

Published in final edited form as:

Mech Ageing Dev. 2008 November ; 129(11): 649–655. doi:10.1016/j.mad.2008.09.002.

Regulation of β -amyloid levels in the brain of cholesterol-fed rabbit, a model system for sporadic Alzheimer's disease

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Abstract

Accumulation of β -amyloid ($A\beta$) peptide in the brain is a major hallmark of Alzheimer's disease (AD). Hypercholesterolemia is a risk factor for AD and has been shown by laboratory studies to cause $A\beta$ accumulation. $A\beta$ levels in the brain are governed by its generation from amyloid precursor protein by β -secretase (BACE1), degradation by the insulin degrading enzyme (IDE), clearance from the brain by the low density lipoprotein receptor-related protein (LRP-1), and transport from circulation into the brain by receptor for advanced glycation end products (RAGE). However, the mechanisms by which hypercholesterolemia causes $A\beta$ accumulation in the brain and contributes to the pathogenesis of AD are still to be determined. In the present study, we determined the extent to which hypercholesterolemia-induced $A\beta$ accumulation is associated with alterations in BACE1, IDE, LRP-1, and RAGE expression levels. We show that hypercholesterolemia increases $A\beta$ production, an effect that is associated with increased levels of BACE1 and RAGE and reduced levels of IDE and LRP-1. These results suggest that reducing $A\beta$ accumulation in the brain may require strategies that combine reduction of generation and transport of $A\beta$ in addition to acceleration of degradation and clearance of this peptide.

Keywords

$A\beta$; BACE1; Cholesterol; IDE; LRP-1

1. Introduction

Alzheimer's disease (AD) is a major health threat affecting approximately 5% of all individuals over 65 years of age and more than 35% of those over 80 (Brookmeyer et al., 1998; Sjogren et al., 2006). The underlying causes of AD in most cases are unknown. Epidemiological (Jick et al., 2000; Wolozin, 2004), animal (Refolo et al., 2000; Fassbender et al., 2001), and cellular (Racchi et al., 1997; Galbete et al., 2000) studies suggest that disturbances in cholesterol homeostasis may contribute to the etiology of AD by increasing beta-amyloid ($A\beta$) generation. There is also evidence showing that cholesterol co-localizes with fibrillar $A\beta$ in the amyloid plaques of transgenic mice (Burns et al., 2003). The first indication of a connection between

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cholesterol and the accumulation of A β plaques has been demonstrated in rabbits (Sparks et al., 1994). However, the mechanisms by which cholesterol causes A β accumulation and contributes to the pathogenesis of AD are still to be determined.

A β is generated from β -amyloid precursor protein (APP) through sequential cleavage by membrane proteases referred to as β - and γ -secretases. The β -secretase, BACE1, first cleaves APP to generate a C-terminus fragment (β -CTF), an immediate substrate for γ -secretase (Busciglio et al., 1993; Haass and Selkoe, 1993), which further cleaves β -CTF to yield A β .

The insulin degrading enzyme (IDE), also known as insulin protease, insulysin, or insulinase (Duckworth et al., 1979; Song et al., 2003) is a 110-kDa thiol zinc metalloendopeptidase located in cytosol, peroxisomes, endosomes, and on the cell surface (Goldfine et al., 1984; Seta and Roth, 1997; Duckworth et al., 1998; Vekrellis et al., 2000). This peptide cleaves numerous proteins that share a propensity to form β -pleated sheet-rich amyloid fibrils including insulin, glucagon, atrial natriuretic factor, transforming growth factor- α , β -endorphin, amylin, APP intracellular domain (AICD), and A β (Duckworth et al., 1998; Selkoe, 2001) with poor substrate selectivity and specificity. IDE was also the first protease to be implicated in the proteolytic degradation of A β (Kurochkin and Goto, 1994). IDE gene was mapped to chromosome 10q23–q25, which made it a candidate gene for the Alzheimer's disease-6 locus (Affholter et al., 1990; Espinosa et al., 1991).

The low density lipoprotein receptor-related protein (LRP-1) is a member of the LDL receptor family. This receptor functions as a multi-functional scavenger and signaling receptor, a transporter, and a metabolizer of cholesterol and apolipoprotein E (apoE)-containing lipoproteins (Herz and Marschang, 2003). The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor which belongs to immunoglobulin superfamily of cell surface molecules. It binds a wide range of molecules including the products of nonenzymatic glycoxidation (advanced glycation end products, or AGEs), A β , proinflammatory cytokine-like mediators, and a DNA-binding protein amphoterin (Yan et al., 2000). RAGE and LRP-1 bind a wide range of ligands including apoE, α_2 -macroglobulin, amyloid precursor protein, and A β (Zlokovic, 2004). While transport of A β from the blood into the brain is mediated by RAGE (Deane et al., 2003, 2004), A β transport from the brain into the blood is mediated by LRP-1 (Shibata et al., 2000). The net flux of A β into or out of the brain depends upon the density and activity of these two receptors (Donahue et al., 2006).

In the present study, we determined the extent to which A β accumulation following hypercholesterolemia alters BACE1, IDE, LRP-1, and RAGE, proteins that are involved respectively in the production, degradation, clearance from the brain, and the transport from the systemic circulation into the brain of A β .

2. Materials and methods

2.1. Experimental design

New Zealand white male rabbits (1.5–2 years old and 3–5 kg) were assigned randomly to group 1 (n=6) fed with the normal chow and group 2 (n=6) fed with 2% (w:w) cholesterol (Harlan Teklad Global Diets, Madison, WI) in chow for 12 weeks and then euthanized. At necropsy, animals were perfused with Dulbecco's phosphate-buffered saline at 37 °C and brains were promptly removed and cut to yield two symmetrical hemispheres; one hemisphere for immunohistochemistry and one for Western blot and ELISA analyses.

2.2. Quantification of A β levels by ELISA

A β levels were quantified in the cortex and hippocampus of control and cholesterol-fed rabbits by ELISA using the Biosource kit as per the manufacturer's protocol. Briefly, to measure the

amount of A β 1–40 and A β 1–42, the wet mass of the brain tissue (100 mg) was homogenized thoroughly with 8X mass of cold 5 M guanidine HCl/50mM Tris HCl. The homogenates were mixed for 3–4 hours at room temperature. The samples were diluted with cold reaction buffer (Dulbecco's phosphate buffered saline with 5% BSA and 0.03% Tween-20 supplemented with 1x protease inhibitor cocktail) and centrifuged at 16,000 \times g for 20 minutes at 4°C. The supernatant was decanted, stored in ice until use, diluted at least 1:2 with standard diluent buffer, and quantified by calorimetric sandwich ELISA kits. For aggregated A β , the wet mass of the brain tissue (100 mg) was homogenized thoroughly with 10X volume of tissue extraction buffer (25 mM Tris-HCl and 150 mM NaCl, supplemented with protease inhibitors; pH 7.4). The samples were sonicated and centrifuged at 100,000 \times g for 1 hour at 4°C. The supernatant was collected and diluted at least 1:2 in standard diluent buffer prior to analysis using the aggregated A β ELISA kit from Biosource. The quantity of A β in each sample was measured in duplicates. Protein concentrations of all samples were determined by standard BCA assay (Pierce). A β levels were normalized to total protein content in the samples.

