Published in final edited form as:

Life Sci. 2012 December 10; 91(23-24): 1159–1168. doi:10.1016/j.lfs.2012.04.039.

Endolysosome involvement in LDL cholesterol-induced Alzheimer's disease-like pathology in primary cultured neurons

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Abstract

Aims—Elevated levels of circulating cholesterol are extrinsic factors contributing to the pathogenesis of sporadic Alzheimer's disease (AD). We showed previously that rabbits fed a cholesterol-enriched diet exhibited blood-brain barrier (BBB) dysfunction, increased accumulation of Apolipoprotein B (ApoB) in brain neurons, and endolysosomes in brain had disturbed structures and functions. These effects were linked to increased amyloid beta ($A\beta$) production, increased tau-pathology, and disrupted synaptic integrity. Because pathological changes to endolysosomes represent a very early event in sporadic AD, we determined here the extent to which ApoB-containing LDL cholesterol altered the structure and function of endolysosomes and contributed to the development of AD-like pathology in primary cultured neurons.

Main methods—Cholesterol distribution and endolysosome morphology were determined histologically. Endolysosome pH was measured ratio-metrically with LysoSensor dye. Endolysosome enzyme activity was measured for acid phosphatase, cathepsin B and D, and BACE-1. AD-like pathologies, including increased production of amyloid beta ($A\beta$), increased tau-pathology, and disrupted synaptic integrity was determined using ELISA, immunoblotting, and immunostaining techniques.

Key findings—Treatment of neurons with ApoB-containing LDL cholesterol increased endolysosome accumulation of cholesterol, enlarged endolysosomes, and elevated endolysosome pH. In addition, ApoB-containing LDL cholesterol increased endolysosome accumulation of BACE-1, enhanced BACE-1 activity, increased $A\beta$ levels, increased levels of phosphorylated tau, and decreased levels of synaptophysin.

Significance—Our findings suggest strongly that alterations in the structure and function of endolysosomes plays a key role in the exhibition of pathological features of AD that result from neuronal exposure to ApoB-containing LDL cholesterol.

Keywords

ApoB; amyloid beta; BACE-1; tau; synaptophysin

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Introduction

Alzheimer's disease (AD) is a persistent and devastating dementing disorder of old age that has associated with it massive health care costs. AD is characterized clinically by progressive disturbances in memory, judgment, reasoning and olfaction, and pathologically by loss of synaptic integrity, increased levels of reactive oxygen species, amyloid plaques composed of amyloid beta (A β) protein and neuronal tangles composed of hyperphosphorylated tau (Blennow et al., 2006, Castellani et al., 2010, Hardy, 2009, Seabrook et al., 2007). Although a small percentage of AD cases are familial and genetically based, the vast majority (>90%) of AD cases are sporadic with unknown etiology; no effective treatments are available.

Altered cholesterol homeostasis has been linked to the pathogenesis of sporadic AD. People bearing the apolipoprotein E4 (ApoE4) allele, the strongest genetic risk factor for sporadic AD (Corder et al., 1993, Wisdom et al., 2011), have increased plasma levels of cholesterol (Corder, Saunders, 1993, Marzolo and Bu, 2009). In contrast, people bearing the ApoE2 allele, a protective factor against sporadic AD (Corder et al., 1996, Corder et al., 1994, Schachter et al., 1994), have lower levels of plasma cholesterol (Scuteri et al., 2001, Wisdom, Callahan, 2011). Moreover, independent of the ApoE gene, elevated levels of plasma cholesterol increases the risk of developing sporadic AD (Solomon et al., 2009). ApoB, the essential apolipoprotein transporting circulating cholesterol in peripheral tissue is not present in normal brain (Pitas et al., 1987) but is present in AD brain and is co-distributed with amyloid plaques and neuronal tangles (Namba et al., 1992, Takechi et al., 2009). Thus, peripherally derived ApoB-cholesterol rather than brain-derived ApoE-cholesterol might contribute more to the pathogenesis of sporadic AD.

