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A High-Cholesterol Diet Increases 27-Hydroxycholesterol and Modifies Estrogen Receptor Expression and Neurodegeneration in Rabbit Hippocampus

Sylwia W. Brooks[a,b,*], **Ava C. Dykes**[c], and **Bernard G. Schreurs**[a,b]

[a]School of Medicine, West Virginia University, Morgantown, WV, USA

[b]Blanchette Rockefeller Neurosciences Institute, Morgantown, WV, USA

[c]Molecular Biology Core Facility, Centers for Disease Control and Prevention/National Institute for Occupational Safety and Health, Morgantown, WV, USA

Abstract

Hypercholesterolemia has been implicated in numerous health problems from cardiovascular disease to neurodegeneration. High serum cholesterol levels in midlife have been associated with an increased risk of developing Alzheimer's disease (AD) later in life which suggests that the pathways leading to AD pathology might be activated decades before the symptoms of the disease are detected. Cholesterol-fed animals, particularly cholesterol-fed rabbits, exhibit brain pathology similar to the changes found in brains of AD patients. Dietary cholesterol, which cannot pass the blood-brain barrier, is thought to influence central nervous system homeostasis by increased transport of its circulatory breakdown product, 27-hydroxycholesterol (27-OHC), into the brain. 27-OHC is an endogenous selective estrogen receptor modulator. Estrogen-mediated non-reproductive functions require estrogen receptors (ERs) and include modulation of mitochondrial function and structure, as well as regulation of synaptogenesis in the brain. ERs are located in brain areas affected early in AD pathogenesis, including the hippocampus. Here we report that increase in serum cholesterol, induced by feeding rabbits a high-cholesterol diet, is associated with higher levels of 27-OHC in the brain as well as increased levels of neurodegeneration in the hippocampus. Furthermore, these results are accompanied by changes in expression of ERs in the hippocampus as well as a decrease in hippocampal mitochondria. These findings provide an important insight into one of the possible mechanisms involved in the development of AD, and shed light on the processes that may antedate amyloid-β and tau phosphorylation changes currently hypothesized to cause AD symptomology and pathology.

Keywords

Alzheimer's disease; cholesterol; cholesterol-fed rabbit; estrogen receptors; ERα; ERβ; 27-hydroxycholesterol; mitochondria; oxysterol; PSD-95; synapse

*Correspondence to: Sylwia W. Brooks, West Virginia University, Blanchette Rockefeller Neurosciences Institute, 8 Medical Center Drive, Morgantown, WV 26506, USA. Tel.: +1 814 207 4188; Fax: +1 304 293 7536; smrowka@mix.wvu.edu.

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INTRODUCTION

As the population of Western countries ages, dementia is becoming a major health concern. Over the last few decades, numerous risk factors contributing to late onset Alzheimer's disease (AD) development and progression have been investigated. These include diabetes mellitus, hypertension, atherosclerosis, and hypercholesterolemia [1]. High serum cholesterol in midlife is associated with an increased risk of AD [2–4]. Moreover, obese individuals with high blood pressure and high cholesterol are six times more likely to develop AD than individuals without these risk factors [5]. Most existing research investigating the role of cholesterol in increasing the risk of AD has focused on how cholesterol affects amyloid-β protein precursor (AβPP) processing and amyloid-β protein (Aβ) clearance [6–9] even though recent findings in middle-aged neurologically healthy subjects indicate that Aβ accumulation might be a reactive process with little mechanistic connection to disease development [10].

A connection between cholesterol and Aβ aggregation characteristic of AD in an animal model was first shown in cholesterol-fed rabbits [11], and numerous experiments since have found that cholesterol increases Aβ in *in-vitro* and *in-vivo* models of AD [12–14]. Treatment of rodents with dietary cholesterol resulted in memory impairment characteristic of AD [15, 16], which we also have shown to be the case in the cholesterol-fed rabbit [17–21]. The inability of cholesterol to cross the blood-brain barrier and the fact that a high-cholesterol diet does not change cholesterol content in the rabbit brain [12, 17] suggest that serum cholesterol by itself does not increase AD risk. This assumption was validated in a study that reported memory impairment in cholesterol-fed mice but not in cholesterol-fed mutant mice lacking the enzyme CYP27A1 that metabolizes cholesterol into 27-OHC [16]. This study suggested that 27-OHC mediated the negative effects of cholesterol on memory. Additionally, increased levels of 27-OHC have been found in AD brains [22]; therefore, increased flux of this cholesterol metabolite into the brain could play an important role in the cascade of events that lead to the development of late-onset AD [23, 24].

A significant insight into a possible mechanism underlying the relationship between 27-OHC and AD came about with the discovery that 27-OHC is an endogenous selective estrogen receptor modulator (SERM) [25, 26]. SERMs are able to act as ligands for different isoforms of the estrogen receptor (ER), including ERα and ERβ, in a tissue-dependent agonist or antagonist manner [27–32].

