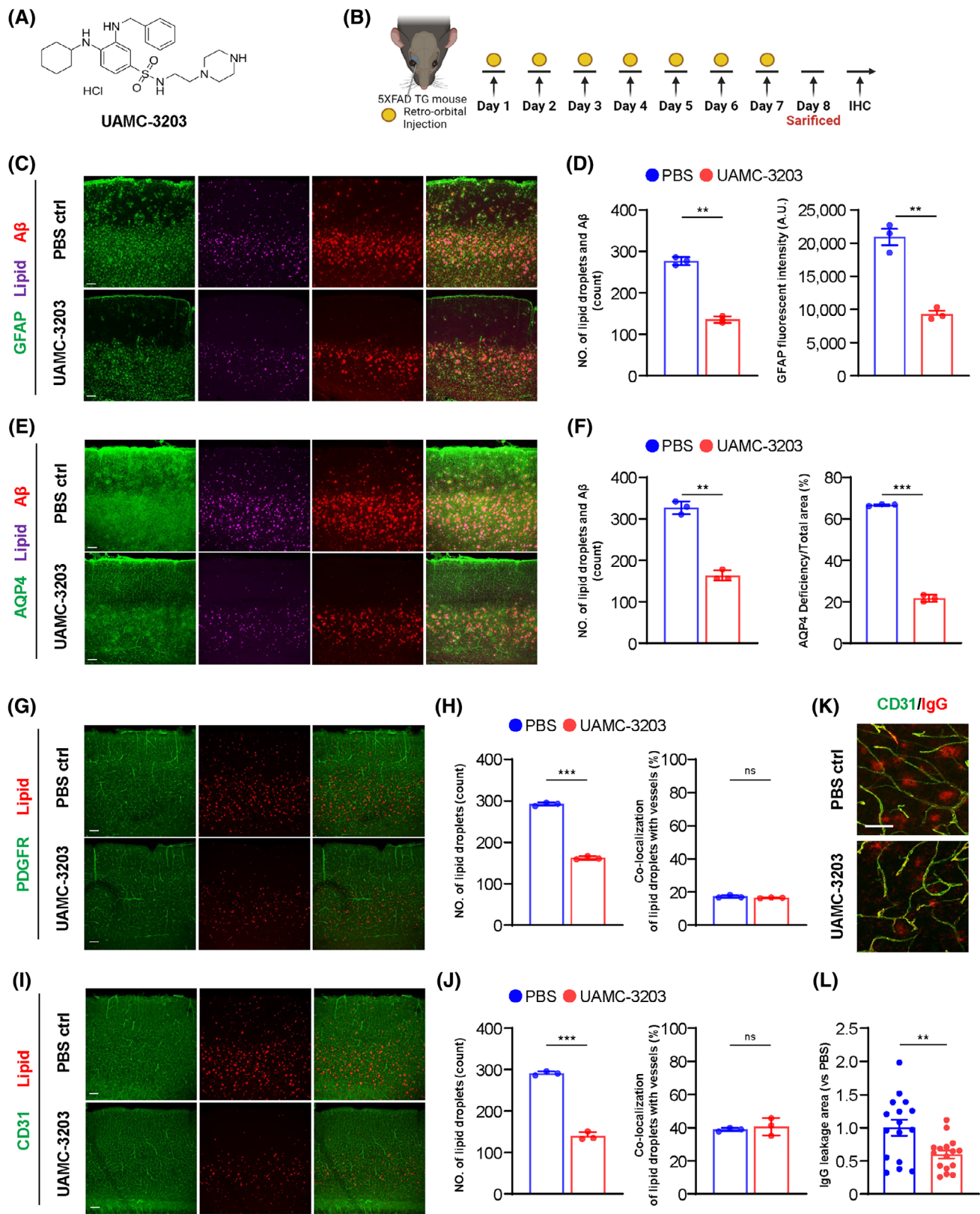


FIGURE 7 Legend on next page.