Under normal conditions, where the blood-brain barrier (BBB) is intact, brain cholesterol is derived from astrocytes (Nieweg et al., 2009) and ApoE-cholesterol secreted by astrocytes is internalized by neurons through receptor-mediated endocytosis using low-density lipoprotein (LDL), LDL receptor-related protein-1 (LRP1), and apoE type-2 receptors. Once internalized, ApoE-cholesterol is transported to endolysosomes where cholesterol esters are hydrolyzed to free cholesterol and this free cholesterol is transported out of endolysosomes via Niemann-Pick type C proteins (Vance et al., 2006). However, when the BBB is leaky, as occurs in sporadic AD (Kalara, 1999, Ujiie et al., 2003, Zipser et al., 2007), circulating ApoB-cholesterol can enter brain where it is internalized and processed similarly to ApoE-cholesterol. Thus, increased brain levels of ApoB-cholesterol may result in increased endocytosis of cholesterol and increased accumulation of cholesterol in neurons where it might affect endolysosome structure and function - one of the very early pathological features of sporadic AD (Boland et al., 2008, Cataldo et al., 2000, Nixon, 2005, Tate and Mathews, 2006, Yuyama and Yanagisawa, 2009).

Using a well-developed rabbit model of sporadic AD that exhibits pathological hallmarks of AD including disrupted synaptic integrity, elevated levels of A β , and tau pathology we demonstrated that the BBB was leaky (Chen et al., 2008a), that levels of brain ApoB-cholesterol were increased and were abnormally accumulated in neuronal endolysosomes (Chen et al., 2010), that the structure and function of neuronal endolysosomes was disturbed (Chen, Wagener, 2010), and that the disturbed structure and function of endolysosomes was linked directly to pathological features of AD including disrupted synaptic integrity, amyloidosis, and tau pathology (Chen, Wagener, 2010). Thus, cholesterol coming from the systemic circulation might be altering neuronal endolysosome function and contributing to the pathogenesis of sporadic AD.

To further elucidate mechanisms whereby elevated levels of cholesterol contribute to AD pathogenesis, the present studies tested the hypothesis that ApoB-containing LDL cholesterol disturbs neuronal endolysosome structure and function and contributes directly to the development of AD-like pathology. The essential findings of the present studies include observations that treatment of primary cultured neurons with ApoB-containing LDL cholesterol increased cholesterol accumulation in neurons, enlarged endolysosomes, and elevated endolysosome pH. More importantly, we found that the altered structure and function of endolysosome was directly involved in elevated A β production, increased phosphorylation of tau, and disrupted synaptic integrity as evidenced by decreased levels of the presynaptic marker protein synaptophysin. Such findings suggest strongly that elevated levels of ApoB-containing cholesterol contribute to sporadic AD pathogenesis by disturbing the structure and function of endolysosomes.

Material and Methods

Primary cultures of rat cerebral cortical neurons

Primary cerebral cortical neurons were cultured from embryonic day 18 rats using a protocol approved by the University of North Dakota Animal Care and Use Committee adherent with the Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23). Briefly, pregnant Sprague Dawley rats (Harlan, WI) were sacrificed by asphyxiation with CO₂ and fetuses were removed, decapitated, and meninges-free cerebral cortex was isolated, trypsinized, and plated onto poly-D-lysine-coated glass-bottom 35-mm tissue culture dishes. Neurons were grown in Neurobasal™ medium with L-glutamine, penicillin/streptomycin/neomycin and B₂₇ supplement, and were maintained at 37°C and 5% CO₂ for 7 to 10 days at which time they were taken for experimentation. Typically the purity of the neuronal cultures was greater than 95% as determined by morphology and staining for neurons with NeuN or MAP-2 antibodies and for astrocytes with GFAP antibodies. Neurons were treated for 3 days with ApoB-containing LDL cholesterol (50 µg/ml, Kalein Biomedical) or PBS prior to being taken for experimentation. The effective concentration of ApoB-containing LDL cholesterol used here was determined experimentally from a series of concentration-dependent and time-dependent studies (data not shown).

