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Endolysosome mechanisms associated with Alzheimer's disease-like pathology in rabbits ingesting cholesterol-enriched diet

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Abstract

Alzheimer's disease (AD) is characterized clinically by progressive disturbances in memory, judgment, reasoning and olfaction, and pathologically by loss of synaptic integrity, extracellular accumulations of amyloid beta (A β) containing plaques, and intraneuronal tangles composed of hyperphosphorylated tau. Endolysosome dysfunction is one of the earliest pathological features of AD and cholesterol, a known risk factor for sporadic AD, is up-taken into neurons via receptor-mediated endocytosis. Accordingly, we determined the extent to which endolysosome dysfunction is associated with pathological features observed in rabbits fed cholesterol-enriched diet; a well-characterized model of sporadic AD. Olfactory bulbs were taken from rabbits fed for 12 weeks a diet enriched with 2% cholesterol and endolysosome morphology and function as well as AD-like pathology were investigated using enzyme activity measurements, immunoblotting and immunostaining techniques. In olfactory bulbs of rabbits fed cholesterol-enriched diet we observed enlarged endolysosomes containing increased accumulations of ApoB containing cholesterol and increased accumulations of synaptophysin, A β and phosphorylated tau. Cholesterol-enriched diet also decreased significantly specific enzyme activities of the endolysosome enzymes acid phosphatase and cathepsin D. Decreased synaptic area was present in olfactory bulbs of cholesterol-fed rabbits as indicated by significant decreases in protein expression levels of the synaptic area marker protein synaptophysin. Our results suggest strongly that elevated circulating cholesterol plays an important role in the pathogenesis of AD, and that alterations in endolysosome structure and function are associated with cholesterol diet-induced AD-like pathology.

Keywords

Cholesterol; endosomes; lysosomes; amyloid beta-protein; tau proteins; olfactory bulb; synaptophysin; cathepsin D; acid phosphatase

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by progressive disturbances in memory, judgment, reasoning, and olfaction. Pathologically, AD is characterized by loss of synaptic integrity, extracellular accumulations of amyloid beta

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(A β) protein containing plaques, and intracellular accumulations of neuronal tangles composed of hyperphosphorylated tau [1–3]. Although a small percentage of AD cases are familial and genetically-based, the vast majority (>90%) of AD cases are sporadic with unknown etiology. Altered cholesterol homeostasis has emerged as an important and recognized risk factor in the pathogenesis of sporadic AD [4–9], and rabbits fed cholesterol-enriched diets exhibit pathological features of AD [10–12]. However, little is known about how increased levels of plasma cholesterol contribute to the pathogenesis of AD.

Increasingly, changes in morphological and functional features of endolysosomes have been identified as early pathological features of AD [13, 14]. It was shown that endosome enlargement was apparent in brains of AD patients and non-demented patients with early signs of AD, in Down's syndrome individuals, and in patients bearing the ApoE4 allele [15, 16]. The hypothesis that endosome enlargement is an early pathogenic event in AD is supported by findings that endosome enlargement largely precedes extracellular deposition of A β in brain [17]. Abnormalities of lysosomes have been noted also in AD; lysosomal components are present in amyloid plaques [14, 18], increased numbers of neuronal lysosomes have been observed, there is increased expression and synthesis of all classes of lysosomal hydroxylase enzymes [19], and residual bodies accumulate as an indicator of lysosome dysfunction [20]. Thus, changes in endolysosomes appear to play an important and early role in the pathogenesis of AD.

Elevated levels of circulating cholesterol is an extrinsic factor that contributes to sporadic AD pathogenesis. Epidemiologically, high plasma levels of cholesterol during mid-life increase the risk of developing AD later in life [9] and apolipoprotein E4 allele (ApoE4), the major genetic risk factor of sporadic AD, has been associated with elevated levels of circulating cholesterol and increased risk of developing AD [21, 22]. Experimentally, diets enriched in cholesterol induced pathological features of AD in mice, rats and rabbits [23–26]. Thus, environmental, genetic and epidemiological evidence supports the linkage between high levels of circulating cholesterol and increased AD pathogenesis. Although it is clear that altered brain cholesterol homeostasis can affect A β production [25, 27–31], less clear is the extent to which and mechanisms by which circulating cholesterol affects A β levels and the development of AD pathology in brain.