The purpose of this study was to explore potential 27-OHC-mediated changes in the hippocampus of rabbits fed a high-cholesterol diet. We chose to focus on the hippocampus because it is an area of the brain important for learning and memory and affected early and profoundly in AD pathology [33]. We examined the levels of 27-OHC in hippocampal tissue of hypercholesterolemic and control animals as well as the expression of target ERs, mitochondria, and the postsynaptic marker PSD-95. We hypothesized that higher levels of cholesterol metabolism in the periphery would result in an increased flux of 27-OHC into the brain and that the surge of this SERM into the hippocampus would affect ER signaling and its downstream targets—mitochondria and synapses.

METHODS

Animals, diet, and tissue collection

The subjects were 32 New Zealand White male rabbits (*Oryctolagus cuniculus*) 3–4 months of age weighing approximately 2 kg upon arrival that were part of a larger study investigating the role of cholesterol on learning and memory [20]. Animals were housed individually with free access to food and water and maintained on a 12-h light–dark cycle. All the experiments followed guidelines of National Institutes of Health and were approved by West Virginia University Animal Care and Use Committee. Rabbits were assigned to two dietary groups: control or a high-cholesterol diet. Control diet animals received Purina 5326 chow, and the high cholesterol diet group received Purina 5326 plus 2% cholesterol chow (T.R. Last Co., Gibsonia, PA). All the rabbits were kept on their respective diets for a total of 11 weeks. At the conclusion of the study, animals were deeply anesthetized with a mixture of ketamine (500 mg/kg) and xylazine (10 mg/kg) and overdosed with Somnasol before transcardial perfusion with either artificial CSF solution or 0.5% paraformaldehyde. Brains were collected and flash frozen or post-fixed in 4% paraformaldehyde solution for further processing. Before euthanasia, transcardial blood was collected in EDTA tubes and centrifuged, and the serum was frozen and stored at –80°C until analysis.

Serum cholesterol levels

Total serum cholesterol levels were assessed at the end of the experiment using a colorimetric kit (BioAssay Systems, ECCH-100) following the manufacturer's instructions.

Neurodegeneration: Fluoro-Jade C staining

Fluoro-Jade C staining was performed based on previously published methods [34]. Brains stored in paraformaldehyde were cryoprotected in sucrose and formaldehyde solution then sectioned using a freezing microtome (Microm HM450). Coronal 50-μm sections of the dorsal hippocampus were mounted on gelatin-coated slides. Every 3rd section was collected, and an average of 15 sections from each subject were processed. Mounted sections were dried overnight and processed with a Fluoro-Jade C staining kit (Histo-Chem Inc). Slides were incubated in 70% ethanol and sodium hydroxide solution (9 : 1 ratio) for 5 min. They were then rinsed for 2 min in 70% ethanol followed by 2 min in distilled water, then incubated in 0.06% potassium permanganate solution for 10 min and again for 2 min in a distilled water rinse. Slides were then transferred to the Fluoro-Jade C solution with fluorescent DAPI counterstain and incubated for 10 min. This was followed by three 1-min distilled water rinses after which slides were placed on a slide warmer at 50°C for at least 5 min. Semi-dried slides were then cleared in xylenes for 2–3 min each and coverslipped using DPX (Sigma) as the mounting medium. Slides were imaged using a confocal microscope (LSM710, Carl Zeiss International). Digital images were collected and stained cells were counted using Image J software (NIH) by a researcher (SWB) blind to the experimental conditions.

Fluorescent antibody staining

Immunofluorescent staining was performed using manufacturer recommended protocols. Brains stored in paraformaldehyde were cryoprotected in sucrose and formaldehyde solution and then sectioned using a sliding microtome. Coronal 50-μm sections of dorsal hippocampus were mounted on gelled slides. Slides underwent an antigen retrieval protocol in 10 mM citrate buffer pH 6.0 for 40 min at 60°C. The sections were washed in phosphate buffered saline (PBS) plus Tween (pH 7.4) six times for 5 min each at room temperature. After incubation in 5% normal goat serum in PBS+tween for 2 h, sections were incubated in primary antibodies (mouse monoclonal anti-mitochondria (ab3298, Abcam) dilution 1 : 500, mouse monoclonal ERα (MA1-27107, ThermoScientific) dilution 1 : 200, rabbit polyclonal ERβ (PA5-16476, ThermoScientific) dilution 1 : 200) for 48 h at 4°C. Again, the sections were washed with PBS, 6 times for 5 min each before secondary antibody incubation (goat anti-rabbit IgG AF488 (ab150077, Abcam) dilution 1 : 1000, goat anti-mouse IgG AF488 (ab150113, Abcam) dilution 1 : 1000) for 4 h at room temperature. After a final series of rinses (PBS 6 times for 5 min each), slides were coverslipped using fluoromount G containing a DAPI counterstain. Slides were imaged on a LSM710 confocal microscope (Carl Zeiss International) and digital images processed as stated above.

27-OHC extraction from serum samples

Oxysterol extraction methods were adapted from Ahonen et al. [35]. Briefly, 1 mL of methyl t-butyl ether (MTBE) was added to a 150 μL of rabbit serum. Sample was vortexed for 1 min and centrifuged at 2000 rpm for 5 min. MTBE phase was filtered into a glass sample vial through a 0.2 μm syringe filter (Corning Incorporated) and evaporated to dryness. Samples were reconstituted in 100 μL of 5% ammonium acetate (50 mM, pH 4.5 with acetic acid): methanol:acetonitrile (1 : 3:6, v/v) and vortexed just before the analysis by the liquid chromatography-mass spectrometry (LC-MS) system.