Intraneuronal staining for cholesterol

Free cholesterol was stained with filipin (Sigma). Briefly, neurons were fixed with 10% formalin and incubated with PBS containing 1.5 mg/ml of glycine to quench the formalin. Fixed neurons were then incubated with filipin working solution for 2 hours at 4°C in the dark; the filipin stock solution was prepared by dissolving 5 mg filipin in 1 ml DMSO and a 100 µg/ml working solution was prepared by dissolving the stock solution 1:50 in PBS (pH = 7.2). Neurons stained with filipin for cholesterol were examined by fluorescence microscopy (Zeiss). To further determine the specificity of LDL cholesterol staining and its intracellular localization, we co-stained with lysoTracker dye (Invitrogen) and Dil-labeled LDL cholesterol (Kalein Biomeccidal). Neurons were loaded with Dil-labeled LDL (10 µg/ml) for 3 days, washed with PBS, and incubated with lysoTracker (100 nM, Invitrogen) for 30 min at 37 °C. Neurons were then examined by confocal microscopy (Olympus).

Immunostaining for endolysosomes, A β , BACE-1, phosphorylated tau and synaptophysin

Neurons were fixed with cold methanol (−20°C) for 10 min, washed with PBS, blocked with 5% goat serum, and incubated overnight at 4°C with primary antibodies targeting early endosome antigen-1 (EEA1, 1:500, rabbit polyclonal, Santa Cruz), lysosome associated membrane protein-1 (LAMP1, 1:500, rabbit polyclonal, Santa Cruz), A β (6E10, 1:500, mouse monoclonal, Signet), A β (4G8, 1:500, mouse monoclonal, Signet), C-terminal APP (1:500, Sigma), N-terminal APP (1:500, Milipore), BACE-1 (1: 500, mouse monoclonal,

Milipore), phosphorylated tau (AT8, 1:500, mouse monoclonal, Pierce) or synaptophysin (1:1000, mouse monoclonal, Sigma). After washing with PBS, neurons were incubated with corresponding fluorescence-conjugated secondary antibodies including Alexa 488-conjugated goat anti-mouse antibodies (Invitrogen) and Alexa 546-conjugated goat anti-rabbit antibodies (Invitrogen). Neurons were examined by confocal microscopy (Olympus). Controls for immunostaining specificity included staining neurons with primary antibodies without fluorescence-conjugated secondary antibodies (background controls), and staining neurons with only secondary antibodies; these controls helped eliminate auto-fluorescence in each channel and bleed-through (crossover) between channels.

Immunoblotting for EEA1, LAMP1, acid phosphatase, cathepsin B, cathepsin D, BACE-1, phosphorylated tau, and synaptophysin

Neurons were lysed with RIPA buffer (Pierce) containing 10 mM NaF, 1 mM Na₃VO₄ and Protease Inhibitor Cocktail (Sigma). After centrifugation (14,000 × g for 10 min at 4°C), supernatants were collected and protein concentrations were determined with a DC protein assay (Bio-Rad). Equal amount of proteins (10 µg) were separated by SDS-PAGE (12% gel), and following transfer polyvinylidene difluoride membranes (Millipore) were incubated overnight at 4°C with antibodies against early endosome antigen-1 (EEA1, 1:1000, rabbit polyclonal, Santa Cruz), lysosome associated membrane protein-1 (LAMP1, 1:1000, rabbit polyclonal, Santa Cruz), acid phosphatase (1:1000, mouse monoclonal, Abcam), cathepsin B (1:500, mouse monoclonal, Sigma), cathepsin D (1:1000, mouse monoclonal, Sigma), BACE-1 (1:1000, mouse monoclonal, Milipore), phosphorylated tau (AT8, 1:1000, mouse monoclonal, Pierce), Tau-5 (1:1000, Calbiochem) or synaptophysin (1:5000, mouse monoclonal, Sigma). GAPDH (1:1000, mouse monoclonal, 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).

Quantification of extracellular Aβ levels

Aβ levels were quantified using human/rat Aβ₁₋₄₀ and Aβ₁₋₄₂ ELISA kits as per the manufacturer's protocol (Wako). Media from cultured neurons were collected, diluted 1:4 with standard diluent buffer, and quantified by the calorimetric sandwich ELISA method. Each sample was measured in duplicate. Protein levels from neurons in each dish were determined by a DC protein assay (Bio-Rad). Aβ levels were normalized to total protein content in each sample.