It is well known that cholesterol and proteins that are involved in cholesterol transport including ApoE and low density lipoprotein receptor related protein (LRP) all play important roles in the pathogenesis of AD [6, 21, 32], and their functions are all dependent on endocytosis. One of the major sources of neuronal cholesterol is uptake of cholesterol containing lipoproteins through receptor-mediated endocytosis. We showed previously that cholesterol-enriched diet markedly increased plasma levels of cholesterol and disrupted the integrity of the blood-brain barrier in olfactory bulb and elsewhere throughout rabbit brain [12]. Indeed, cholesterol-enriched diet has been shown to change brain cholesterol homeostasis, increase cholesterol accumulation in neurons, and increase cholesterol levels in brain [7, 26]. Thus, increased plasma levels of cholesterol may result in enhanced cholesterol endocytosis and increased accumulation of cholesterol in endolysosomes of neurons, thereby affecting neuronal endolysosomal structure and function.

Accordingly, the present studies tested the hypothesis that changes in endolysosome structure and function contribute to the AD-like pathology in brain of rabbits fed cholesterol-enriched diet. The essential findings of our studies of olfactory bulbs from this rabbit model for sporadic AD include observations of enlarged endolysosomes containing increased accumulations of apolipoprotein B (Apo B) containing cholesterol, significant decreases in specific enzyme activities of the endolysosome enzymes acid phosphatase and cathepsin D, and increased accumulations of synaptophysin, A β and phosphorylated tau.

Synaptic area loss was also present as indicated by significant decreases in protein expression levels of the synaptic area marker protein synaptophysin. These studies support the concept that endolysosome dysfunction might be targeted for therapeutic intervention against AD.

Material and Methods

Animals

Male New Zealand white rabbits (1.5 to 2 years old) weighing 3 to 4 kg were used in the present study. Rabbits were randomly assigned into two groups and fed with either normal chow (n = 6) or 2% cholesterol-enriched diet (n = 9). After 12 weeks on the respective diets, animals were anesthetized and perfused with PBS. Olfactory bulbs were dissected, frozen on a liquid nitrogen-cooled surface, and stored at -80°C until taken for experimentation. All experiments were approved by the Committee for Animal Care and Use at the University of North Dakota.

Immunohistochemistry

Snap-frozen olfactory bulbs were embedded in Tissue-Tek optimum cutting temperature (OCT) compound in cryomolds. Horizontal cryostat sections (thickness $14\text{ }\mu\text{m}$) were mounted on superfrost plus slides (Fisher). For immuno-fluorescent staining studies, sections were air-dried at room temperature for 40 minutes, fixed in ice-cold acetone for 10 minutes, air-dried for 30 minutes, and washed 3-times with PBS. Sections were then blocked with 5% donkey serum and incubated overnight at 4°C with primary antibodies targeting synaptophysin (1:1000, mouse monoclonal, Sigma), EEA1 (1:500, goat polyclonal, Santa Cruz), LAMP2 (1:500, goat polyclonal, Santa Cruz), Cathepsin D (1:1000, mouse monoclonal, Sigma), acid phosphatase (1:1000, mouse monoclonal, Abcam), apolipoprotein B (1:500, mouse monoclonal, Santa Cruz), A β (4G8, 1:500, mouse monoclonal, Signet), A β_{1-40} (1:500, mouse monoclonal, Wako), A β_{1-42} (1:500, mouse monoclonal, Wako), and phospho-tau (AT8, 1:500, Pierce). After 3 washes with PBS, sections were incubated with corresponding fluorescence-conjugated secondary antibodies including Alexa 546-conjugated donkey anti-mouse antibodies (Invitrogen), Alexa 488-conjugated donkey anti-mouse antibodies (Invitrogen), and Alexa 546-conjugated donkey anti-goat antibodies (Invitrogen). Neurons were identified with fluorescence-based NeuroTrace Nissl stain (1:1000, Invitrogen). Sections were examined using a Zeiss LSM 510 Meta confocal microscope. Controls for specificity included staining olfactory bulb sections with primary antibodies without fluorescence-conjugated secondary antibodies (background controls), and staining olfactory bulb sections with only secondary antibodies – these controls eliminated auto-fluorescence in each channel and bleed-through (crossover) between channels. Fluorescence intensity was quantified using Image J software. For detection of free cholesterol, olfactory bulb sections were fixed with formalin and stained with Filipin (Sigma). The Filipin stock solution was prepared by dissolving 5 mg Filipin in 1 ml DMSO. A 100 $\mu\text{g}/\text{ml}$ working solution prepared by dissolving the stock solution 1:50 in PBS (pH = 7.2) was applied to sections for 1 h at 4°C . Sections were examined by conventional fluorescence (Leica) microscopy. For immunohistochemistry studies, horizontal olfactory bulb sections ($14\text{ }\mu\text{m}$) were fixed in ice-cold acetone for 10 minutes, treated with 1% hydrogen peroxide in methanol, and incubated with a blocking solution of 5% normal goat serum in PBS. Subsequently, sections were incubated overnight at 4°C with 4G8 (1:500), a monoclonal antibody that stains A β . After washing with PBS and incubating with the biotinylated secondary antibody, sections were processed with a Vectastain Elite avidin–biotin complex kit (Vector Laboratories) and visualized by liquid diaminobenzidine/hydrogen peroxide (Vector Laboratories) with light hematoxylin counterstaining.