and observed lipid droplet-like structures in the brain ECs, stained with BODIPY. We also detected ferritin, a marker of intracellular iron accumulation, around the vicinity of the brain ECs. Interestingly, the co-occurrence of lipid droplets and ferritin-positive vesicles in ECs as well as pericytes, astrocytic endfeet, and microglia is seemingly similar to atherosclerotic changes observed in patients where oxidized lipid accumulation in the brain ECs is the initiating factor for the development of atherosclerotic changes and the additional influx of lipid droplets in the macrophages [63, 64]. Such vascular and lipid-related alterations, together with perivascular A β deposition, suggest that the pathological features we observed may represent an early stage of CAA-related endothelial injury, linking vascular ferroptosis to both cerebrovascular and amyloid pathologies in AD. Consistent with this interpretation, recent studies have demonstrated that endothelial ferroptosis contributes to vascular injury and plaque formation in atherosclerosis [19, 65, 66]. These findings support the idea that ferroptotic endothelial dysfunction represents a shared mechanism of lipid peroxidation-driven vascular damage across both neurodegenerative and cardiovascular diseases, with A β acting as a disease-specific trigger within the brain microvasculature.

4 | CONCLUSION

Our findings from both in vitro and in vivo experiments support the essential roles of A β in promoting lipid peroxidation and its subsequent damage to the BBB, with downstream effects on surrounding brain cells. These results suggest that lipid-related abnormalities triggered by A β aggregates may act as upstream events that contribute to atherosclerotic-like changes observed in brain ECs and the broader neuropathology observed in AD, including the vascular manifestations characteristic of cerebral CAA, where A β accumulation along vessel walls and endothelial dysfunction play key roles. While these vascular alterations appear to be initiated by A β and are therefore specific to AD pathology, similar ferroptosis-related endothelial dysfunction and lipid peroxidation processes have also been described in

systemic atherosclerosis. This suggests that the mechanisms identified here may represent a shared pathway linking lipid dysregulation to vascular injury across both neurodegenerative and cardiovascular contexts. Notably, prior studies have shown that large-vessel atherosclerosis involves intraluminal plaque formation [67, 68], whereas in small vessels such as those in the brain plaque-like structures tend to form at the vessel periphery of the vasculature, as we have observed in our study. This distinction underscores the need for future studies to clarify how vascular architecture influences the manifestation and progression of lipid-associated pathology in the CNS.

As the pathological basis of neurodegenerative diseases remains poorly defined, and considering evidence that A β might not be the single causal factor in AD, our findings highlight the importance of lipid droplet accumulation and possible ferroptosis-like mechanisms in disease progression. This study provides novel insights into the involvement of ECs dysfunction in AD and may offer new therapeutic targets centered on lipid metabolism dysregulation.

Although our pharmacological data demonstrate that inhibition of ferroptosis effectively reduces A β -induced lipid peroxidation and lipid droplet accumulation in both in vitro and in vivo models, the systemic delivery of the radical-trapping agent in vivo precludes precise determination of the relative contributions of endothelial cells versus other neurovascular cell types. Our findings strongly support endothelial ferroptosis as a major driver of the observed vascular pathology; however, additional endothelial-cell-specific approaches will be required to quantify the extent to which other cellular components may modulate these responses. Furthermore, although lipid droplet accumulation was clearly observed in postmortem human AD brain tissue, the analysis was limited to a single case, preventing comparisons across different pathological stages or clinical severities. Given that lipid droplet accumulation emerges at early disease stages in 5xFAD mice, larger human cohorts spanning diverse stages of AD will be essential to determine whether these lipid-related abnormalities represent early initiating events or later consequences of disease progression.

