Free Radical Biology and Medicine 75 (2014) 48–59

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Oxidized LDL lipids increase β-amyloid production by SH-SY5Y cells through glutathione depletion and lipid raft formation

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ARTICLE INFO

Article history:
Received 31 March 2014
Received in revised form
17 June 2014
Accepted 9 July 2014
Available online 15 July 2014

Keywords:
OxLDL
GSH
Redox
Aging
BACE1
Lipid raft
Low-density lipoprotein
Cholesterol
Lipid oxidation
Free radicals

ABSTRACT

Elevated total cholesterol in midlife has been associated with increased risk of dementia in later life. We have previously shown that low-density lipoprotein (LDL) is more oxidized in the plasma of dementia patients, although total cholesterol levels are not different from those of age-matched controls. β-Amyloid (Aβ) peptide, which accumulates in Alzheimer disease (AD), arises from the initial cleavage of amyloid precursor protein by β-secretase-1 (BACE1). BACE1 activity is regulated by membrane lipids and raft formation. Given the evidence for altered lipid metabolism in AD, we have investigated a mechanism for enhanced Aβ production by SH-SY5Y neuronal-like cells exposed to oxidized LDL (oxLDL). The viability of SH-SY5Y cells exposed to 4 μg oxLDL and 25 μM 27-hydroxycholesterol (27OH-C) was decreased significantly. Lipids, but not proteins, extracted from oxLDL were more cytotoxic than oxLDL. In parallel, the ratio of reduced glutathione (GSH) to oxidized glutathione was decreased at sublethal concentrations of lipids extracted from native and oxLDL. GSH loss was associated with an increase in acid sphingomyelinase (ASMase) activity and lipid raft formation, which could be inhibited by the ASMase inhibitor desipramine. 27OH-C and total lipids from LDL and oxLDL independently increased Aβ production by SH-SY5Y cells, and Aβ accumulation could be inhibited by desipramine and by *N*-acetylcysteine. These data suggest a mechanism whereby oxLDL lipids and 27OH-C can drive Aβ production by GSH depletion, ASMase-driven membrane remodeling, and BACE1 activation in neuronal cells.

Alzheimer's disease (AD)[1] is the most common neurodegenerative disease and is characterized by progressive decline in cognitive performance with loss of memory, orientation, and judgment [1]. Loss of synapses and cholinergic neurons, accumulation of extracellular Aβ plaques, and intraneuronal neurofibrillary tangles of hyperphosphorylated tau are major hallmarks of the AD brain [1].

Aβ is derived from amyloid precursor protein (APP), an abundant type I membrane protein that is a substrate for at least three proteolytic ("secretase") activities designated α, β, and γ. The major proteolytic pathway, undergone by ~95% of the APP in neurons, is α–γ, i.e., APP is first cleaved by an α-secretase within the Aβ region and subsequently by the γ-secretase. The second proteolytic pathway, which leads to the formation of Aβ, is the β–γ pathway. In this case, APP is first cleaved by the β-secretase β-site amyloid cleaving enzyme (BACE1), a membrane-spanning aspartic protease, with further processing by the γ-secretase to produce the 4-kDa Aβ peptide [2]; the initial cleavage of APP by BACE1 is the rate-limiting step for Aβ production in AD brains.

Lipids are key regulators of brain function and have been increasingly implicated in neurodegenerative disorders, including AD; a major risk factor for late-onset AD is the ε4 allelic variant of ApoE, which encodes a protein involved in cholesterol metabolism and lipid transport [3]. Importantly, a variety of genes have been

Abbreviations: AD, Alzheimer disease; Aβ, β-amyloid; APP, amyloid precursor protein; ASMase, acid sphingomyelinase; BACE, β-secretase β-site amyloid cleaving enzyme; BBB, blood–brain barrier; BCA, bicinchoninic acid; BHT, butylated hydroxytoluene; BSO, buthionine sulfoximine; CSF, cerebrospinal fluid; CTB, cholera toxin B; E-64, proteinase inhibitor E-64; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ESI–MS, electrospray ionization mass spectrometry; ELISA, enzyme-linked immunosorbent assay; GCL, glutamate–cysteinyl ligase; GSSG, oxidized glutathione; GSH, glutathione; LDL, low-density lipoprotein; MDA, malondialdehyde; NAC, *N*-acetylcysteine; 27OH-C, 27-hydroxycholesterol; oxLDL, oxidized low-density lipoprotein; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 1% Tween 20; PVDF, polyvinylidene fluoride; SM, sphingomyelin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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http://dx.doi.org/10.1016/j.freeradbiomed.2014.07.012

recently linked to late-onset AD through genome-wide association studies and are directly or indirectly connected to lipid metabolism or cellular membrane dynamics [4,5].

In support of a role for lipids in AD, Chan et al. [6] analyzed the prefrontal cortex, entorhinal cortex, and cerebellum of late-onset AD patients and found an elevation of diacylglycerol and sphingolipids in the prefrontal cortex of AD patients. Enrichment of lysobisphosphatidic acid, sphingomyelin, the ganglioside GM3, and cholesterol esters was observed in the affected entorhinal cortex but no change in lipids occurred within the cerebellum. Most recently, a fingerprint of 10 plasma lipids that defines AD presence has been identified in older adults [7]; however, it is unknown whether they play a mechanistic role in disease development or progression.