2.3. Western blot analysis

Cortex and hippocampus from control and cholesterol-fed rabbit brains were homogenized in T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors. Protein concentrations were determined with BCA protein assay. Proteins (10 μ g) were separated in SDS-PAGE gels followed by transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) and incubation with antibodies to BACE1 (1:100, Chemicon International, Temecula, CA), RAGE (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), IDE (1:100, Chemicon International, Temecula, CA), and LRP-1 (1:100, Biotools International, Saco, Maine). β -actin was used as a gel loading control. The blots were developed with enhanced chemiluminescence (Immun-star HRP chemiluminescent kit, Bio-Rad, Hercules, CA). Bands were visualized on a polyvinylidene difluoride membrane and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland). Quantification of results was performed by densitometry and the results analyzed as total integrated densitometric values (arbitrary units).

2.4. Confocal microscopy studies

Coronal frozen sections (14 μ m) at the level of hippocampus and cortex were air-dried, fixed with cold acetone, blocked with 5% normal goat serum, and reacted overnight at 4°C with antibodies to BACE1 (1:250), RAGE (1:250), IDE (anti-mouse, 1:250, Signet laboratories), LRP-1 (1:500), or 6E10 (1:250, Signet laboratories Inc., Dedham, MA). According to the manufacturer, the region of the A β peptide where the 6E10 epitope resides is completely homologous between human and rabbit. Sections were then washed and incubated with secondary antibodies conjugated to Alexa fluor-488 (Molecular Probes, Inc., Eugene, OR) for one hour at room temperature in the dark and washed with PBS. The sections were then incubated in autofluorescence eliminator reagent (Chemicon International, Temecula, CA) for 5 minutes, washed with 70% ethanol, and mounted with vectasheild containing DAPI (Vector laboratories, Inc., Burlingame, CA). The sections were visualized with a Zeiss LSM 510 META confocal system coupled to a Zeiss Axiophot 200 inverted epifluorescence microscope. Imaging was performed with a 63X oil immersion objective. The dimensions of all images displayed on the monitor were 1024 \times 1024 pixels. For single scanning detection of the fluorescence of Alexa-fluor(R) 488, a 488 nm Argon laser with wavelength filter, a 488/543 HFT and 545 NFT primary dichroic beam splitter, and a 505–530 nm band pass filter were used. The typical laser power at the sample was around 10% transmission. The DAPI cube was used for both viewing and taking images of the nucleus. The emission was a LP 420, which was combined with the mercury bulb to take the images.

2.5. Statistical Analysis

Quantitative data are presented as mean values \pm SEM. The significance of differences between the control and cholesterol-fed group was assessed using the Student's *t* test, with $p < 0.05$ considered statistically significant. Statistical analysis was performed with GraphPad Prism software 4.01.

3. Results

3.1. Cholesterol-enriched diet leads to increase in A β levels

ELISA assay was used to determine the extent to which cholesterol-enriched diets influence A β levels. We found that the total amount of guanidine-solubilized A β 1–40 and A β 1–42 and aggregated A β levels were significantly increased in the cortex of cholesterol-fed rabbits in comparison to control rabbits (Fig. 1A). In the cortex, A β 1–40 levels were 138 ± 5.6 pg/mg of protein in the control animals and 190 ± 37 pg/mg of protein in the cholesterol-fed rabbits, A β 1–42 levels were 55.7 ± 20 pg/mg of protein in controls and 162 ± 69 pg/mg of protein in the cholesterol-fed rabbits, and aggregated A β levels were 3.8 ± 0.7 ng/mg protein in controls and 5.6 ± 1.4 ng/mg protein in the cholesterol-fed rabbits (Fig. 1A). In the hippocampus, A β 1–40 levels were 480 ± 33 pg/mg of protein in the control animals and 550 ± 41 pg/mg of protein in the cholesterol-fed rabbits, A β 1–42 levels were 590 ± 80 pg/mg of protein in the controls and 860 ± 204 pg/mg of protein in the cholesterol-fed rabbits, and aggregated A β levels were 2.5 ± 0.2 ng/mg protein in the controls and 2.3 ± 0.2 ng/mg protein in the cholesterol-fed rabbits (Fig. 1B).

Immunohistochemistry and confocal microscopy using 6E10 antibody showed an increase in the immunoreactivity to A β antibody in both hippocampus and cortex of cholesterol-fed rabbits in comparison to control brains (Fig. 2). The immunostaining imaging in the hippocampus was carried out in CA1, CA2, and CA3. Results in CA1 and adjacent cortex are shown throughout the results section. A β staining in the CA1 appears as perinuclear dots and also in a filamentous pattern suggestive of axonal staining. In the cortex, most of A β staining is confined to small aggregates.

3.2. Increased A β levels are associated with increased BACE1 and RAGE

Levels of BACE1 and RAGE are significantly increased in the hippocampus and cortex of the cholesterol-fed rabbits in comparison to control levels. Western blot (Fig. 3a) and densitometric analysis (Fig. 3b) show an increase in BACE1 and RAGE in the hippocampus and cortex of cholesterol-fed rabbits compared to controls.

The increased levels of BACE1 and RAGE proteins is confirmed by confocal microscopy, where expression levels of BACE1 (Fig. 4a) and RAGE (Fig. 4b) increased dramatically in the hippocampus and moderately in the cortex of cholesterol-fed rabbits compared to control brains.

3.3. Increased A β levels are associated with decreased IDE and LRP-1 levels

Hypercholesterolemia caused a substantial decrease in levels of IDE and LRP-1 in the hippocampus and cortex as shown by Western blot (Fig. 5a) and densitometric analysis (Fig. 5b). Confocal studies further confirmed the changes in the expression of LRP-1 and IDE showing a reduced immunoreactivity to LRP-1 (Fig. 6) and IDE (Fig. 7) antibodies in both hippocampus and cortex of cholesterol-fed rabbits.