FIGURE 7 Radical-trapping agent significantly reduces the accumulation of the lipid droplets and A β plaques at 6-months old in 5xFAD mouse. (A, B) Chemical structure of UAMC-3203 (A) and schematic representation depicting timeline of the UAMC-3203 by retro-orbital injection into the 5xFAD mice and subsequent procedure (B). (C–J) Representative images and bar graph showing the amount of lipid droplets and A β with several components of BBB; astrocytes (C, D) and end-feet (E, F), pericytes (G, H), endothelial cells (I, J) in the cortex region of 5xFAD. The brain treated with UAMC-3203 showed a prominent decrease in lipid droplets and A β plaques compared with the untreated brain. Moreover, UAMC-3203 reduced the activation of GFAP and recovered the AQP4 deficiency in the 5xFAD mice. Moreover, the lipid droplets within blood vessels were more than twice as often on vascular endothelial cells as on pericytes. All data were statistically analyzed by one-way ANOVA with Tukey post hoc test for multiple comparisons. An asterisk represents the statistical difference compared with the control or treatment. Error bars represent the mean $\pm$ SD ($N = 3$, biological replicates). Ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bar, 1000 μ m. (K, L) Representative images and bar graph showing the IgG leakages in the cortex region of 5xFAD. The brain treated with UAMC-3203 showed a prominent decrease in IgG leakages compared with the PBS-treated group. All data were statistically analyzed by unpaired two-tailed Student's t -test. Error bars represent the mean $\pm$ SD (4 areas were measured each animal $N = 4$, biological replicates). Ns, not significant; ** $p < 0.01$. Scale bar, 50 μ m.

5 | MATERIALS AND METHODS

5.1 | Chemicals and antibodies

Antibodies are used as follows: Rab5 (Cell Signaling, Cat #3547S), Rab7 (Cell Signaling, Cat #9367S), Lamp1 (Santa Cruz, Cat #sc-20,011), beta-amyloid (Abcam, Cat #ab11132; Thermo Fisher Scientific, Cat #700254), 4-HNE (Abcam, Cat #ab46545), Ferritin (Abcam, Cat #ab75973), GPX4 (Cell Signaling, Cat #59735S), Beta-actin (Abcam, Cat #ab16039), GAPDH (Cell Signaling, Cat #2118S), Perilipin 1 (Cell Signaling, Cat #9349S), Perilipin 2 (Bioss, Cat #BS-10780R), Perilipin 3 (Abcam, Cat #ab224344), GFAP (Cell Signaling, Cat #12389S), PDGFR- β (Cell Signaling, Cat #3169S), CD31 (Santa Cruz, Cat #sc-376,764), AQP4 (Cell Signaling, Cat #59678S), LipidSpot™ 488 (Biotium, Cat #70065), BOD-IPY™ 581/591-C11 (Invitrogen, Cat #D3861), Rabbit IgG-488 (Invitrogen, Cat #A32731), Rabbit IgG-568 (Invitrogen, Cat #A11011), Rabbit IgG-647 (Invitrogen, Cat #A32733), Mouse IgG-488 (Invitrogen, Cat #A11001), Mouse IgG-568 (Invitrogen, Cat #A11004), Mouse IgG-647 (Invitrogen, Cat #A21235), Rat IgG-647 (Invitrogen, Cat #A21247), Lysotracker™ Deep Red (Invitrogen, Cat #L12492). Chemicals are used as follows: Triacsin-C (Tri-C; MedChemExpress, Cat #HY-N6707), PF-04620110 (Sigma, Cat #PZ0207), PF-06424439 (Sigma, Cat #PZ0233), TVB-3664 (TVB; MedChemExpress, Cat #HY-120062), 10,12-Tricosadiynoic acid (10,12-TDYA; MedChemExpress, Cat #HY-135425), UAMC-3203 (Selleckchem, Cat #S8792), RSL3 (MedChemExpress, Cat #HY-100218A), and Palmitic acid-13C16 (Sigma, Cat #605573).

5.2 | Cell culture

Human brain microvascular endothelial cells (HBMVECs) (Cell Systems, Cat #ACBRI 376), passages 6 through 9, were cultured in a complete classic medium (Cell Systems, Cat #4Z0-500) with 1.5% culture boost and 1% Penicillin–Streptomycin solution (Gibco, Cat #15140-122) and were grown for 4–5 days at 37°C and 5% CO₂. All experiments were performed in 12-well and 24-well plates coated with the attachment factor (Cell Systems, Cat #4Z0-500).