Immunoblotting

Olfactory bulbs were gently homogenized using a teflon homogenizer (Thomas) in 10 volumes of cold suspension buffer (20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepstatin, and 12.5 mg/ml of *N*-acetyl-Leu-Leu-Norleu-Al). Protein concentrations were determined with a Bradford protein assay (Bio-Rad). Proteins (10 µg) were separated by SDS-PAGE (12% gel), and following transfer to polyvinylidene difluoride membranes (Millipore) were incubated overnight at 4°C with anti-synaptophysin (1:1000), EEA1 (1: 500), Cathepsin D (1: 1000), acid phosphatase (1: 1000), N-terminal AβPP (1: 1000), and C-terminal AβPP (1: 500, Sigma), or AT8 (1:1000). β-actin (1: 10000, Abcam) was used as a gel loading control. The blots were developed with enhanced chemiluminescence, and bands were visualized and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland). Quantification of results was performed by densitometry and the results were analyzed as total integrated densitometric volume values (arbitrary units).

Acid phosphatase activity measurement

The enzyme activity of acid phosphatase was determined using an Acid Phosphatase Assay kit (Sigma); a luminescence-based assay that uses 4-nitrophenyl phosphate as substrate. After olfactory bulbs were lysed, protein concentrations were determined using a Bradford assay (Bio-Rad) and samples were diluted to a concentration of 1 µg/µl with the 1× extraction buffer. Equal amounts of protein (50 µg) were added into wells of 96-well plates, 50 µl of Substrate Solution were added to each well, and plates were mixed using a horizontal shaker and incubated 10 minutes at 37°C. Each sample was assayed in duplicate and included on each plate were blank reactions (Substrate Solution without enzyme), positive controls containing acid phosphatase control enzyme, and the *p*-nitrophenol standard solution. Reactions were stopped by adding 0.2 ml of Stop Solution to each well except for the wells containing the Standard Solution. Absorption was read with a microplate reader (Molecular Devices) at 405 nm. Acid phosphatase activity was expressed as optical density per 50 µg of protein. Specific activities of acid phosphatase were expressed as a ratio of acid phosphatase activity to protein levels of acid phosphatase as determined by immunoblotting.

Cathepsin D activity measurement

The enzyme activity of cathepsin D was determined using a Cathepsin D Activity Assay kit (BioVision), a fluorescence-based assay that used the preferred cathepsin-D substrate sequence GKPILFFRLK (Dnp)-DR-NH₂ labeled with MCA. Cathepsin-D catalyzes the cleavage of the synthetic substrate to release a fluorescence signal which was then quantified using a fluorescence plate reader (Molecular Devices). After olfactory bulbs were lysed protein concentrations were determined using a Bradford protein assay (Bio-Rad) and samples were diluted to the same concentration (1 µg/µl) with the 1× extraction buffer. Equal amounts of protein (50 µg) were added into wells of 96-well plates, 50 µl of Reaction Buffer and 2 µl of substrate were then added into each well and incubated at 37°C for 2 h. Each sample was assayed in duplicate and fluorescence was determined using a fluorescence plate reader (Molecular Devices) with 328 nm excitation and 460 nm emission filters. Cathepsin D activity was expressed as relative fluorescence units (RFU) per 50 µg of protein. Specific activities of cathepsin D were expressed as a ratio of cathepsin D activity to protein levels of cathepsin D as determined by immunoblotting.

Statistical Analysis

All data were expressed as mean and SEM. Statistical significance was determined with a Student's t-test. *p* < 0.05 was considered to be a statistically significant difference.