4. Discussion

The present study was designed to determine the extent to which hypercholesterolemia leads to alterations in levels of A β and proteins that are involved in the accumulation of this peptide. We demonstrated that hypercholesterolemia increases A β levels, an effect that is associated with changes in levels of proteins that are involved in production, degradation, clearance, and transport processes of A β . Our experiments were carried out in the cholesterol-fed rabbit as this model system displays pathologies consistent with certain features of the AD brain (Sparks, 2004).

Our data showed increased levels of all forms of A β (i.e. soluble A β 1–40, soluble A β 1–42 and aggregated A β) in the cortex of cholesterol-fed rabbit brains. In hippocampus, soluble A β 1–40 and soluble A β 1–42 were increased but not aggregated A β as shown by ELISA assay. Currently, the molecular mechanisms by which dietary cholesterol promotes A β peptide accumulation are not fully understood. The BACE1 and γ -secretase complexes, that sequentially cleave amyloid precursor protein to yield A β , are dependent on cholesterol metabolism. BACE1 appears to be particularly sensitive to membrane cholesterol content and is located in lipid rafts. BACE1 activity has also been demonstrated to be increased in lipid microdomains (Marlow et al., 2003; Cordy et al., 2003), and is upregulated in sporadic cases of Alzheimer's disease (Holsinger et al., 2002; Yang et al., 2003). Our previous studies showed co-localization of cholesterol with BACE1 in neurons and a dramatic increase in BACE1 levels and activity in brains from cholesterol-fed rabbits (Ghribi et al., 2006). The increased A β levels caused by hypercholesterolemia may be due, at least in part, to increased expression levels of BACE1 that we demonstrated in the present study. In Tg2576 transgenic mice however, although a cholesterol diet influenced APP metabolism, BACE1 levels were not different in these mice compared to mice on a control basal diet (George et al., 2004).

We also show that high cholesterol levels led to increased expression of RAGE, a receptor that transports A β from the blood to the brain. This suggests that increased transportation of A β from the circulation may be also involved in accumulation of this peptide in the brain. Plasma RAGE levels were decreased in hypercholesterolemic patients compared to normocholesterolemic subjects (Santilli et al., 2007). However, whether reduced RAGE levels in plasma were associated with increased levels of RAGE in the brain in the latter study remain to be determined.

We also demonstrated that levels of LRP-1, a receptor that clears A β out of the brain, are decreased in cholesterol-fed rabbits, suggesting that reduced clearance may play a role in the increased levels of A β in the brain. Several studies have provided strong support for the hypothesis that RAGE and LRP-1 perform opposite functions in transporting A β (Shibata et al., 2000; Deane et al., 2003). The fact that RAGE transports A β into the brain and LRP-1 transports A β out of the brain (Donahue et al., 2006) demonstrates that AD is associated with significant changes in the relative distribution of RAGE and LRP-1 receptors at the BBB in the human hippocampus.

Proteolysis of A β peptide by degrading enzymes is one way to lower peptide levels in the brain. There is ample evidence of the role of IDE in A β degradation. Kurochkin and Goto (1994) showed the first evidence that IDE is involved in A β degradation. IDE was found to be the main soluble β -amyloid degrading enzyme at neutral pH in rat brain and in normal human brain (Kurochkin and Goto, 1994). The highest β -amyloid degrading activity occurs at pH 4–5, indicating that IDE may act as an “amyloidase” that prevents the accumulation of amyloidogenic derivatives (McDermott and Gibson, 1997). In rat cortical cell system, IDE was shown to eliminate the neurotoxic effects of both A β 1–40 and A β 1–42 (Mukherjee et al., 2000; Hoyer, 2002). We demonstrate in the present work that the expression level of IDE is

decreased by hypercholesterolemia in both the hippocampus and cortex. Our data suggests that the reduction in IDE levels may lead to the accumulation of A β in the brain.

In summary, our results demonstrated that hypercholesterolemia-induced A β accumulation is associated with (1) an increase in levels of BACE1, an enzyme that initiates A β production; (2) a decrease in levels of IDE, an enzyme that degrades A β ; (3) a decrease in levels of LRP-1, a protein that transports A β from brain to blood; and (4) an increase in levels of RAGE, a receptor that transports A β from blood to brain. The changes in expression levels of these proteins may have contributed to the cholesterol-induced A β accumulation. The potential mechanisms by which high cholesterol diet may increase A β levels in cholesterol-fed rabbit brains are summarized in Fig 8. We propose that maintaining normal blood cholesterol levels would prevent changes in proteins that regulate A β levels, thus precluding A β accumulation and potentially protecting against Alzheimer's disease.

Acknowledgements

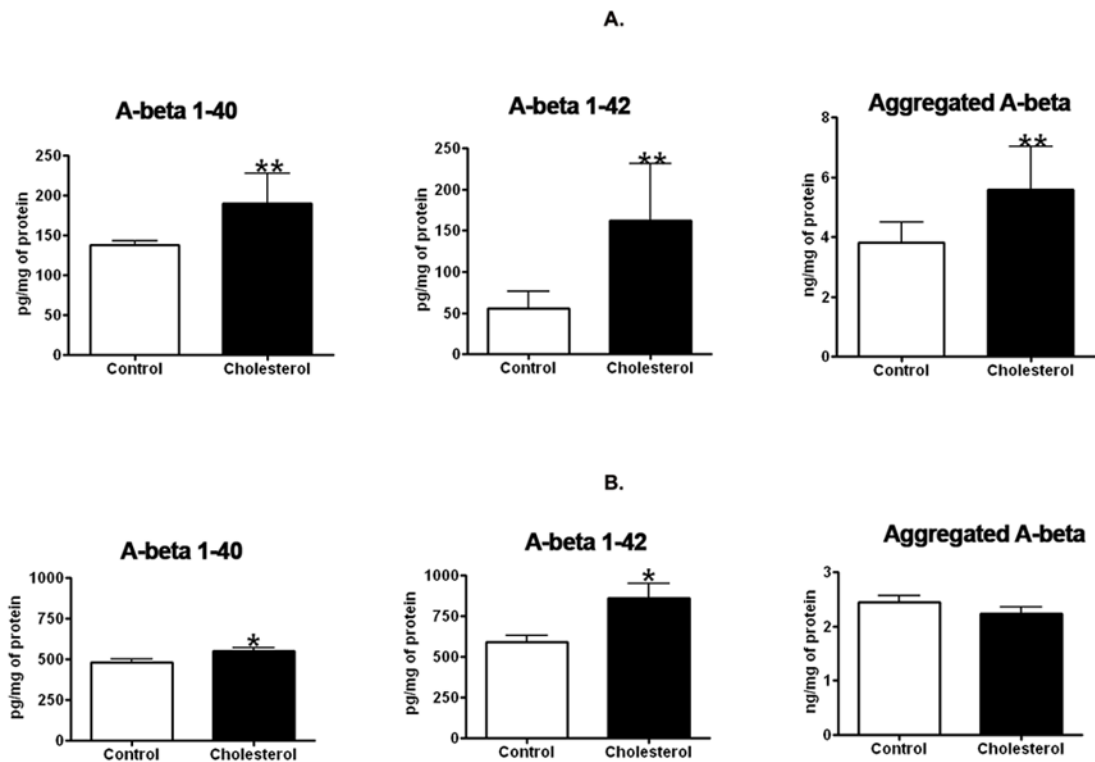
This work was supported by a Grant from the National Center for Research Resources (5P20RR017699, Centers of Biomedical Research Excellence).