5.3 | Inhibitors treatment assay

For the in vitro assay, several inhibitors were dissolved in DMSO (Sigma, Cat #34869) and diluted in DPBS (Dulbecco's Phosphate-Buffered Saline, HYCLONE, Cat #SH30028.02) or complete classic media. The final concentration of Triacsin-C was 5 μ M; PF-04620110 and PF-06424439 were 10 μ M; TVB-3664 was 10 nM; 10,12-Tricosadiynoic acid was 3 μ M; UAMC-3203 was 2 μ M; RSL3 was 200 nM.

5.4 | A β oligomerization

Amyloid beta (A β 1-42; Enzo Life Sciences, Cat #ALX-151-002) was dissolved in DMSO (Sigma, Cat #34869) at a concentration of 1 mM and was stored at –80°C. Prior to treatment, a 1 mM stock which was diluted in DPBS to a concentration of 100 mM was incubated in a 37°C water bath for 24 h for A β oligomerization. The oligomeric A β stock was stored at –80°C or used directly on the cells.

5.5 | LC–MS analysis of brain ECs

The samples were sent from the Korea Brain Research Institute to Human Metabolome Technologies (HMT) and were treated at the Korea Brain Research Institute according to the protocol provided by HMT (Sample Preparation Protocol Document: Ver. ACJ.1.0.0). Metabolome analysis was performed in the HBMCEV using Lipid Chromatography Fourier Transform Mass Spectrometry (LC-FTMS). Peaks detected in LC-FTMS analysis were extracted using automatic integration software (MasterHands ver. 2.20.0.1 developed at Keio University) to obtain peak information including m/z, migration time (MT), retention time (RT), and peak area. The peak area was then converted to relative peak area by the following equation: Relative peak area = Metabolite peak area/(Internal standard peak area $\times$ Sample amount). From the measurement, peaks were detected and annotated according to HMT's metabolite standard library. All the metabolite concentrations were calculated by normalizing the peak area of each metabolite, and the profile of peaks with putative metabolites was represented on the metabolite cluster using VANTED (Visualization and Analysis of Networks containing Experimental Data) software.

5.6 | Immunohistochemistry

Mice were perfused with PBS and were fixed with 4% paraformaldehyde (T&I, Cat #BPP-9004-001). Brains were extracted and coronally sectioned using a vibratome and stored in PBS at 4°C or used directly for IHC staining. Cryo-sections were washed with PBS and permeabilized with 1% TritonX-100 (Sigma, Cat #X100-500ML; diluted in PBS) for 5 min. The sections were incubated with a blocking buffer containing 5% Normal Goat Serum (Jackson ImmunoResearch, Cat #005-000-121; diluted in PBS) for 1 h at room temperature. These sections were then incubated overnight at 4°C with primary antibodies in a blocking buffer. Next, sections were washed thoroughly with PBS and incubated with secondary antibodies for 1 h at RT. Following washes with PBS, these sections were mounted on slides and coverslipped using DAPI Fluoromount-G (Electron Microscopy Sciences, Cat #17984-24).

5.7 | Immunocytochemistry

HBMVECs were grown on cover glass in 24-well plates for appropriate treatment. Cells were replaced with pre-warmed media containing 1 nM of LysoTracker (Invitrogen, Cat #L12492) and incubated for 1 h at 37°C in a 5% CO₂ incubator. Then, cells were quickly fixed with cold 4% paraformaldehyde (T&I, Cat #BPP-9004-001) and washed with DPBS (Hyclone, Cat #SH30028.02). Cells were permeabilized with 0.2% TritonX-100 (Sigma, Cat #X100-500ML; diluted in PBS) for 1 min. After that, the cells were blocked with 5% Normal Goat Serum (Jackson ImmunoResearch, Cat #005-000-121; diluted in PBS) and were incubated overnight with primary antibodies at 4°C. Post-incubation, the cells were washed and incubated with secondary antibodies (Invitrogen, Alexa Fluor-488, -568, -647) diluted in a blocking buffer at room temperature for 1 h. Followed by washing with DPBS, the samples were mounted by DAPI Fluoromount-G (Electron Microscopy Sciences, Cat #17984-24) onto slides. The stained cells were imaged on a high-resolution confocal microscope (Leica, TCS SP8) with Z-stack imaging and were reconstructed with maximal projection intensity options using LAS X image software.