Results

Effects of cholesterol-enriched diet on endolysosome structure

We published previously that cholesterol-enriched diet disrupts the blood-brain barrier [12]. Thus, elevated levels of plasma cholesterol may enter brain parenchyma and may disturb brain cholesterol homeostasis. Indeed, increased levels of brain cholesterol and increased levels of cholesterol in neurons have been reported in cholesterol-fed rabbits [7, 26]. Cholesterol is linked to the pathogenesis of sporadic AD, cholesterol accumulates in neurons by receptor-mediated endocytosis (uptake), and one of the earliest structural changes noted in brains of people with AD is to endolysosomes. Thus, increased levels of brain cholesterol resulting from ingestion of cholesterol-enriched diet could increase accumulation of free cholesterol in endolysosomes and may affect endolysosome structure and function in neurons. Here, we examined first the effects of cholesterol-enriched diet on endolysosome structure in olfactory bulb neurons. We found that cholesterol-enriched diet not only significantly increased the immunoreactivity (Figure 1B, C, $p<0.05$) and protein levels (Figure 1D, $p<0.01$) of early endosome antigen 1 (EEA1) in periglomerular cells of olfactory bulb, but also increased the size and number of endosomes (Figure 1B) and lysosomes (data not shown).

Next, we examined the extent to which cholesterol-enriched diet affected the accumulation of free cholesterol in endolysosomes. Using double immunofluorescence staining methods, we demonstrated that the cholesterol-enriched diet markedly increased the accumulation of free cholesterol in EEA1-positive endosomes (Figure 2A) and LAMP2-positive lysosomes (Figure 2B) in periglomerular cells of olfactory bulb.

Furthermore, we determined the extent to which plasma cholesterol in the peripheral circulation might enter brain and accumulate in endolysosomes. For this we used Apo B as a marker of peripheral cholesterol because Apo B is not normally found in brain [33]. We found that the cholesterol-enriched diet markedly increased the immunoreactivity of Apo B in olfactory bulb (Figure 3A). Using double immunofluorescence staining methods, we demonstrated that cholesterol-enriched diet increased the accumulation of Apo B in EEA1-positive endosomes (Figure 3B) and LAMP2-positive lysosomes (Figure 3C). These observations indicate that elevated levels of peripheral cholesterol can enter brain where it can disrupt brain cholesterol homeostasis via enhanced cholesterol endocytosis.

Effects of cholesterol-enriched diet on endolysosome function

With clear results that the cholesterol-enriched diet affected endolysosome structure, we next examined the effects of the diet on endolysosome function. For this purpose we used two lysosomal enzymes, cathepsin D and acid phosphatase, as marker enzymes to evaluate endolysosome function and determined for these two enzymes their enzyme activity and protein expression levels. Specific enzyme activities of each lysosomal enzyme were expressed as the ratio of enzyme activities to enzyme protein levels as determined by immunoblotting. We found that the cholesterol-enriched diet increased cathepsin D immunoreactivity (Figure 4A) and protein levels (Figure 4B, $p<0.05$), as well as acid phosphatase immunoreactivity (Figure 4D) and protein levels (Figure 4E, $p<0.01$) in olfactory bulb. The cholesterol-enriched diet decreased significantly the specific enzyme activities, as indicated by the ratio of total enzyme activity to protein levels of cathepsin D (Figure 4C, $p<0.05$) and acid phosphatase (Figure 4F, $p<0.01$). Thus, both morphological data and functional data indicate that the cholesterol-enriched diet changes significantly the structure and function of endolysosomes in olfactory bulb.

Endolysosomes are involved in cholesterol-enriched diet-induced decreases in total synaptic area

Pathological changes in endolysosomes, olfactory dysfunction and synaptic loss have been identified as early pathological features of AD [13, 14, 34–37]. In examining the effects of the cholesterol-enriched diet on synaptic integrity, we noted that synaptophysin immunoreactivity decreased significantly ($p < 0.001$) in the glomerular layer of olfactory bulb (Figure 5A, B). The cholesterol-enriched diet also changed markedly the distribution of synaptophysin (Figure 5A). The distribution pattern of synaptophysin immunoreactivity changed from an even distribution within individual glomeruli from control rabbits to decreased immunostaining in the center of glomeruli from cholesterol-fed rabbits; intense granule-shaped immunoreactivity was observed very close to the cell body of periglomerular cells in cholesterol-fed rabbits (Figure 5A). Cholesterol-enriched diet also decreased significantly ($p < 0.01$) protein levels of synaptophysin in olfactory bulb (Figure 5C). Using double fluorescent staining, we found that the granule-shaped immunoreactivity of synaptophysin was co-distributed with EEA1 (Figure 5D) and with LAMP 2 (Figure 5E). Thus, this dietary treatment results in reduced synaptic area and abnormal distribution patterns of synaptophysin in olfactory bulb endolysosomes.