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**Fig. 1.**

ELISA assay demonstrated that the 2% cholesterol-enriched diet significantly increased A β 40 and A β 42 in the cortex (A) and in the hippocampus (B); Aggregated A β levels were significantly increased in cortex and unchanged in hippocampus. Values from control (n=6) and cholesterol-fed (n=6) rabbits were expressed as mean \pm standard error: A β 1-40 (p = 0.005), A β 1-42 (p = 0.002), and aggregated A β (p = 0.004) in cortex. A β 1-40 (p = 0.0461) and A β 1-42 (p = 0.0431) in hippocampus tissue. *p < 0.05; **p < 0.01 (Student *t* test)

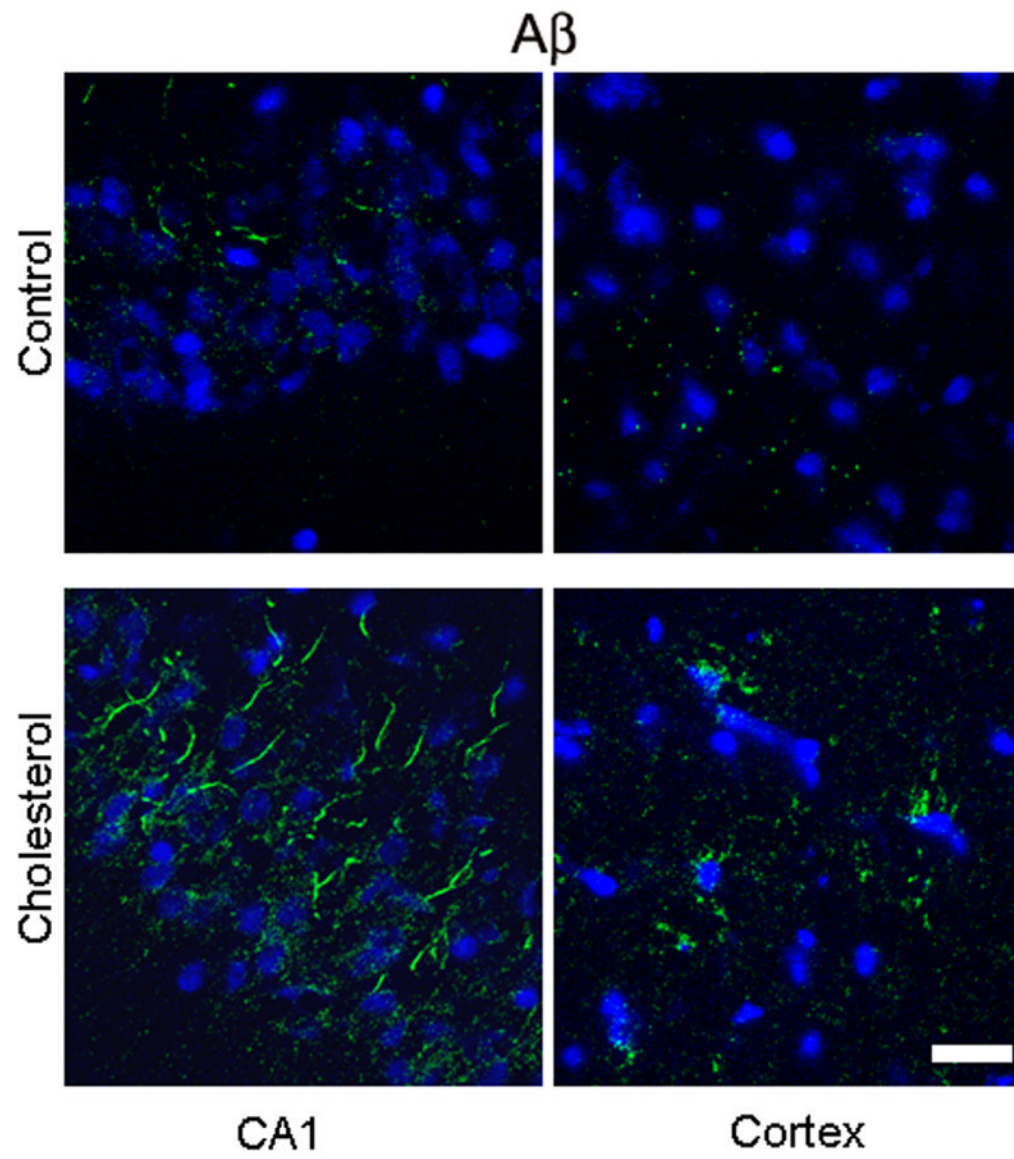


Fig. 2. Immunofluorescence staining showing increased expression of A β levels (green) as detected by 6E10 antibody in the CA1 region of the hippocampus and in the adjacent cortex of a cholesterol-fed rabbit brain compared to a control rabbit. DAPI (blue) was used to stain nuclei. Bar= 20 μ m.