5.8 | Immunoblot analysis

For immunoblot, cells were lysed in RIPA buffer (Rockland, Cat #MB-030-0050) containing the protease inhibitor cocktail solution (Thermo, Cat #18611281) and 0.5 M EDTA solution (Thermo, Cat #1861274). The Pierce™ BCA protein assay kit (Thermo, Cat #23225) was used to quantify the concentration of cell lysate. Proteins were separated on SDS-PAGE and transferred to nitrocellulose transfer membranes (Pall Corporation, Cat #66485). Then, blots were blocked in 1% BSA/PBS buffer and labeled with the following primary antibodies overnight at 4°C. The appropriate HRP-conjugated secondary antibodies were used to incubate the blots for 1 h at RT. SuperSignal™ West Pico PLUS Luminol/Enhancer Solution (Thermo, Cat #1863096) was used at a ratio of 1:1 for blotting.

5.9 | Animal experiments

This study used 5xFAD mice (B6SJL-Tg(APPs^wFILon,-PSEN1*^{M146L}*^{L286V})6799Vas/Mmjax) obtained from The Jackson Laboratory. Male hemizygous 5xFAD mice were crossed with inbred female mice to maintain the 5xFAD and WT mice. Mice were housed under standard conditions at 22°C and a 12-h light/dark cycle with food and water ad libitum, and the experimental protocols were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory

Animals of the National Institutes of Health. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Brain Research Institute (IACUC-23-00031). Only male mice were used in this study and a total of 4 animals died unexpectedly due to AVERTIN-induced anesthesia before drug administration and were therefore excluded from the analysis. These deaths were not related to the experimental treatment. Mice were assigned to the treatment and control groups. The control group mice were injected with PBS buffer, and the treatment group was given the radical-trapping agent UAMC-3203 (Selleckchem, Cat #S8792, 1 mg/1 mL) by retro-orbital injection (12.5 mg/kg, daily until the 7th day). After that, the mice were sacrificed at a time point, and their brains were post-fixed in 4% paraformaldehyde and 100% methanol for further processing.

5.10 | Postmortem human brain sample sources

Human brain samples were obtained at autopsy provided by the Institutional Review Board of Konkuk University (Seoul, Korea; IRB7001355-202103-E-135). Cortical brain tissues were collected from the frozen sections, a process performed by the Korea Brain Bank Network (KBBN, Daegu, Korea). For this study, we selected one healthy control (KBBN03003046, 46 years old, 9.5 h post-mortem delay) and one patient with Alzheimer's disease (KBBN03003049, 82 years old, Braak stage VI [B3], 5 h post-mortem delay), and used the brain sections for immunohistochemistry assays.

5.11 | RNA sequencing and phospho-protein array analysis

Human brain microvascular endothelial cells (HBMVECs) were cultured and treated with A β aggregates for the indicated time points. Untreated cells were used as controls, and each condition was prepared in triplicate.

5.12 | RNA sequencing

Total RNA was extracted from HBMVECs using TRIzol reagent according to the manufacturer's instructions (Thermo Fisher Scientific, Cat #15596026). RNA purity and concentration were measured with a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, USA), and RNA integrity was verified using a TapeStation RNA ScreenTape system (Agilent Technologies, Cat #5067-5576). Samples with an RNA integrity number (RIN) >7.0 were used for sequencing. RNA-seq libraries were prepared using the TruSeq Stranded Total RNA

Library Prep Gold Kit (Illumina, Cat #20020599) and sequenced on an Illumina NovaSeq 6000 platform (2 × 100 bp paired-end reads) by Macrogen Inc. (Seoul, Korea).