Endolysosome involvement in cholesterol-induced intraneuronal depositions of A β

A hallmark of AD is increased levels of A β in brain and intraneuronal deposition of A β precedes extracellular deposition of A β [38, 39]. Here, we examined effects of cholesterol-enriched diet on intraneuronal deposition of A β in olfactory bulb and found that the cholesterol diet increased markedly 4G8 immunopositive staining of A β in olfactory bulb (Figure 6A). Furthermore, intracellular A β accumulated in neuronal endosomes (Figure 6B) and lysosomes (Figure 6C). To investigate this further, specific antibodies against A β_{1-40} and A β_{1-42} were used to stain for A β , and we found that cholesterol-enriched diet increased significantly ($p < 0.05$) intraneuronal deposition of both A β_{1-40} (Figure 6D) and A β_{1-42} (Figure 6E). Our observations are consistent with findings by others that A β accumulates in endolysosomes in AD brain [15, 40]. Because A β originates from A β precursor protein (A β PP), we examined protein levels of A β PP in total lysates of olfactory bulb and found that the cholesterol-enriched diet did not change significantly protein levels of A β PP in olfactory bulb (Figure 6F). Furthermore, we examined the effects of cholesterol-enriched diet on A β PP processing by measuring levels of C99 and C83 fragments using an antibody against C-terminal A β PP; C99 is the cleavage product of A β PP by β -site A β PP cleavage enzyme (BACE-1) and C83 is the product of the non-amyloidogenic α -secretase pathway. The cholesterol-enriched diet increased significantly ($P = 0.03$) levels of C99 fragments, but did not change significantly ($P = 0.8$) levels of C83 fragments (Figure 6F). These results indicate that the cholesterol-enriched diet affects A β PP processing in favor of A β production in olfactory bulb. Our results indicate that cholesterol-enriched diet affects A β PP processing and increases A β generation by affecting endolysosome function.

Endolysosome involvement in cholesterol-induced intraneuronal depositions of phosphorylated tau

Neurofibrillary tangles composed of phosphorylated tau are another pathological feature of AD and here we examined the extent to which the cholesterol-enriched diet affected intraneuronal deposition of phosphorylated tau in olfactory bulb. We found that cholesterol-enriched diet increased significantly ($p < 0.05$) protein levels of phosphorylated tau in total lysates of olfactory bulb (Figure 7A). Accompanied by Nissl staining, the increases in deposition of phosphorylated tau was found to occur in olfactory bulb neurons (Figure 7B). Thus, high dietary cholesterol increased levels of phosphorylated tau, an observation consistent with our previous report that cholesterol-enriched diet increased tau phosphorylation in rabbit brain [26]. Furthermore, we demonstrated that phosphorylated tau

accumulated in lysosomes (Figure 7C), and this observation is consistent with reports of others that tau is degraded in lysosomes [41–43] and that lysosomal dysfunction induces tau-pathology [44–46]. Thus, cholesterol-induced increases in tau-pathology appear to be the result of altered endolysosome function.

Discussion

Elevated levels of circulating cholesterol has emerged as an extrinsic factor that contributes to sporadic AD pathogenesis [4–7] and proteins that are responsible for neuronal cholesterol transport, such as Apo E and LRP, all have been implicated in the pathogenesis of AD [6, 21, 32]. Several lines of evidence from environmental, genetic, and epidemiological studies support the importance of elevated levels of circulating cholesterol in the pathogenesis of sporadic AD. Others and we have shown that cholesterol-enriched diets induce AD-like features in rabbits including learning deficits, blood-brain barrier breakdown, and accumulations of A β and phosphorylated tau [7, 10–12, 25, 26, 47]. However, mechanisms underlying cholesterol-induced AD-like pathology are not fully understood. Here, we report that cholesterol-enriched diet increased the accumulation of Apo B containing cholesterol in endolysosomes of neurons from olfactory bulb, that this diet increased sizes and numbers of endolysosomes, that endolysosome function was affected, and that changes in endolysosome structure and function was involved in AD-like pathological features including decreased synaptic area and increased levels of A β and phosphorylated tau.