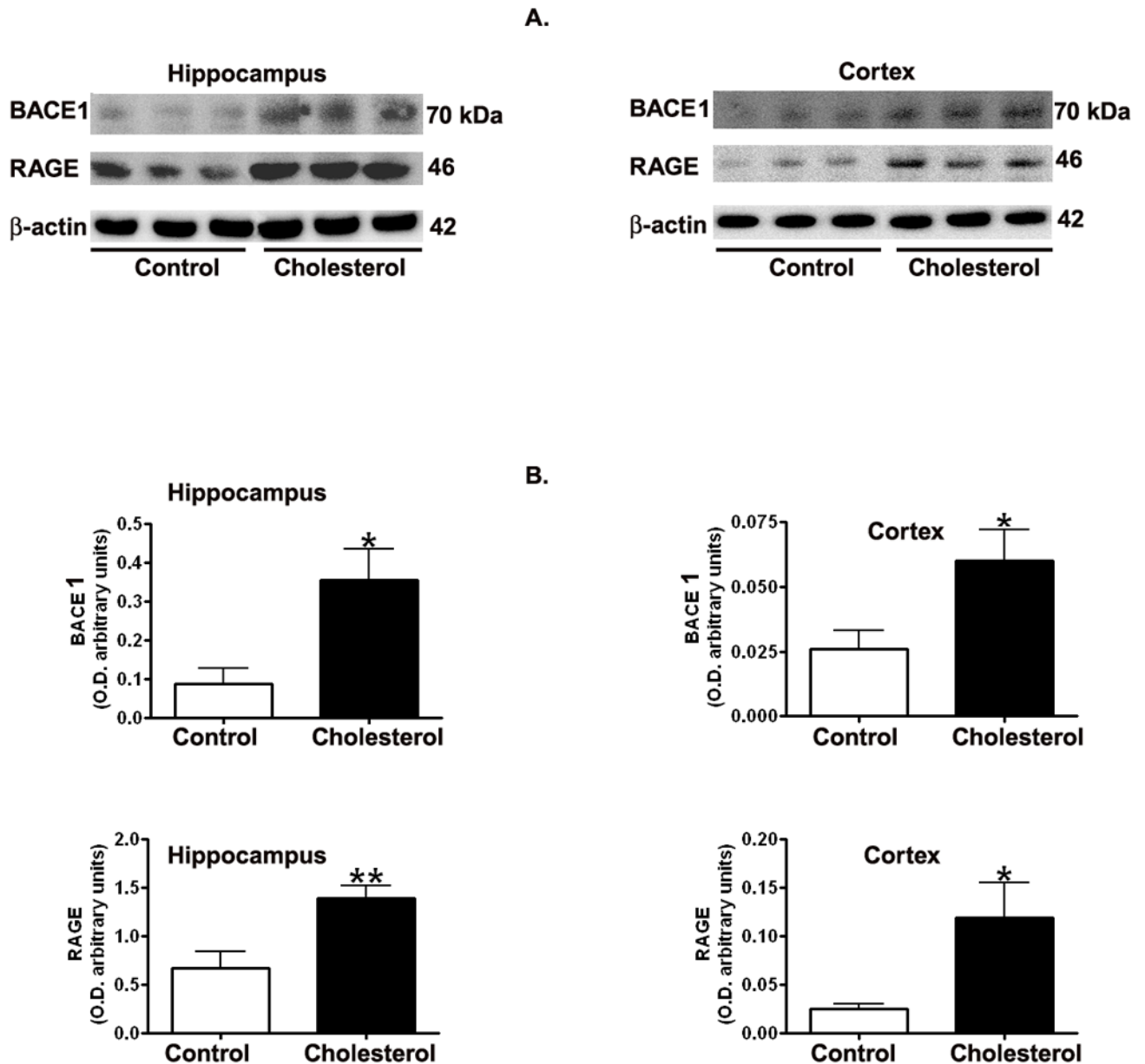
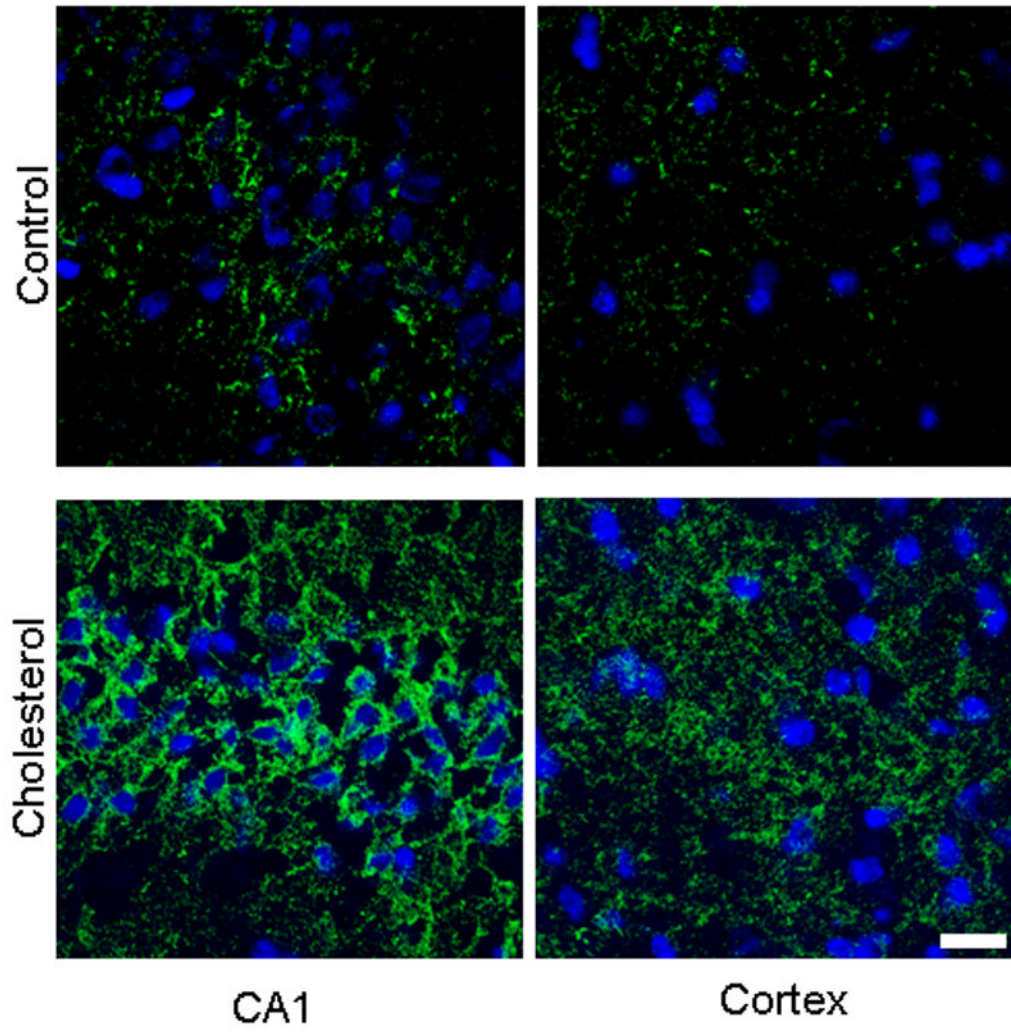