27-OHC extraction from hippocampus

Methods of oxysterol extraction from the brain were developed based on Ahonen et al. [35]. Briefly, the intact left hippocampi were weighed and homogenized using ultrasonication, and 0.5 mL dichloromethane (DCM): methanol mixture (1 : 1, v/v) was added to tissue and sonicated on an ice bath for 1 min. Samples were centrifuged at 13200 rpm for 5 min, then supernatants removed and the procedure repeated. After the second extraction, supernatants were collected and evaporated to dryness. Immediately before analysis by LC-MS, the samples were reconstituted in 100 μL of methanol, centrifuged at 13200 rpm for 5 min and the supernatants collected into glass sample vials.

27-OHC levels: Liquid chromatography-mass spectrometry

Extracts from hippocampal tissue and rabbit's serum (1 μL) were injected into a Dionex UltiMate 3000RS Nano LC system (ThermoScientific) using a custom made 2.5 μm XBridge BEH C8 column, 300 μm × 150 mm (Waters) with a flow rate of 5 μL/min. 27-OHC was eluted using a gradient of 20% A (water with 5 mM of ammonium formate) and 80% B (100% methanol with 5 mM ammonium formate) for 10 min. The gradient was then transitioned from 80 to 99% B for 5 min, then maintained at 99% B for 10 min, followed by

a re-equilibration period when the column was returned to 80% B in 5 min and maintained to the end of the 35-min run. 27-OHC eluted at 20.56 min.

Western analysis

Protein levels were quantified using an automated Simple Western "Wes" system from ProteinSimple [36, 37]. It is a capillary electrophoresis assay that automatically loads, separates and detects proteins. Procedures were performed with the manufacturer's reagents following the manufacturer's protocol. Briefly, the lysate was mixed with fluorescent standard master mix and heated at 95°C for 5 min. The samples, blocking reagents, primary and secondary antibodies, and chemiluminescent substrate were dispensed into a microplate included in manufacturer's kit. The prepared microplate and the capillary cartridge were placed into a Wes instrument (ProteinSimple), and the program was run using default settings. During the electrophoresis, proteins were separated by size and immobilized into the capillary wall, and chemiluminescent signals were read by Compass software (version 2.6.5 ProteinSimple) which analyzed the area under the curve for each antibody. The area under the curve represents the signal intensity of the chemiluminescent reaction and is proportional to the amount of target protein in a respective capillary [38]. The data were analyzed by a blinded researcher (SWB) and normalized to β-actin levels (mouse monoclonal anti-β-actin (sc-47778, Santa Cruz Biotechnology, Inc.)).

Antibodies used for western analysis include: mouse monoclonal anti-mitochondria (ab3298, Abcam) dilution 1 : 50, mouse monoclonal ERα (MA1-27107, ThermoScientific) dilution 1 : 50, rabbit polyclonal ERβ (PA5-16476, ThermoScientific) dilution 1 : 50, mouse monoclonal PSD-95 (MA1-046, ThermoScientific) dilution 1 : 50.

Data analysis

All the data analysis was performed by a researcher blinded to experimental conditions (SWB). For statistical analysis one-way ANOVA was performed with significance set at $p < 0.05$. Data are presented as mean ± SEM.

RESULTS

Serum cholesterol and hippocampal 27-OHC

Feeding rabbits a high-cholesterol diet for 11 weeks significantly increased serum cholesterol. The mean serum cholesterol level for cholesterol-fed animals was significantly higher than for animals in the control group. The control group had a mean serum cholesterol level of 15.6 mg/dL whereas the cholesterol-fed group had a mean serum total cholesterol of 992.6 mg/dL as measured by a colorimetric kit [$F(1,8) = 61.11$, $p < 0.001$] (Fig. 1).

Spectrometric analysis of 27-OHC levels in serum as well as hippocampal tissue showed the level of 27-OHC in serum was significantly increased following a high-cholesterol diet, from 15.37 ng/mL in the control group to 34.71 ng/mL in the cholesterol-fed group [$F(1,8) = 47.61$, $p < 0.001$] (Fig. 2A). Similarly the hippocampal level of 27-OHC was significantly

increased in the cholesterol-fed group, from 6.82 ng/mg in the control group to 26.94 ng/mg in the cholesterol-fed group [$F(1,8) = 32.11$, $p < 0.001$] (Fig. 2B).

Neurodegeneration

Analysis of Fluoro-Jade C staining in hippocampal tissue indicated that the high-cholesterol diet was associated with significantly increased levels of neurodegeneration in CA1/2 [$F(1,8) = 9.60$, $p < 0.05$] and CA3 of hippocampus [$F(1,8) = 22.70$, $p < 0.001$] but not in the dentate gyrus (DG) [$F(1,8) = 0.85$, $p = 0.384$] (Fig. 3).