Measurement of endolysosome enzyme activity

Acid phosphatase enzyme activity was determined using an Acid Phosphatase Assay kit (Sigma); a luminescence-based assay that uses 4-nitrophenyl phosphate as the substrate (Chen, Wagener, 2010). Enzyme activities of cathepsin D and cathepsin B were determined using two separate assay kits (BioVision); fluorescence-based assays that use preferred MCA-labeled substrates for cathepsin-D and cathepsin B (Chen, Wagener, 2010). Enzyme activity was expressed as optical density per 10 µg of protein, and specific enzyme activity was expressed as a ratio of enzyme activity to enzyme protein levels as determined by immunoblotting.

Measurement of BACE-1 enzyme activity

BACE-1 enzyme activity was determined with a BACE-1 activity kit (Calbiochem) according to the manufacturer's protocol. BACE-1 activity was measured using synthetic peptide substrates containing the BACE-1 cleavage site (MCA-Glu-Val-Lys-Met-Asp-Ala-

Glu-Phe-(Lys-DNP)-OH); 50 mM in reaction buffer consisting of 50 mM acetic acid pH 4.1 in 100 mM NaCl. Equal amounts of protein (50 µg) were used from sample lysates and fluorescence was measured using a fluorescent microplate reader with an excitation wavelength of 320 nm and an emission wavelength of 383 nm. As a control for specificity, BACE-1 activity was tested in the absence and the presence of the BACE-1 inhibitor H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Stat-Val-Ala-Glu-Phe-OH, (Calbiochem). BACE-1 activity will be expressed as fluorescent intensity per 50 µg of protein, and specific enzyme activity will be expressed as a ratio of enzyme activity to enzyme protein levels as determined by immunoblotting.

Quantitative RT-PCR measurement of BACE-1 mRNA

Total RNA was extracted with TRIzol-Reagent (Invitrogen) and levels were determined spectrophotometrically. Reverse transcription reactions were carried out using a SuperScript® III First-Strand Synthesis supermix (Invitrogen). The primers for BACE-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: f: 5'-TACACCCAGGGCAAGTGG-3' and r: 5'-GCCTGTGGATGACTGTGA-3' for BACE-1; f: 5'-TGCACCACCAACTGCTTAG-3' and r: 5'-GGATGCAGGGATGATGTTC-3' for GAPDH. Samples were run with our iCycler IQ™ Multicolor Real-Time PCR Detection System (Bio-Rad) that monitors fluorescence as a direct indication of PCR product (Chen et al., 2008b). All samples were run in triplicate and the averaged values were used for the relative quantification of gene expression. BACE-1 mRNA expression levels were calculated as the ratio of their expression compared with that of GAPDH.

Statistical analysis

All data were expressed as means ± SEM. Statistical significance was determined by two-tailed Student t-test. * P < 0.05 was considered to be statistically significant.

Results

Neuronal cholesterol is dependent on ApoE being synthesized *in situ* by astrocytes and then transported into neurons by receptor-mediated endocytosis by multiple receptor systems including LDLR, LRP and apoER2. These same receptors also control the uptake of ApoB-containing cholesterol, which is present in AD-brain, where and when there is a leaky BBB. To examine the extent to which exogenously added ApoB-containing LDL cholesterol is accumulated in endolysosomes, we first determined ApoB-containing LDL cholesterol uptake in cultured neurons. As determined by staining for free cholesterol with filipin, we found that LDL cholesterol (50 µg/ml) treatment for 3 days markedly altered the distribution profile of free cholesterol in primary cultured neurons; cholesterol was distributed near the plasma membrane in control neurons, but was accumulated inside neurons treated with ApoB-containing LDL cholesterol (Figure 1A). To determine further the intracellular distribution of accumulated LDL cholesterol, we treated neurons with fluorescence-labeled LDL cholesterol (Dil-LDL) and found that Dil-LDL co-distributed with endolysosomes as identified with lysoTracker (Figure 1B). These findings suggest that elevated levels of ApoB-containing LDL cholesterol enhance neuronal cholesterol endocytosis and result in increased accumulation of cholesterol in endolysosomes.