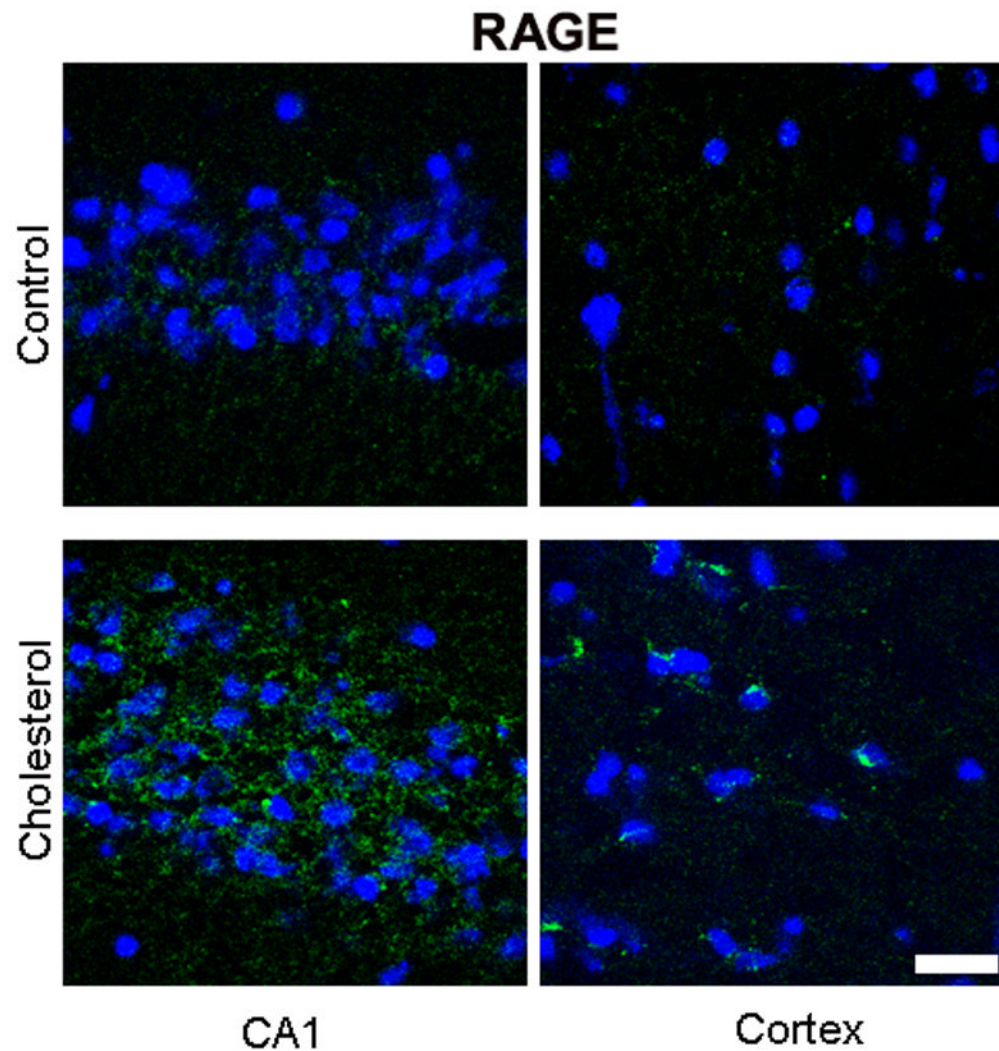