Hippocampal estrogen receptors and their downstream targets

Figure 4A shows representative fluorescent confocal images of the hippocampus of a control rabbit labeled with anti-ERα antibody in green and DAPI staining nuclei blue. Figure 4B depicts the same antibody labeling in the hippocampus of a cholesterol-fed rabbit. In rabbits fed a high-cholesterol diet, immunofluorescent staining of ERα was decreased significantly in CA1/2 [$F(1,14) = 55.89$, $p < 0.001$] and DG [$F(1,14) = 6.36$, $p < 0.05$] (Fig. 4C). Western blot analysis of whole hippocampal homogenate showed a significant decrease in ERα [$F(1,6) = 18.62$, $p < 0.05$] (Fig. 4D).

Figure 5A shows representative fluorescent confocal images of labeling of ERβ (green) and the nucleus marker DAPI (blue) in the hippocampus from a control rabbit (left) and in the hippocampus from a cholesterol-fed (right). There was an increase in ERβ immunofluorescent staining in CA3 [$F(1,15) = 5.85$, $p < 0.05$] and DG [$F(1,14) = 5.68$, $p < 0.05$] in cholesterol-fed rabbits compared to controls (Fig. 5B). In area CA1/2, there was a trend for increased levels of staining for ERβ [$F(1,15) = 4.15$, $p = 0.06$] in cholesterol-fed rabbits. Western blot analysis of whole hippocampus homogenates suggested somewhat higher levels of ERβ in hypercholesterolemic subjects although this difference was not statistically significant.

Finally, cholesterol-fed rabbits exhibited lower levels of post-synaptic marker PSD-95 in hippocampus [$F(1,6) = 10.40$, $p < 0.05$] (Fig. 6) as well as a decrease in levels of mitochondria [$F(1,6) = 17.057$, $p < 0.01$] (Fig. 7) compared to controls.

DISCUSSION

In this study, we were able to show for the first time that high serum cholesterol in a cholesterol-fed rabbit is accompanied by increased levels of 27-OHC in the hippocampus and provide an indication of possible mechanisms responsible for AD-like pathology related to hypercholesterolemia. Based on our results, we suggest that there may be an association between the levels of 27-OHC, an endogenous estrogen modulator, and expression of ERs in the rabbit hippocampus. Moreover, we report that a high-cholesterol diet is associated with higher levels of neurodegeneration in the hippocampus as well as decreased levels of mitochondria and synaptic marker protein PSD-95, which could be a downstream result of aberrant ER signaling.

The cholesterol-fed rabbit model of AD shows a multitude of pathological findings similar to those seen in AD patients including Aβ deposits, neurofibrillary tangles, apoptosis,

microglia activation, and increased ventricular volume [12, 13, 39, 40] as well as cognitive deficits [17–21]. Here we also demonstrated that a high-cholesterol diet is associated with significant levels of neurodegeneration in the hippocampus. This is an important finding because hippocampal neurodegeneration is one of the hallmarks of AD neuropathology and an aspect often lacking in transgenic models of the disease based on autosomal dominant forms of AD [41, 42]. Mutations of AβPP [43, 44], presenilin-1 [45–47], or presenilin-2 [48] all result in an abnormally high Aβ burden as well as some cognitive deficits characteristic in patients with familial AD. Transgenic rodents containing mutations in genes encoding for AβPP and enzymes involved in AβPP processing present with Aβ plaques accompanied by cognitive deficits, but most of these models fail to show a loss of synapses and neurons in the hippocampus [41, 42]. Consequently, clinical trials based on research with genetically modified animals targeting Aβ have been discouraging [49, 50]. Moreover, studies utilizing those models provided very few answers regarding the etiology of late onset (sporadic) AD. This suggests that there might be important mechanistic differences between early and late onset forms of dementia, and there is a need for non-transgenic models such as the cholesterol-fed rabbit for studying the causes of more common sporadic AD.

Many studies have investigated the correlation between oxysterol levels and neurodegenerative disease but their findings are somewhat inconsistent [3, 22, 51–55]. Some of these inconsistencies arise from the fact that subject cohorts comprised different disease stages and subtypes. The appropriate time for clinical assessment and treatment is especially important as midlife cardiovascular risk factors confer increased risk for developing AD, but once dementia begins, these risk factors diminish [56], a finding that might account for inconsistencies in studies of diet affecting AD patients. In this study we used a cholesterol-fed rabbit model of sporadic AD that removes many of these inconsistencies to investigate the role of 27-OHC at a relatively early time point during the progression of well-characterized AD-like pathology [11, 57].

An increase of 27-OHC in hippocampus in this study is especially important in light of recent findings regarding the function of this oxysterol. This biologically-active molecule has the potential to bind to estrogen receptors and either activate or inhibit ER-dependent pathways. Since its identification as the first endogenous estrogen receptor modulator [26], 27-OHC has been extensively studied in known estrogen-regulated tissues and cell types. It has been shown to act as a partial agonist of ERα in breast cancer cells [26, 29] and bone tissue [27, 58], but in the cardiovascular system, 27-OHC competitively antagonizes estrogen's actions on ERα and ERβ [30] and promotes inflammation and atherosclerosis via ERα signaling [32]. Not much is known at present about the biological actions of 27-OHC in the brain although it has been shown that 27-OHC adversely affects cognition in rodents [15, 16]. Here we report that an increase in 27-OHC in the hippocampus is accompanied by changes in ER expression, a finding that could indicate a mechanistic link between oxysterols and neurodegeneration.