We next determined the extent to which ApoB-containing LDL cholesterol treatment affected the morphology of endolysosomes in primary cultured neurons. Using lysoTracker to identify endolysosomes in living neurons, we found that endolysosomes were markedly enlarged in neurons treated with ApoB-containing LDL cholesterol (Figure 2A). We found that endosomes as identified by EEA1 antibody staining and lysosomes as identified by LAMP1 antibody staining were relatively small and homogeneous in size and were

distributed fairly evenly in control neurons (Figure 2B). However, in neurons treated with ApoB-containing LDL cholesterol for 3 days, endosomes and lysosomes were markedly enlarged and clumped together (Figure 2B). Furthermore, ApoB-containing LDL cholesterol treatment significantly increased protein levels of EEA1 and LAMP1 (Figure 2C).

Next, we determined the extent to which ApoB-containing LDL cholesterol treatment affected endolysosome function. Because pH is of central importance to physiological functions of endolysosomes, we first measured endolysosome pH using LysoSensor; a dye that permits ratiometric assessment of pH changes in acidic organelles independent of dye concentration. We found that treatment of neurons with ApoB-containing LDL cholesterol increased significantly ($p < 0.001$) endolysosome pH (Figure 3A). Next we determined expression levels and activities of endolysosome enzymes because of their pH dependence. Treatment of neurons with ApoB-containing LDL cholesterol for 3 days increased significantly ($p < 0.01$) protein levels of all three endolysosome enzymes examined (Figure 3B); acid phosphatase, cathepsin B, and cathepsin D. On the other hand, the specific activities of cathepsin B and D but not acid phosphatase were decreased significantly (Figure 3C, D, E).

Disturbances in the structure and function of endolysosomes are early pathological features of sporadic AD, and our *in vivo* studies demonstrated that elevated levels of circulating cholesterol lead to the development of pathological hallmarks of AD including elevated A β production, tau pathology, and disrupted synaptic integrity (Chen, Wagener, 2010). Here, using primary cultured neurons we found that ApoB-containing LDL cholesterol treatment for 3 days increased significantly levels of both A β_{1-40} ($p < 0.01$) and A β_{1-42} ($p < 0.001$) (Figure 4A). Furthermore, ApoB-containing LDL cholesterol treatment increased A β (4G8, Figure 4B) and APP (N-terminal APP, Figure 4C) accumulation in endosomes (EEA1) and in lysosomes (LAMP1). We determined next the extent to which ApoB-containing LDL cholesterol affected expression levels, enzyme activity, and intracellular distribution of beta-site APP-cleaving enzyme 1 (BACE-1), the rate-limiting enzyme in A β production. We found that ApoB-containing LDL cholesterol treatment for 3 days did not affect mRNA or protein levels of BACE-1 (Figure 5A, B). However, ApoB-containing LDL cholesterol enhanced significantly ($p < 0.05$) the specific activity of BACE-1 (Figure 5C) and increased markedly the accumulation of BACE-1 in endosomes (Figure 5D) and in lysosomes (Figure 5E). ApoB-containing LDL cholesterol treatment for 3 days increased significantly ($p < 0.05$) protein levels of phosphorylated Tau as compared with total Tau probed with Tau-5 antibody (Figure 6A) and increased markedly the accumulation of phosphorylated Tau in endosomes (Figure 6B) and to a lesser extent in lysosomes (Figure 6C). ApoB-containing LDL cholesterol treatment for 3 days decreased significantly ($p < 0.01$) protein levels of synaptophysin (Figure 7A) and increased markedly accumulation of synaptophysin in endosomes (Figure 7B) and to a lesser extent in lysosomes (Figure 7C).

Discussion

Several lines of evidence suggest strongly that elevated levels of circulating cholesterol is an extrinsic factor that contributes to the pathogenesis of sporadic AD (Chen, Wagener, 2010, Crisby et al., 2004, Reiss et al., 2004, Sparks, 2008, Sparks et al., 2000). In humans, elevated plasma levels of cholesterol during mid-life increases the risk of developing AD later in life (Solomon, Kivipelto, 2009). People carrying the ApoE4 allele have an increased risk of developing AD (Corder, Saunders, 1993, Wisdom, Callahan, 2011) and have elevated levels of plasma cholesterol (Corder, Saunders, 1993, Marzolo and Bu, 2009). On the other hand, people carrying the ApoE2 allele have decreased incidence of AD (Corder, Lannfelt, 1996, Corder, Saunders, 1994, Schachter, Faure-Delanef, 1994) and have lower levels of plasma cholesterol (Dallongeville et al., 1992). Results from animal studies support the link