Fig. 3. Representative Western blots (A) and densitometric analysis (B) showing increased levels of BACE1 and RAGE in hippocampus and cortex from cholesterol-fed rabbits in comparison to control animals. * $p < 0.05$; ** $p < 0.01$ (Student *t* test).

BACE 1



**Fig. 4.**

(A) Immunofluorescence staining demonstrating a dramatic increase in expression of BACE1 (green) in the CA1 region of the hippocampus and adjacent cortex in sections from a cholesterol-fed rabbit brain compared to section from a control rabbit. DAPI (blue) was used as nuclear counterstain. Bar= 20 μ m.

(B) Immunostaining for RAGE is low in sections from CA1 region of hippocampus or adjacent cortex in a control rabbit. In section from CA1 and adjacent cortex from a cholesterol-fed rabbit, immunoreactivity to RAGE (green) is increased in hippocampus and in cortex. Nuclei were stained with DAPI (blue). Bar= 20 μ m

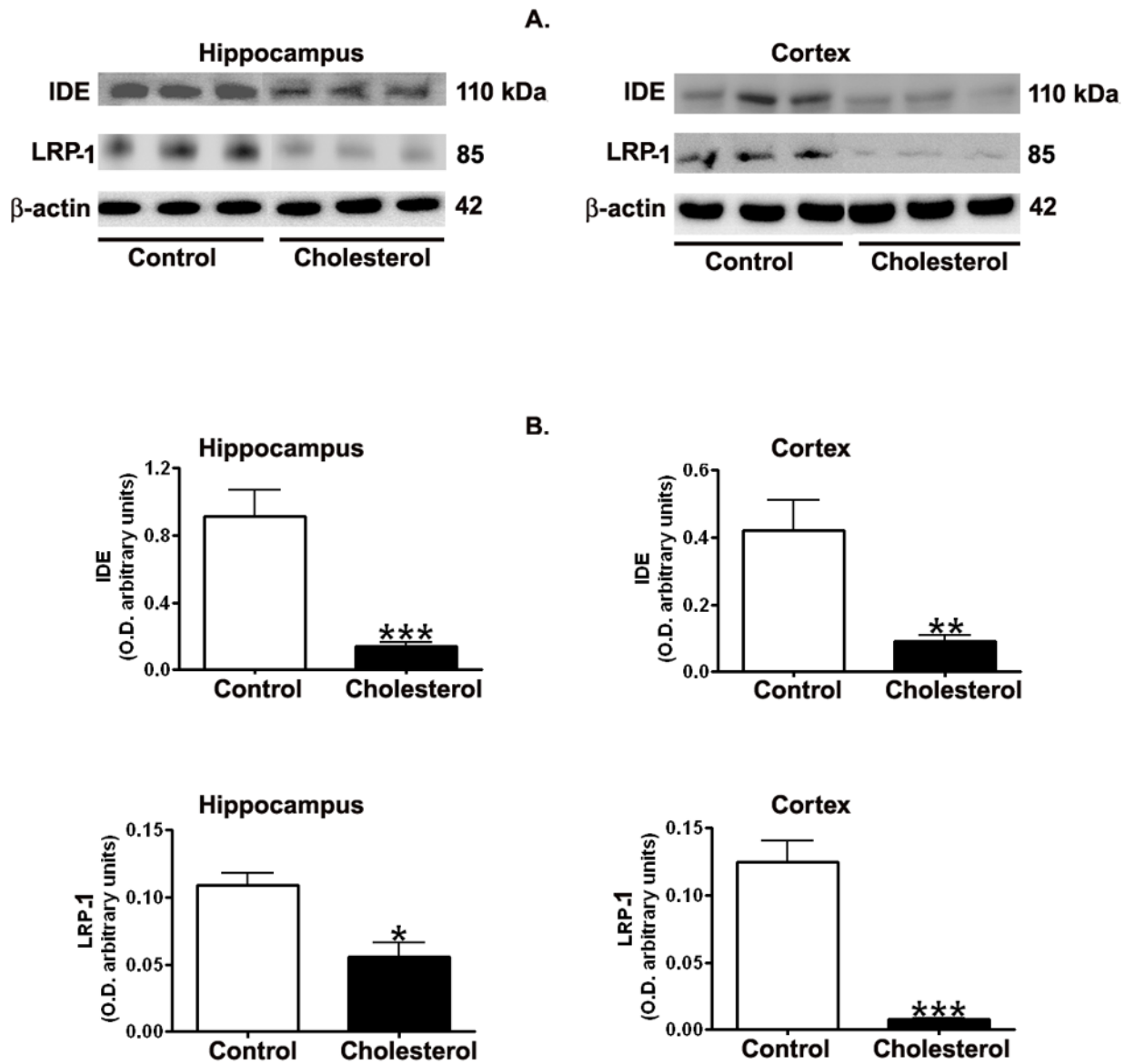


Fig. 5. Representative Western blots (A) and densitometric analysis (B) showing decreased levels of IDE and LRP-1 in hippocampus and cortex from cholesterol-fed rabbits in comparison to control animals. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student *t* test).

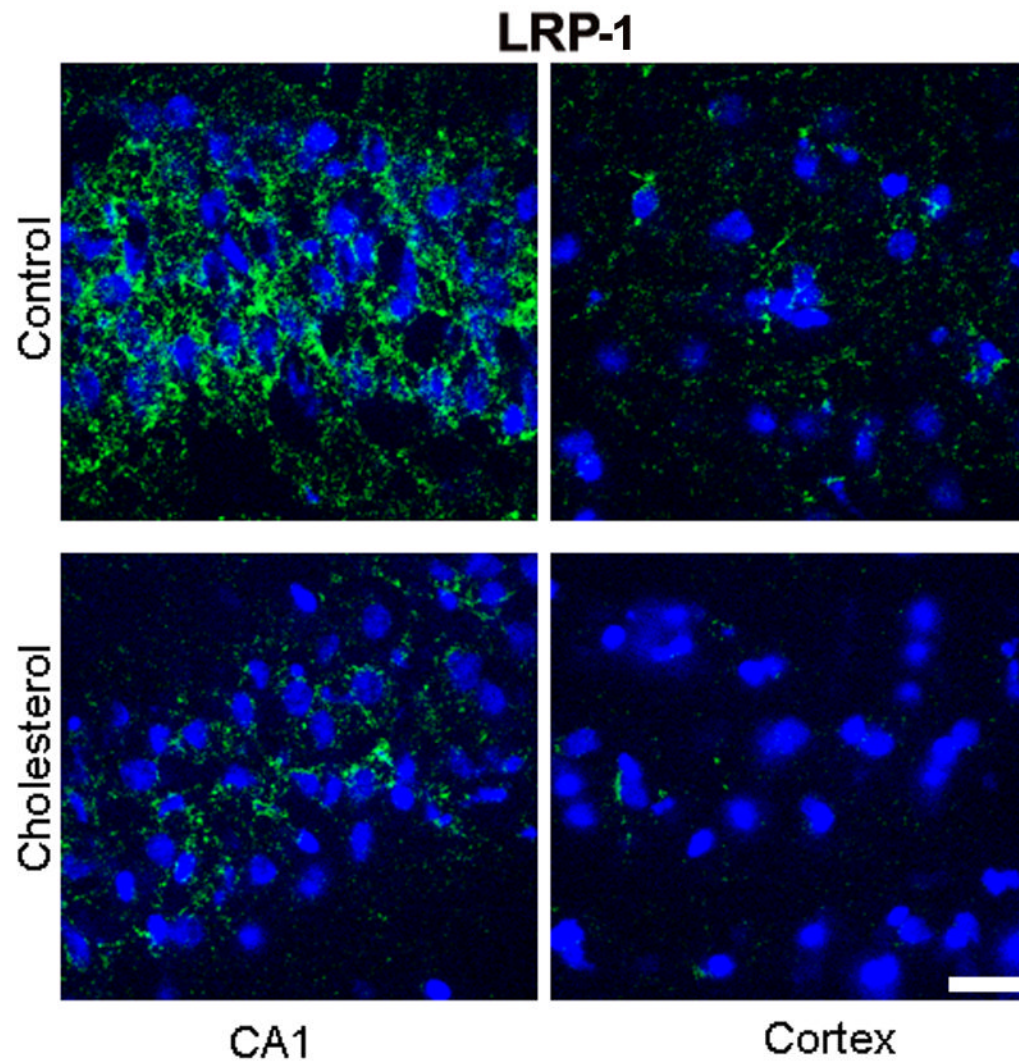


Fig. 6. Immunofluorescence staining for LRP-1 (green) showing a marked immunoreactivity in the CA1 region of the hippocampus and a lesser reactivity in cortex from a control rabbit. In a cholesterol-fed rabbit, the immunoreactivity to LRP-1 antibody is greatly reduced in both hippocampus and cortex. DAPI (blue) was used as nuclear marker. Bar=20 μ m.