In our study we used male rabbits in order to eliminate the protective effects of estrogen seen in female rabbits with elevated cholesterol [59]. Both ERα and ERβ are localized throughout the hippocampal formation, and there are no significant sex differences in ER distribution in the hippocampus of rodents, primates, or humans [60–64] making our study

relevant to both males and females. It has been shown that AD is more common in women [65], and women experience more severe behavioral and cognitive symptoms during the progression of the disease [66, 67]. Early research into possible reasons for this sex difference was based on the fact that women tend to live longer and hence have a higher susceptibility to developing the disease. However, more recent studies indicate that there must be other factors predisposing women to a higher risk for AD [68], and one of these is the loss of neuroprotection mediated by the loss of estrogen at menopause.

Downregulation of ERα in the hippocampus of hypercholesterolemic rabbits in our study could indicate that 27-OHC antagonizes the beneficial effects of estrogen signaling through ERα receptor. A decrease in hippocampal ERα has been associated with estrogen depletion in rodents while ERβ levels remained unchanged [69, 70] implicating ERα as an important element in estrogen-mediated neuroprotection. Neuroprotective roles of estrogen have been extensively studied, and there are many reports that estrogen affects cognitive function in humans and in animal models [61, 62, 71]. The importance of estrogen signaling in the hippocampus came to light with the discovery of estrogen-induced synapse formation [72]. ERα and ERβ receptors function differently in regulating synaptic connectivity in the hippocampus: activating ERα increases the density of dendritic spines in CA1 [73] while treatment with ERβ agonists has the opposite effect [74] suggesting the importance of ERα in synaptogenesis. Moreover, in an organotypic hippocampal culture, estrogen treatment prevented Aβ-induced neuronal death and that same neuroprotective effect was achieved with the use of an ERα selective agonist [75]. The same study also showed that neuronal death was correlated with a decrease in the post-synaptic marker PSD-95. Therefore, we suggest that low levels of ERα associated with the high-cholesterol diet in our study could be responsible for a reduction in the post-synaptic marker PSD-95. Additionally, upregulation of ERβ has been shown to have a negative effect on synaptic function by downregulating synaptopodin, an actin associated post-synaptic protein [76]. The increase in ERβ levels and the decreased number of mitochondria observed in our study are presumably connected because it has been shown that mitochondrial ERβ can have detrimental effects on mitochondrial function [77].

In summary we demonstrate here that diet-induced hypercholesterolemia causes an increase in 27-OHC in hippocampus. Our results suggest that 27-OHC is an active molecule that is associated with downregulation in expression of ERα and the synaptic marker PSD-95, and increased levels of ERβ possibly linked to decreased mitochondria in hippocampal cells. We suggest that 27-OHC modulates ER signaling that leads to the loss of estrogen-related neuroprotection which might explain one of the mechanisms of 27-OHC-related neurodegeneration described in both *in-vivo* [52] and *in-vitro* systems [24, 78].

Sporadic AD is a heterogeneous disorder, and there are many well-characterized risk factors influencing an individual's chance of developing this disease as well as its progression. We conclude that there is sufficient evidence linking life style risk factors like hypercholesterolemia with a biological predisposition to develop AD. In this study we examined some of the elements that could be involved in mechanisms leading to dementia, suggesting a role for high levels of 27-OHC in the hippocampus and associated ER signaling dysfunction.

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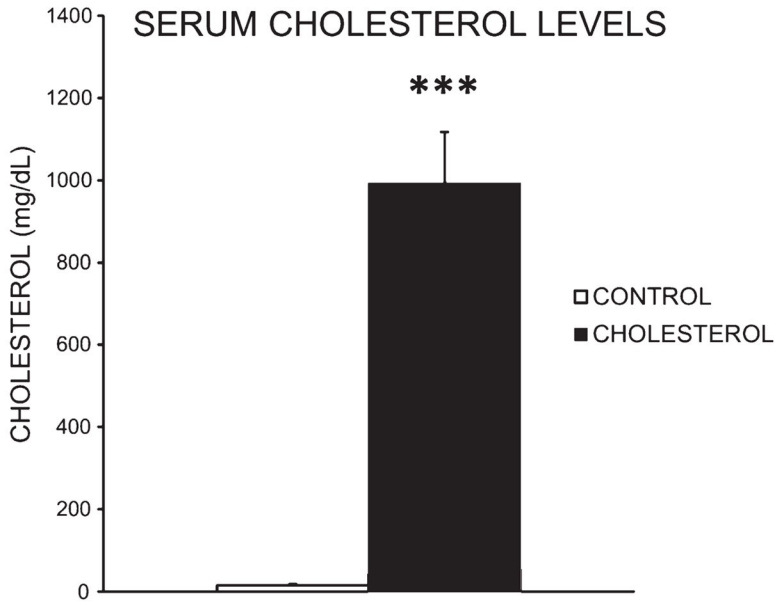

Fig. 1.