between diets enriched in cholesterol and pathological features of AD (Chen, Wagener, 2010, Ghribi et al., 2006, Granholm et al., 2008, Sparks et al., 1994, Thirumangalakudi et al., 2008). Rabbits fed a diet enriched in cholesterol develop pathological features resembling sporadic AD including disruption of the BBB, ApoB accumulation in endolysosomes, structural and functional changes to endolysosomes, disrupted synaptic integrity, increased A β levels, and tau pathology (Chen, Wagener, 2010). Here we found that primary cultured neurons treated with ApoB-containing LDL cholesterol exhibited increased cholesterol accumulation in endolysosomes, enlarged endolysosomes, elevated endolysosome pH, enhanced production of A β , increased levels of phosphorylated tau, and decreased levels of the presynaptic protein synaptophysin. Thus, endolysosome structure and function might be linked mechanistically to the development of AD induced by elevated levels of circulating cholesterol.

Brain *in situ* synthesized ApoE-cholesterol is up-taken by neurons via receptor-mediated endocytosis – this includes internalization of lipoproteins bound to receptors, lipoprotein receptor transport to endolysosomes, hydrolysis of bound to free cholesterol, and cholesterol transport to cellular compartments via Niemann-Pick type C proteins (Maxfield and Tabas, 2005, Sleat et al., 2004, Vance, Karten, 2006). ApoB, the main apolipoprotein that transports cholesterol to peripheral tissues, is not present in normal brain (Pitas, Boyles, 1987) but is present in AD brain (Namba, Tsuchiya, 1992, Takechi, Galloway, 2009). Thus, when the BBB is leaky, as occurs in AD, circulating ApoB-containing cholesterol enters brain and is transported to neuronal endolysosomes via the same mechanisms for ApoE cholesterol. This notion is consistent with our *in vivo* findings in a cholesterol-fed rabbit model of sporadic AD and our current *in vitro* findings.

Neurons are long-lived post-mitotic cells that possess an elaborate endolysosome system for quality control, and altered structure and function of neuronal endolysosomes are early pathological features of AD (Boland, Kumar, 2008, Tate and Mathews, 2006). Endosome enlargement is present in brains of AD patients and non-demented patients with early signs of AD, in Down's syndrome individuals, in patients bearing the ApoE4 allele (Arriagada et al., 2007, Cataldo et al., 2004), and it precedes extracellular deposition of A β (Cataldo, Peterhoff, 2000). Lysosome abnormalities occur as well in AD and include increased numbers and co-distribution in amyloid plaques (Boland, Kumar, 2008, Cataldo et al., 1994, Cataldo et al., 1990, Nixon, 2007). We showed here that internalized ApoB-containing LDL cholesterol increased intra-neuronal accumulation of cholesterol and enlarged markedly neuronal endolysosomes. Our observations are consistent with findings that neuronal endosomes are increased in size when loaded with cholesterol (Cossec et al., 2010) and that this might be due to the accumulated LDL-cholesterol exceeding the ability of neurons to export cholesterol out of endolysosomes or suppressing cholesterol export from endolysosomes. Consistent with this notion are findings that lysosome accumulation of cholesterol in Niemann-Pick type C diseased brain is associated with alterations in the structure of lysosomes (Bi and Liao, 2007, Distl et al., 2003, Liao et al., 2007).

Endolysosome pH is central to enzymatic activity in endolysosomes. We demonstrated here that ApoB containing LDL cholesterol treatment of neurons increased significantly endolysosome pH and decreased enzyme activity of acid phosphatase, cathepsin B, and cathepsin D, all of which are pH-sensitive enzymes. Although, it is not known how increased LDL cholesterol affects endolysosome pH, it is likely that it suppresses the activity of vacuolar H⁺-ATPase, an enzyme that maintains a low pH by pumping H⁺ into endolysosomes (Cox et al., 2007).