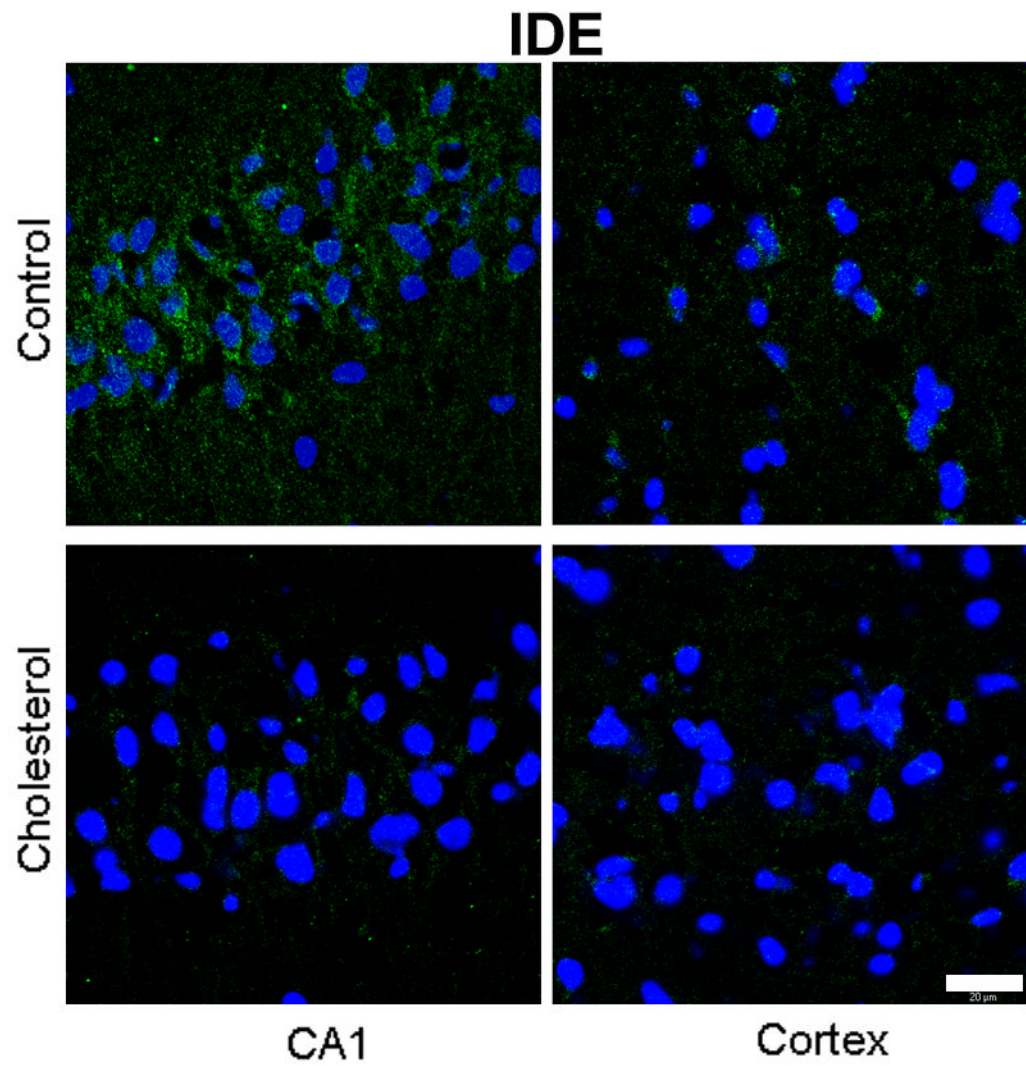


Fig. 7. Immunofluorescence staining for IDE (green) is higher in the CA1 region of the hippocampus than the cortex of a control rabbit. IDE immunostaining is greatly reduced in both hippocampus and cortex from a cholesterol-fed rabbit. DAPI (blue) was used as nuclear marker. Bar=20μm.

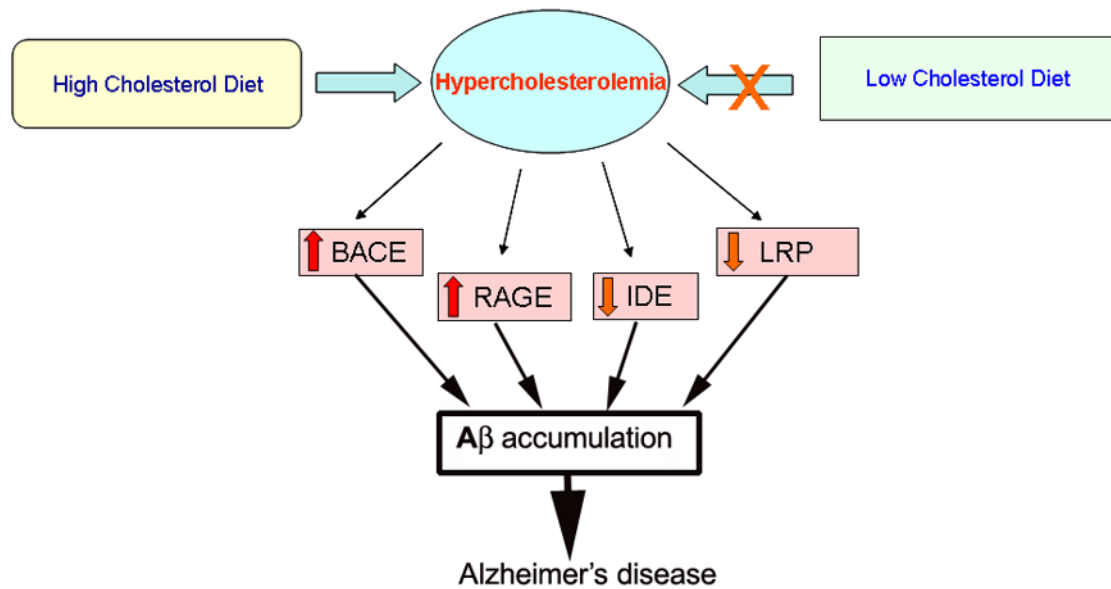


Fig. 8.

A schema showing the possible mechanisms involved in hypercholesterolemia-induced A β accumulation. A high cholesterol diet increases blood cholesterol levels (hypercholesterolemia) and leads to increased levels of BACE1 and RAGE, and decreased levels of IDE and LRP-1. Increased levels of BACE1 accelerate processing of APP to A β , and increased levels of RAGE result in increased transport of A β from the circulation into the brain. Decreased levels of IDE in the brain lead to a decreased cleavage of A β and decreased levels of LRP-1 result in reduced clearance of A β from the brain out to the circulation. All these changes triggered by hypercholesterolemia may contribute to increased levels of A β in the brain. Accumulation of A β potentially causes neurodegeneration and may ultimately contribute to the pathogenesis of Alzheimer's disease. Reducing hypercholesterolemia or maintaining normal levels of cholesterol in the blood would prevent changes in BACE1, RAGE, IDE and LRP-1, thereby precluding A β accumulation and subsequent deleterious effects.