Serum cholesterol. Mean serum cholesterol levels for control group ($n = 5$) was 15.6 mg/dL while high-cholesterol animals ($n = 5$) average serum total cholesterol was 992.6 mg/dL as measured by a colorimetric kit (***$p < 0.001$).

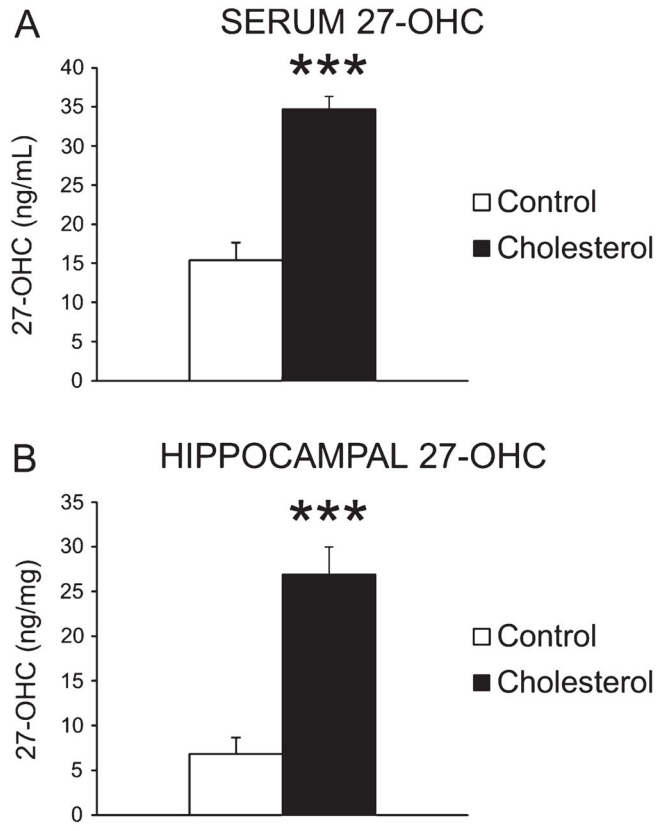

Fig. 2.

27-OHC levels in serum and hippocampus. Serum level of 27-OHC was significantly increased following a high-cholesterol diet, from 15.37 ng/mL in control group ($n = 5$) to 34.71 ng/mL in cholesterol-fed animals ($n = 5$, ***$p < 0.001$) (A), and similarly the hippocampal level of 27-OHC increased from 6.82 ng/mg in control group ($n = 5$) to 26.94 ng/mg in cholesterol-fed group ($n = 5$, ***$p < 0.001$) (B).

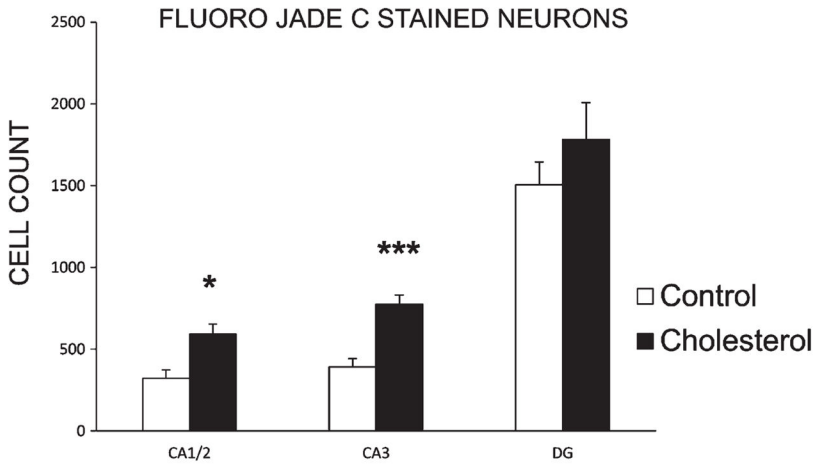

Fig. 3.

Neurodegeneration. Fluoro Jade C staining of hippocampal sections. Data are mean (±SEM) counts of stained cells for control ($n = 4$) and cholesterol-fed ($n = 6$) groups in each respective region of dorsal hippocampus (CA1/2; *$p < 0.05$), CA3; ***$p < 0.001$) and dentate gyrus (DG).