A rather large body of literature suggests strongly that AD pathogenesis and A β production is linked to endolysosome structure and function. A β PP and its cleavage products are

present in clathrin-coated vesicles (Ferreira et al., 1993, Harris and Milton, 2010). A β production is decreased when the C-terminal endocytic targeting signal of A β PP was removed (Perez et al., 1999, Soriano et al., 1999). A β production is decreased when endocytosis is prevented (Chyung and Selkoe, 2003). BACE-1, the rate-limiting enzyme in the production of A β , is located in endosomes (Rajendran et al., 2008, Shimizu et al., 2008, Vassar et al., 1999). A β is accumulated in endolysosomes of neurons from AD brain (Cataldo, Petanceska, 2004). Here, we showed that neurons treated with ApoB-containing LDL cholesterol exhibited increased A β production, increased accumulation of BACE-1 in endolysosomes, and increased BACE-1 enzyme activity. Because the activity of BACE-1 is optimal at a pH $\approx$ 5 (Rajendran, Schneider, 2008, Shimizu, Tosaki, 2008, Vassar, Bennett, 1999) and is degraded in lysosomes at a pH $\approx$ 4 (Koh et al., 2005), LDL cholesterol-induced increases in endolysosome pH could have resulted in decreased degradation of BACE-1 and increased accumulation and activity of BACE-1 in endolysosomes, thus increasing A β production. On the other hand, A β can be degraded by lysosome cathepsin D whose activity is decreased with increasing pH (Hamazaki, 1996, Higaki et al., 1996, Lador et al., 1994, Saftig et al., 1996) thus contributing to increased levels of A β . γ -secretase too is present in endolysosomes (Frykman et al., 2010, Pasternak et al., 2003, Refolo et al., 1995, Vetrivel et al., 2004), is pH sensitive (Pasternak, Bagshaw, 2003, Pasternak et al., 2004), and is degraded in lysosomes (He et al., 2007), however it was not studied here.

Endolysosomes have been implicated in the development of another pathological hallmark of AD, tau-pathology. Tau is degraded by cathepsin D in autophagosomes-lysosomes (Hamano et al., 2008, Kenessey et al., 1997, Oyama et al., 1998, Wang et al., 2009), and increased levels of cholesterol in lysosomes causes lysosome dysfunction and tau-pathology in brains of patients with Niemann-Pick type C disease (Bi and Liao, 2007, Bu et al., 2002, Distl, Treiber-Held, 2003, Liao, Yao, 2007, Sawamura et al., 2001, Vance, 2006). Consistent with our previous *in vivo* findings (Chen, Wagener, 2010), here we found that ApoB containing LDL cholesterol decreased cathepsin D activity, and increased endolysosome accumulation of phosphorylated tau. These findings provide further evidence connecting increased levels of LDL cholesterol with tau pathology and AD.

Endolysosomes recycle synaptic proteins (Blumstein et al., 2001, Kuromi and Kidokoro, 1998, Murthy and Stevens, 1998), lysosome dysfunction has been linked to synaptic pathology in AD brain (Bahr and Bendiske, 2002, Callahan et al., 1999), and inhibition of lysosome acidification with chloroquine results in synaptic dysfunction and synaptic loss (Bendiske and Bahr, 2003, Bendiske et al., 2002, Kanju et al., 2007). Consistent with our previous *in vivo* findings (Chen, Wagener, 2010), we demonstrated here that ApoB containing LDL cholesterol, increased endolysosome accumulation of presynaptic protein synaptophysin, and decreased total protein levels of synaptophysin. Thus, endolysosomes are implicated strongly in the development of disrupted synaptic integrity, a pathological hallmark of AD that correlates best with dementia (Selkoe, 2002, Terry et al., 1991).

Conclusion

Taken together, data from others and us suggest strongly that elevated levels of ApoB-containing cholesterol and changes to the structure and function of neuronal endolysosomes contribute to the pathogenesis of AD.

Acknowledgments

This work was supported by P20RR17699 from the National Center for Research Resources, a component of the NIH.

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Figure 1.

LDL cholesterol increased cholesterol accumulation in neurons. (A) Neurons treated with LDL cholesterol (50 $\mu\text{g}/\text{ml}$) for 3 days exhibited altered free cholesterol (filipin staining) distribution; cholesterol was distributed near the plasma membrane in control neurons and in the cytosol of LDL cholesterol treated neurons. Bar = 10 μm . (B) DiI-labeled LDL (red) co-distributed with endolysosomes (green, lysoTracker). Bar = 10 μm .