Membrane lipids are involved in the trafficking and/or activity of the key membrane-bound proteins controlling Aβ levels, including APP, BACE1, and presenilins; first, the regulation of BACE1 activity is determined by its access to APP, which is in turn lipid-dependent and involves lipid raft formation; three groups of lipids that stimulate proteolytic activity of BACE are (1) neutral glycosphingolipids (cerebrosides), (2) anionic glycerophospholipids, and (3) cholesterol [8]; second, γ-secretase activity is regulated by membrane levels of cholesterol and sphingomyelin (SM) [9]. The enzymes most strongly implicated in regulation of these lipids are HMG-CoA reductase and sphingomyelinase. Third, lipids such as ganglioside GM1 modulate the pathogenic potential of Aβ by affecting its propensity to aggregate [10]. Cholesterol is highly enriched in the brain and is a major constituent of normal neuronal membranes. Whereas brain cholesterol homeostasis is regulated through de novo synthesis and is normally segregated from peripheral circulation owing to the impermeability of the blood–brain barrier (BBB) [11,12], oxysterols such as 27-hydroxycholesterol (27OH-C) are known to cross the blood–brain barrier more readily than cholesterol [13,14]. Moreover, 27OH-C has been shown to accumulate in AD brains [15] and aberrant lipid homeostasis is implicated in AD [16]. In contrast, the related 24OH-C is produced within the brain and is found at raised levels in plasma from AD patients

Not only cholesterol is transported by lipoproteins; for example, SMs are transported by low-density lipoproteins (LDL) [17] and high-density lipoproteins carry carotenoids and ApoA1, and lower concentrations of either are independent risk factors for AD [18,19]. We have previously shown in a population of older adults that oxidized LDL is increased in AD patients with vascular risk factors compared to age-matched control subjects [11]. Levels of protein carbonyls on LDL were associated with cognitive impairment, although total cholesterol levels were not different from those of age-matched controls [11].

Exposure of a variety of cells to oxLDL can trigger intracellular oxidative stress and glutathione (GSH) depletion [20]. GSH is consumed in the detoxification of lipid peroxidation products, catalyzed by GSH peroxidase. According to the extent of lipid peroxide burden, GSH loss is usually transient and after several hours is restored through de novo synthesis. Lipid raft formation is also accelerated during oxidative stress, as the activity of sphingomyelinase, an enzyme responsible for cleaving membrane sphingolipids, is increased by GSH depletion [21]. This illustrates a relationship between extracellular lipoprotein oxidation, intracellular redox imbalance, and membrane remodeling, although the nature of the molecules in oxLDL that mediate this effect is unknown.

Others have shown recently that 27OH-C increases BACE1 levels in hippocampal organotypic slices from adult rabbits [22] and in human SH-SY5Y neuroblastoma cells [23]. These studies have also demonstrated that Gadd153 and nuclear factor-κB regulate BACE1 expression in a concerted fashion in response to 27OH-C [24]. These studies provide evidence that BACE1 expression is important for Aβ formation; however, they do not examine the effect of 27OH-C on the pathway, e.g., in membrane reorganization that is required for BACE1 activation, nor has the (patho) physiological source of 27OH-C been investigated.

Recognizing the association of systemic hypercholesterolemia in midlife with AD in later life, we have therefore investigated the hypothesis that systemically oxidized 27OH-C and oxidized lipids in general derived from oxLDL in plasma deplete GSH in neuronal cells, promote lipid raft formation through enhanced sphingomyelinase activity, and increase Aβ formation. Our approach to identifying novel pathways for reducing toxic Aβ formation through improved understanding of 27OH-C effects and production upstream of Aβ is an important strategy in the search for new therapeutic targets.

Materials and methods

Cell culture

The neuroblastoma cell line SH-SY5Y from the American Type Culture Collection was maintained in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum, 1% nonessential amino acids, and 200 U/ml penicillin and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. All reagents were from Sigma unless otherwise stated.

Isolation, modification, and characterization of LDL

Blood for lipoprotein isolation was drawn from the antecubital vein of normolipemic healthy volunteers into EDTA after 12 h of fasting. Control subject LDL for in vitro oxidation and analysis was from healthy controls recruited at Aston University who showed no evidence of cognitive impairment. Ethical approval was obtained from the Aston University ethics committee. None of the volunteers were taking antioxidant supplements.

AD subjects were recruited from the Unit of Cognitive Frailty, Neurology Outpatient Clinic, Cologne, Germany, after diagnosis of AD using NINCDS–ADRDA criteria [7]. Informed consent was obtained from the patients or their caregivers according to severity of disease by the local ethics committee.

LDL was isolated from plasma by density-gradient ultracentrifugation with potassium bromide (KBr) as previously described [25,26]. To remove residual KBr and EDTA before starting the in vitro oxidation reaction, the LDL was passed through a PD-10 column (GE Healthcare, Little Chalfont, UK). OxLDL was prepared by incubating with 10 μM $CuSO_4$ for 1 h at 37 °C and then the reaction was stopped by adding 10 °μM EDTA as previously described [26]. Copper and EDTA were then removed by passing the samples through PD-10 columns (GE Healthcare) against phosphate-buffered saline (PBS). To sterilize and remove aggregates, both LDL and oxLDL were filtered through a 0.22-μm filter (Millipore), stored under nitrogen in the dark at 4 °C, and used within 2 weeks of preparation. To ensure the LDL did not undergo further oxidation, the MDA concentration was measured as thiobarbituric acid-reactive substances. MDA concentration was not affected significantly (2.5 ± 0.1 versus 2.6 ± 0.08 nmol MDA/mg for LDL and 3.8 versus 3.9 ± 0.6 nmol MDA/mg for oxLDL) after storage. The final protein concentration was determined by bicinchoninic acid assay (BCA assay) against a bovine serum albumin standard and the amounts of LDL used in experiments are described by protein content. The purity and charge of LDL and oxLDL were evaluated by examination of electrophoretic migration in a 1% agarose gel. 8-Isoprostane F2α levels in LDL and oxLDL were measured by ELISA (Cayman Chemicals, Ann Arbor, MI, USA).