Neuronal cholesterol is up-taken by receptor-mediated endocytosis, a process where lipoproteins bound to its receptors are internalized, transported to endolysosomes, hydrolyzed to free cholesterol, and from where free cholesterol is transported to various intracellular compartments via a mechanism involving the Niemann-Pick typeC (NPC) proteins type-1 (NPC1) and -2 (NPC2) [48–50]. Although under normal conditions plasma cholesterol is largely excluded from the brain, others and we have shown that cholesterol-enriched diets disrupt the integrity of the blood-brain barrier [12] and thereby can affect the levels of brain cholesterol. Indeed, cholesterol-enriched diets increase cholesterol levels in brain and increase the accumulation of free cholesterol in neurons [7, 26]. Here, we showed that increased levels of cholesterol enhanced receptor-mediated cholesterol endocytosis, increased the accumulation of free cholesterol in endolysosomes, and affected endolysosome function. Importantly, the cholesterol-enriched diet increased the accumulation of Apo B, an apolipoprotein that is not normally present in brain [33], but is present in AD brain [51, 52]. These latter results indicate clearly that the accumulated cholesterol originated from peripheral sources. Moreover, the cholesterol-enriched diet increased the size and number of endolysosomes and affected their function as evidenced by decreased specific enzyme activities of two lysosomal enzymes, cathepsin D and acid phosphatase. These results are consistent with findings of others that altered endolysosomes play a role in AD pathogenesis [17, 19, 20, 53, 54].

Olfaction is an ancient and primal sense in animals. The first level of central olfactory information processing occurs in spherical structures of the olfactory bulb called glomeruli where axons of olfactory sensory neurons that are located on the epithelium in the roof of the two nasal cavities synapse onto projection neurons; mitral and tufted cells. In addition to synapses between olfactory sensory neurons and the dendritic tufts of projection neurons, glomeruli contain processes from a shell of interneurons called periglomerular cells, which send their dendrites into the glomerulus and form dendrodendritic reciprocal synapses with the dendrites of mitral, tufted and other periglomerular cells. The axons of periglomerular cells are also presynaptic to neurons of neighboring glomeruli [55, 56]. The periglomerular cells modulate glomerular processing and play a very important role in odor decoding [57]. After information processing at glomeruli, the projection neurons, mitral and tufted cells of

the olfactory bulb pick up information and transmit it to higher olfactory centers for further processing. Accordingly, the present study focused on periglomerular cells of olfactory bulb.

Deficits in olfaction are commonly reported in neurodegenerative diseases generally and AD particularly, and olfactory dysfunction has been deemed to be the earliest symptom of AD [34, 35]. Deficits in odor identification have been shown to increase the likelihood of subsequent cognitive decline, especially in patients carrying one or two copies of the $\epsilon 4$ allele of ApoE [34, 58, 59], the major genetic risk factor of sporadic AD. Thus, altered cholesterol homeostasis could contribute to the development of olfactory dysfunction in sporadic AD. Pathologically, oxidative damage, the presence of A β plaque, and accumulation of phosphorylated tau as neurofibrillary tangles all have been documented in olfactory epithelium and olfactory bulbs of AD patients [37, 60–66]. As mentioned earlier, we demonstrated that elevated levels of circulating cholesterol disrupts the blood-brain barrier in olfactory bulb [12] and increased accumulations of cholesterol in endolysosomes of neurons in olfactory bulb originated from peripheral sources. Here, we further investigated, in olfactory bulb, the extent to which and mechanisms by which elevated levels of circulating cholesterol contributed to the development of AD-like pathology including synaptic loss, elevated A β production, and increased tau phosphorylation.

In AD patients, synapse loss and not neuronal loss correlates best with dementia [67, 68]. In mouse models of AD, there is hardly any neuronal loss but synaptic loss and dendritic spine abnormalities have been described [69–71]. Here we found that the cholesterol-enriched diet decreased significantly protein levels of synaptophysin, a presynaptic marker, in the glomerular layer of olfactory bulbs. Furthermore, we found that the cholesterol-enriched diet markedly increased synaptophysin accumulation in endolysosomes of periglomerular cells. Thus, our data are consistent with that of others that endolysosomes are responsible for recycling synaptic proteins [72–74], that lysosome dysfunction is linked to synaptic pathology in AD brain [75, 76], and that inhibiting lysosomal function with chloroquine, a lysosomotropic agent that blocks acidification, results in synaptic dysfunction and synaptic loss in hippocampal slices [77–79].

Next, we examined endolysosome involvement in cholesterol-enriched diet induced increases in A β production. We found that the cholesterol-enriched diet did not change protein levels of full length A β PP, but increased significantly levels of C99 fragment, a cleavage product of A β PP by BACE-1. The cholesterol-enriched diet markedly increased intracellular deposition of immunopositive A β in olfactory bulb, which was largely restricted to endolysosomes of neurons. These data are consistent with reports that A β accumulates in the endolysosomal system in AD brain [15, 40], however it is not clear how endolysosomes participate in accelerated A β PP processing and amyloidogenesis.