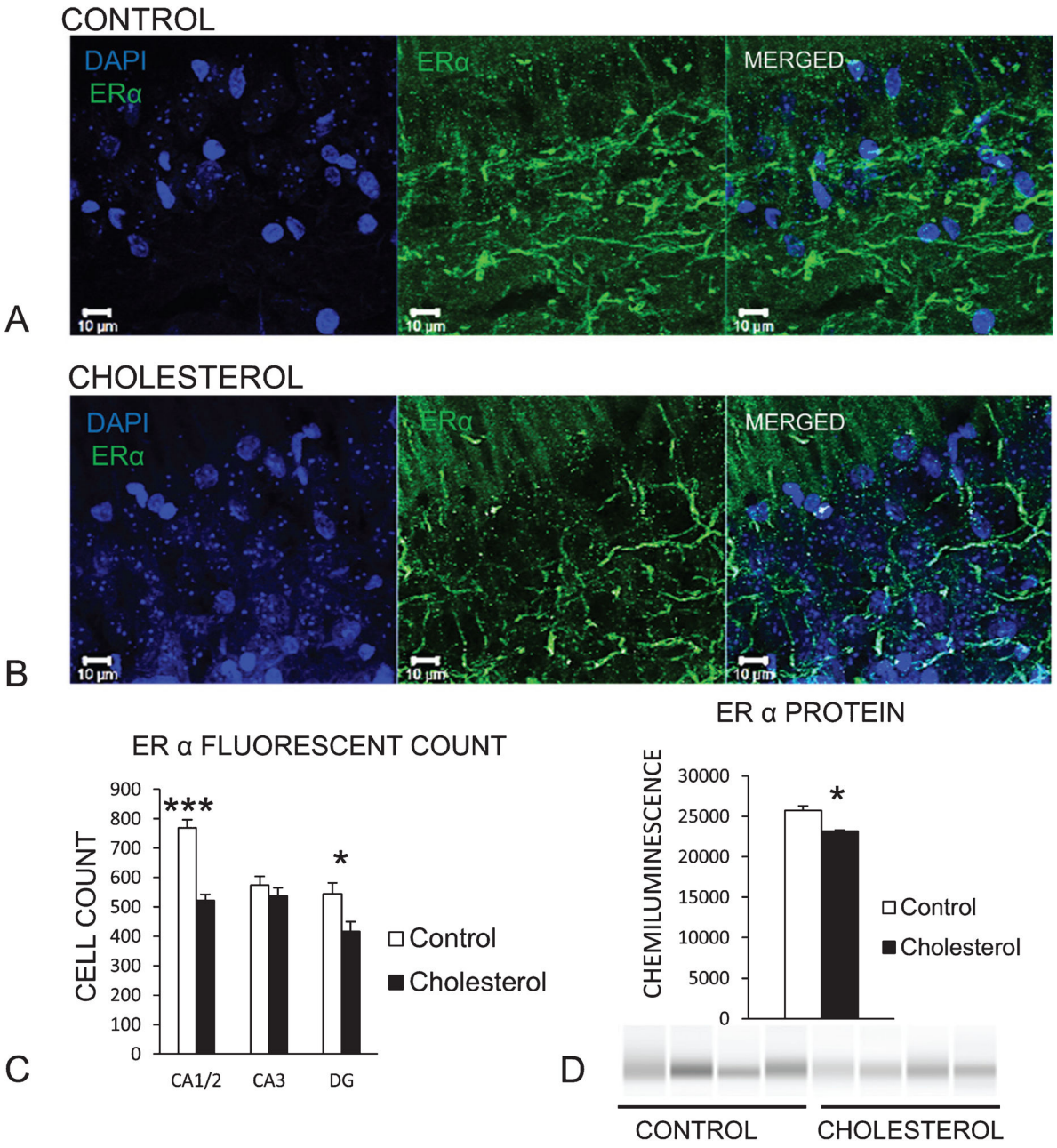

Fig. 4.

High-cholesterol diet reduces ERα in the hippocampus. Representative confocal images of (A) control hippocampus section with DAPI staining nuclei blue and green fluorescence labeling ERα. B) ERα labeling decreased in rabbits fed a high-cholesterol diet. C) Mean count of stained cells for control ($n = 7$) and cholesterol ($n = 9$) groups in each respective region of dorsal hippocampus showed a decrease in the amount of staining in rabbits fed a high-cholesterol diet (CA1/2 (***$p < 0.001$), CA3 and dentate gyrus (DG) (*$p < 0.05$)). D) Western blot showed significant downregulation of ER alpha (*$p < 0.05$) in hippocampal tissue (control group $n = 4$, cholesterol $n = 4$).