Raw sequencing reads were quality-checked using FastQC and trimmed with Trimmomatic (v0.38) to remove adapter sequences and low-quality bases. Clean reads were aligned to the human reference genome (GRCh38) using HISAT2 (v2.1.0) [69], and gene-level counts were obtained using StringTie (v2.1.3b) [70, 71]. Differential expression analysis was performed using DESeq2 (v1.38.3) [72] in R (v4.2.2). Genes with low counts were filtered out prior to normalization, and the median-of-ratios normalization method was applied. Statistical significance of differential expression was assessed with the Wald test, followed by Benjamini–Hochberg false discovery rate (FDR) correction for multiple testing. Genes with $|\log_2(\text{fold change})| \geq 1$ and adjusted p -value < 0.05 were considered significantly differentially expressed. Gene Ontology and KEGG pathway enrichment analyses were performed using g:Profiler [73] with FDR correction applied.

5.13 | Phospho-array analysis

For phospho-protein profiling, total protein was extracted from HBMVECs using RIPA buffer (Rockland, Cat #MB-030-0050) containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Cat #78440). Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Cat #23225). Phospho-protein explorer antibody array analysis was conducted by Full Moon Biosystems, Inc. (Sunnyvale, CA). Signal intensities were normalized to internal controls, and values were expressed relative to untreated controls. Statistical comparisons between groups were performed using unpaired Student's t -tests in GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA), and data are presented as means $\pm$ SD.

5.14 | Cranial window preparation and Intravital imaging

5xFAD transgenic mice and age-matched WT control (6 months old) were used for cranial window implantation and intravital two-photon imaging following the manufacturer's protocol (IVIM Technology, Daejeon, Korea).

Mice were anesthetized with Avertin and secured in a stereotaxic frame on a heating pad. After scalp removal and skull exposure, a circular craniotomy (3 mm in diameter) was performed over the somatosensory cortex while preserving the dura. A preassembled cranial window, consisting of a 3 mm coverslip bonded using UV-curable optical adhesive, was placed over the craniotomy and

secured with cyanoacrylate glue and dental acrylic. A custom-made titanium or stainless-steel head-bar was affixed posterior to the window to allow for head fixation during imaging. Mice received 0.5 mL sterile saline subcutaneously post-operatively and were monitored until full recovery. Mice received Carprofen (Sigma, Cat #PHR1452, 10 mg/kg) subcutaneously post-operatively and were monitored until full recovery for 2 weeks.

After a recovery period, mice were re-anesthetized with isoflurane (4% for induction, 3.5% for maintenance). One hundred microliters of a mixture containing Methoxy-X04 (amyloid- β marker; Tocris, Cat #4920; stock 100 mM, used at 1/17 dilution), SF44 (lipid droplet marker; SPARK, Cat #BP00202; stock 83.5 mM, used at 1/50 dilution), and CD31-647 (blood vessel marker; IVIM, Cat #IVITM-991-2004; 1 mg/mL) was administered via retro-orbital injection prior to imaging. The intravital imaging was performed at excitation wavelengths depending on the fluorescence dye, with z-stack images acquired at 5 μ m steps. Image reconstructed using IVIM Studio Program.

5.15 | Statistics

Statistical analyses were conducted with GraphPad Prism 9 software. All data are presented as mean $\pm$ SD. Quantified data were analyzed using ordinary one-way ANOVA or unpaired two-tailed Student's t -test and differences were considered statistically significant at $p < 0.05$.

AUTHOR CONTRIBUTIONS

Do-Geun Kim conceived and supervised this project, and Seung-Yeol Nah provided the human samples. Suhyeon Son, Suji Kim, Suji Lee, Min-Tae Jeon, and Moonseok Choi performed in vitro experiments. Suhyeon Son, Min-Tae Jeon, Kyu-Sung Kim, and Moonseok Choi performed, and Suhyeon Son analyzed biological in vivo experiments. Do-Geun Kim and Suhyeon Son analyzed data and wrote manuscripts. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The RNA-seq data generated in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE310780. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Brain Research Institute (IACUC-23-00031). Human postmortem tissues were provided by the Institutional Review Board of Konkuk University (Seoul, Korea; IRB7001355-202103-E-135).

ORCID

Do-Geun Kim  <https://orcid.org/0000-0002-0066-884X>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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