A variety of findings suggest strongly that pathological changes in endolysosomes contribute to A β production and AD pathogenesis. A β PP and its cleavage products have been identified in clathrin-coated vesicles that are part of the endocytic pathway [80]. A β production was decreased in cultured cells that were stably transfected with an A β PP construct where the C-terminal endocytic targeting signal was removed [81, 82]. A β production was decreased in cells transfected with dominant negative dynamin, which prevents endocytosis [83]. BACE-1, a key enzyme for amyloidogenesis, is localized in endosomes and its activity is optimal at a pH of about 5.0 [84–86]. It was also reported that A β accumulated in endolysosomes of neurons from AD brain [15], abnormal endosomes were observed before extracellular A β was deposited in brain [17], and intraneuronal deposition of A β preceded extracellular deposition of A β [39]. Consistent with these results we showed here that the cholesterol-enriched diet affected both endolysosome function and A β generation. Although the sequence of events are not yet clear, it is likely that high levels

of cholesterol disrupt the function of endolysosomes and subsequently affect A β production [87]. It could be that receptor-mediated endocytosis of cholesterol promotes A β PP internalization and processing [22, 88–93]. It is possible that decreased lysosomal function prevents BACE-1 degradation, because BACE-1 is degraded via the lysosomal pathway [94]. It is also possible that the observed decreased specific activity of cathepsin D slows down the degradation of A β in lysosomes because cathepsin D has been implicated in the catabolism of A β [95–98]. However, it is unlikely that the observed endolysosome dysfunction results from A β overproduction because altered endolysosomes are not apparent in familial AD brain that exhibit extensive A β deposition [17]. Thus, our results suggest that increased levels of cholesterol accelerate amyloidogenesis by affecting endolysosomes.

Lastly, we examined endolysosome involvement in cholesterol-enriched diet induced increases in tau phosphorylation. Here we showed that the cholesterol-enriched diet increased protein levels and immunopositive staining of phosphorylated tau in olfactory bulbs. This confirms and extends our previous findings that high dietary cholesterol increases levels of phosphorylated tau in rabbit brain [26]. Although the underlying mechanisms for this are not yet clear, tau is degraded in the autophagy-lysosome system [41–43], and cholesterol storage in lysosomes of Niemann-Pick type C diseased brain induces lysosomal dysfunction and tau-pathology [44–46]. Those findings in combination with our observations of increased accumulations of phosphorylated tau in lysosomes of neurons suggest that elevated levels of cholesterol induce tau-pathology by affecting endolysosomal function of neurons. Together, our data strongly suggest that increased levels of circulating cholesterol play an important role in the pathogenesis of sporadic AD and that AD-like pathology occurs mechanistically because of changes to endolysosomes.

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Figure 1. Effects of cholesterol-enriched diet on endolysosome structure

(A) Schematic of a horizontal section of olfactory bulb; the blue square indicates a single glomeruli an expanded view of which is shown on the left. Glomeruli are roughly spherical bundles of dendritic processes; mitral cells (MC) and tufted cells (not shown) receive inputs from olfactory sensory neurons (OSN) and periglomerular cells (PG). (B) Cholesterol-enriched diet markedly increased immunoreactivity of EEA1 (red) in neurons (Nissl, green) of olfactory bulb glomeruli (bar = 20 μ m) and increased the sizes and numbers of endosomes (EEA1, red) as can be seen in the expanded images (bar = 10 μ m). (C) Cholesterol-enriched diet increased significantly ($p = 0.022$) fluorescence intensity of EEA1 in the glomerular layer of olfactory bulb. (D) Cholesterol-enriched diet significantly increased ($p = 0.0022$) protein levels of EEA1 in olfactory bulb. (Control diet, $n = 6$; Cholesterol diet, $n = 9$)

Figure 2. Effects of cholesterol-enriched diet on free cholesterol accumulation in endolysosomes
 (A) Cholesterol-enriched diet increased markedly the accumulation of free cholesterol (Filipin, blue) in endosomes (EEA1, red) of neurons (Nissl, green) in olfactory bulb glomeruli (bar = 20 μ m). (B) Cholesterol-enriched diet increased markedly the accumulation of free cholesterol (Filipin, blue) in lysosomes (LAMP2, red) of neurons (Nissl, green) in olfactory bulb glomeruli (bar = 20 μ m).