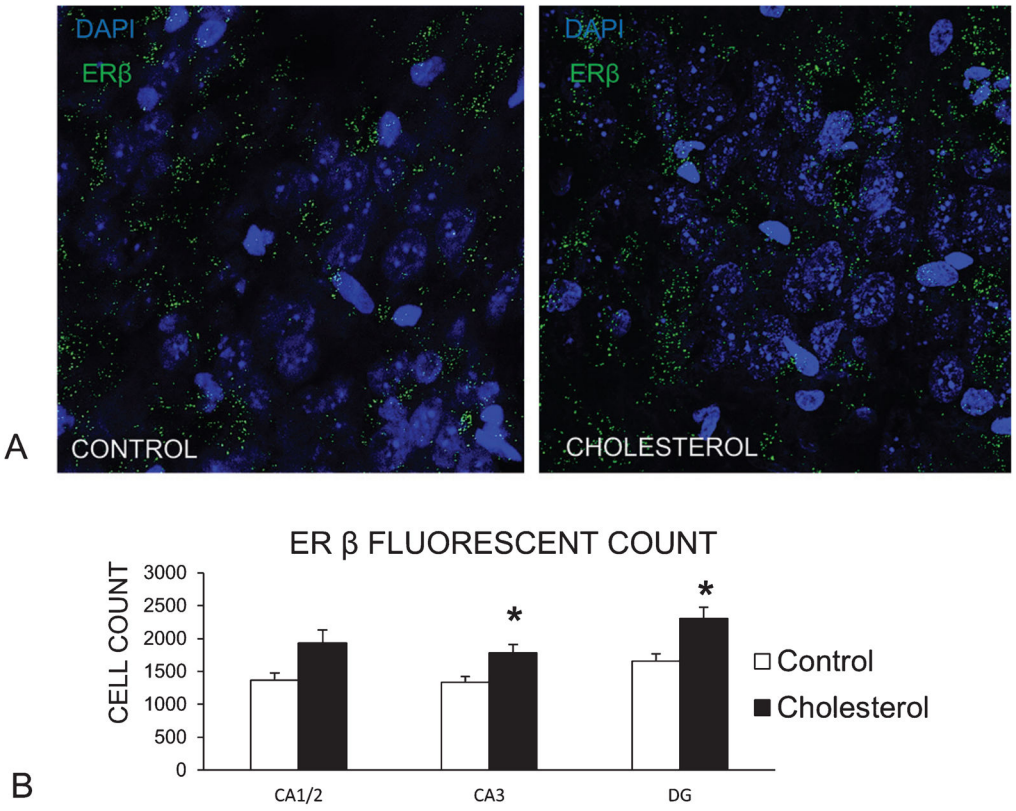

Fig. 5.

ERβ labeling in hippocampus increased following a high-cholesterol diet. A) Representative images of ERβ (green) fluorescent labeling in hippocampus. Cholesterol-fed subject (right) shows and increase in ERβ staining relative to control (left) (DAPI – blue – stains nuclei). B) Fluorescent labeling for ERβ was quantified using Image J and a one-way ANOVA showed a significant effect of the high-cholesterol diet on the number of ERβ in areas CA3 and DG of the hippocampus ($p < 0.05$). Data from CA1 were also consistent with these findings although not statistically significant ($p = 0.06$) (control group $n = 6$, cholesterol $n = 11$).

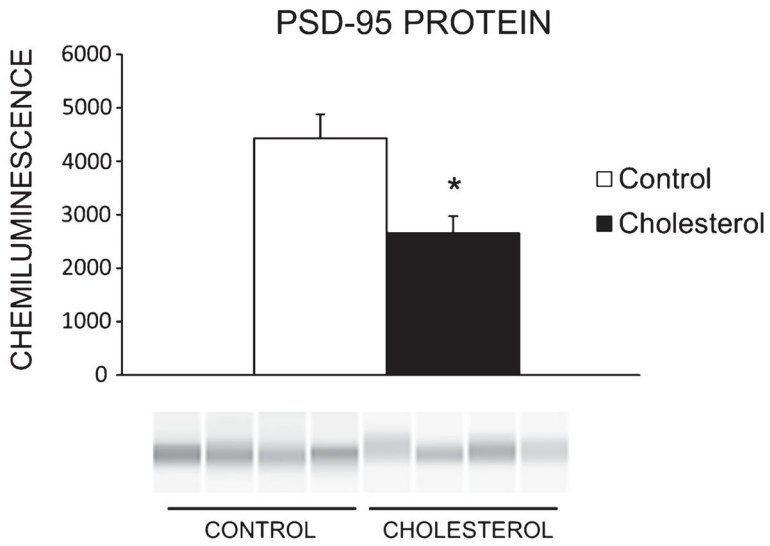

Fig. 6.

Post-synaptic protein PSD-95 in hippocampus. Western blot showed significant downregulation of post-synaptic marker PSD-95 in rabbits fed the high-cholesterol diet (*$p < 0.05$) (control group $n = 4$, cholesterol $n = 4$).

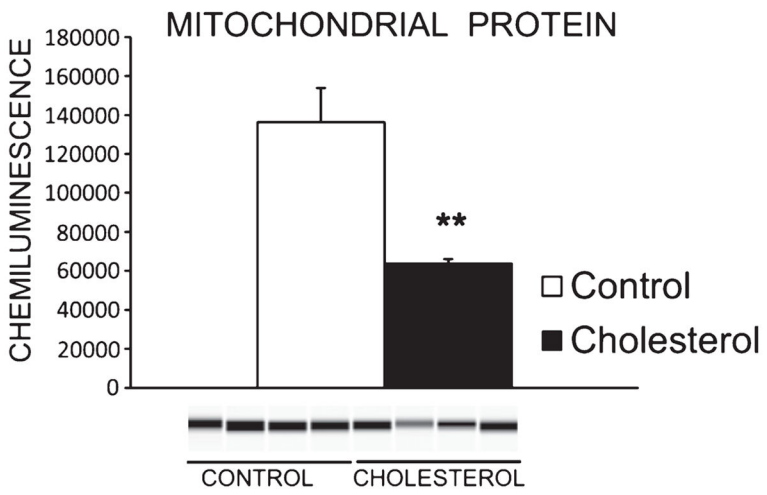

Fig. 7.

Mitochondrial protein content in hippocampus. Western blot showed significant downregulation of mitochondrial protein in rabbits fed the high-cholesterol diet (**$p < 0.01$) (control group $n = 4$, cholesterol $n = 4$).