Figure 2.

LDL cholesterol altered the structure of endolysosomes. (A) LDL treatment (50 $\mu\text{g/ml}$) for 3 day increased the size of endolysosomes (LysoTracker) in primary cultured cortical neurons. Bar = 10 μm . (B) LDL cholesterol treatment for 3 days markedly altered the structure of endosomes (EEA1) and lysosomes (LAMP1); the sizes of endosomes and lysosomes were relatively small, homogeneous, and evenly distributed in control neurons, whereas they were markedly enlarged and clumped together in neurons treated with LDL cholesterol. (C) LDL cholesterol (50 $\mu\text{g/ml}$) treatment for 3 days increased significantly protein levels of EEA1 and LAMP1. GAPDH was used as a loading control (n = 4; ** p < 0.01).

Figure 3. LDL cholesterol disturbed the function of endolysosomes. (A) Endolysosome pH was measured ratio-metrically with a LysoSensor dye. LDL cholesterol treatment (50 μ g/ml) for 3 days increased significantly neuronal endolysosome pH ($n = 8$; *** $p < 0.001$). (B) LDL cholesterol treatment (50 μ g/ml) for 3 days increased protein levels of acid phosphatase, cathepsin B, and cathepsin D ($n = 4$; ** $p < 0.01$; *** $p < 0.001$). GAPDH was used as a loading control. (C, D, E) LDL cholesterol treatment (50 μ g/ml) decreased specific enzyme activities of cathepsin B, cathepsin D, and acid phosphatase, when compared with controls ($n = 6$; * $p < 0.05$; *** $p < 0.001$).

Figure 4.

LDL cholesterol increased Aβ production. (A) LDL cholesterol treatment (50 μg/ml) for 3 days increased significantly levels of Aβ₁₋₄₀ and Aβ₁₋₄₂, when compared with controls (n = 8; **p < 0.01; ***p < 0.001). (B) LDL cholesterol treatment (50 μg/ml) for 3 days increased the co-distribution of Aβ (4G8) with endosomes (EEA1) and lysosomes (LAMP1). Bar = 10 μm. (C) LDL cholesterol treatment (50 μg/ml) for 3 days increased the co-distribution of APP (N-terminal APP) with endosomes (EEA1) and lysosomes (LAMP1). Bar = 10 μm

Figure 5.

LDL cholesterol increased BACE-1 activity. (A) LDL cholesterol treatment did not change significantly mRNA levels of BACE-1 ($n = 8$). (B) LDL cholesterol treatment did not change significantly protein level of BACE1 ($n = 6$). (C) LDL cholesterol treatment increased significantly BACE1 specific enzyme activity ($n = 8$; $*p < 0.05$). (D) LDL cholesterol treatment increased co-distribution of BACE1 with endosomes (EEA1). Bar = 10 μ m. (E) LDL cholesterol treatment increased the co-distribution of BACE1 with lysosomes (LAMP1). Bar = 10 μ m.

Figure 6.

LDL cholesterol altered the expression and distribution of phosphorylated tau. (A) LDL cholesterol treatment (50 $\mu\text{g/ml}$) for 3 days increased significantly the ratio of phosphorylated tau (AT8) to total tau as probed with tau-5 antibody ($n = 6$, *** $p < 0.05$). (B) LDL cholesterol treatment (50 $\mu\text{g/ml}$) for 3 days increased the co-distribution of phosphorylated tau (AT8) with endosomes (EEA1). Bar = 10 μm . (C) LDL cholesterol treatment (50 $\mu\text{g/ml}$) for 3 days increased the co-distribution of phosphorylated tau (AT8) with lysosomes (LAMP1). Bar = 10 μm .

Figure 7.

LDL cholesterol altered the expression and distribution of synaptophysin. (A) LDL cholesterol treatment (50 μg/ml) for 3 days decreased protein levels of synaptophysin (n = 6; **p < 0.01). (B) LDL cholesterol treatment (50 μg/ml) for 3 days increased the co-distribution of synaptophysin with endosomes (EEA1). Bar = 10 μm. (C) LDL cholesterol treatment (50 μg/ml) for 3 days increased the co-distribution of synaptophysin with lysosomes (LAMP1). Bar = 10 μm.