Figure 3. Effects of cholesterol-enriched diet on Apo B accumulation in endolysosomes
 (A) Cholesterol-enriched diet markedly increased immunoreactivity of Apo B (red) in neurons (Nissl, green) in olfactory bulb glomeruli (bar = 50 μ m). (B) Cholesterol-enriched diet markedly increased accumulation of Apo B (green) in endosomes (EEA1, red) in olfactory bulb glomerular layer (bar = 20 μ m). (C) Cholesterol-enriched diet markedly increased accumulation of Apo B (green) in lysosomes (LAMP2, red) in olfactory bulb glomeruli (bar = 20 μ m).

Figure 4. Effects of cholesterol-enriched diet on endolysosome function

(A) Cholesterol-enriched diet increased markedly cathepsin D-positive immunoreactivity (red) in neurons (Nissl, green) in olfactory bulb glomeruli (bar = 50 μ m). (B) Cholesterol-enriched diet increased significantly ($p = 0.017$) cathepsin D protein levels in olfactory bulb. (C) Cholesterol-enriched diet decreased significantly ($p = 0.037$) cathepsin D specific enzyme activity (ratio of total activity to cathepsin D protein level). (D) Cholesterol-enriched diet increased markedly acid phosphatase-positive immunoreactivity (red) in neurons (Nissl, green) in olfactory bulb glomeruli (bar = 50 μ m). (E) Cholesterol-enriched diet increased significantly ($p = 0.0011$) protein levels of acid phosphatase in olfactory bulb. (F) Cholesterol-enriched diet decreased significantly ($p = 0.005$) acid phosphatase specific enzyme activity (ratio of total activity to acid phosphatase protein levels). (Control diet, $n = 6$; Cholesterol diet, $n = 9$)

Figure 5. Effects of cholesterol-enriched diet on levels and distribution patterns of synaptophysin (A) Cholesterol-enriched diet changed the distribution pattern of synaptophysin-positive immunoreactivity in olfactory bulb glomeruli (bar = 50 μ m). (B) Cholesterol-enriched diet decreased significantly ($p = 0.0001$) fluorescence intensity of synaptophysin-positive immunoreactivity in glomeruli of olfactory bulb. (C) Cholesterol-enriched diet decreased significantly ($p = 0.0002$) protein levels of synaptophysin in olfactory bulb. (D) In olfactory bulbs from cholesterol-fed rabbits, synaptophysin was co-distributed with EEA1 (bar = 1 μ m). (E) In olfactory bulbs from cholesterol-fed rabbits, synaptophysin was co-distributed with LAMP2 (bar = 1 μ m).

Figure 6. Effects of cholesterol-enriched diet on intraneuronal deposition of Aβ

(A) Cholesterol-enriched diet increased intracellular deposition of Aβ in olfactory bulb when detected with 4G8 antibody. 10X, bar = 100 μm; 63X, bar = 20 μm. (B) 4G8-positive staining of Aβ was co-distributed with EEA1 in neurons (Nissl) of olfactory bulb (bar = 1 μm). (C) 4G8-positive staining of Aβ was co-distributed with LAMP2 in neurons (Nissl) of olfactory bulb (bar = 1 μm). (D) Cholesterol-enriched diet increased significantly ($p = 0.031$) deposition of Aβ₁₋₄₀ in neurons (Nissl stain, blue, bar = 20 μm). (E) Cholesterol-enriched diet increased significantly ($p = 0.025$) deposition of Aβ₁₋₄₂ in neurons (Nissl stain, blue, bar = 20 μm). (F) Cholesterol-enriched diet did not change ($p = 0.726$) protein levels of full length AβPP. Cholesterol-enriched diet increased significantly ($p = 0.0304$) levels of C99 fragments, but did not change significantly ($p = 0.810$) levels of C83 fragments. (Control diet, $n = 6$; Cholesterol diet, $n = 9$)

Figure 7. Effects of cholesterol-enriched diet on intraneuronal deposition of phosphorylated tau
 (A) Cholesterol-enriched diet increased significantly ($p = 0.017$) phosphorylated tau protein levels in brain. (B) Cholesterol-enriched diet increased markedly the deposition of phosphorylated tau (AT8) in neurons (Nissl stain, bar = 50 μ m). (C) In olfactory bulbs from cholesterol-fed rabbits, phosphorylated tau was co-distributed with lysosome marker LAMP2 (bar = 10 μ m). (Control diet, $n = 6$; Cholesterol diet, $n = 9$)