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Long-term high-dose atorvastatin decreases brain oxidative and nitrosative stress in a preclinical model of Alzheimer disease: A novel mechanism of action

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ABSTRACT

Alzheimer disease (AD) is an age-related neurodegenerative disorder characterized by progressive memory loss, inability to perform the activities of daily living and personality changes. Unfortunately, drugs effective for this disease are limited to acetylcholinesterase inhibitors that do not impact disease pathogenesis. Statins, which belong to the class of cholesterol-reducing drugs, were proposed as novel agents useful in AD therapy, but the mechanism underlying their neuroprotective effect is still unknown. In this study, we show that atorvastatin may have antioxidant effects, in aged beagles, that represent a natural higher mammalian model of AD. Atorvastatin (80 mg/day for 14.5 months) significantly reduced lipoperoxidation, protein oxidation and nitration, and increased GSH levels in parietal cortex of aged beagles. This effect was specific for brain because it was not paralleled by a concomitant reduction in all these parameters in serum. In addition, atorvastatin slightly reduced the formation of cholesterol oxidation products in cortex but increased the 7-ketocholesterol/total cholesterol ratio in serum. We also found that increased oxidative damage in the parietal cortex was associated with poorer learning (visual discrimination task). Thus, a novel pharmacological effect of atorvastatin mediated by reducing oxidative damage may be one mechanism underlying benefits of this drug in AD.

1. Introduction

Under physiological conditions, cell homeostasis is finely regulated by a balance between pro-oxidant and anti-oxidant stimuli; however, certain environmental factors, stressors, or diseases may affect this equilibrium and increase production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both ROS and RNS may react with biomolecules including proteins, lipids, carbohydrates, DNA and RNA [1] leading to their oxidative damage resulting in cellular dysfunction [2–5]. Several lines of evidence have shown that oxidative stress levels are elevated in the brains from subjects with Alzheimer disease (AD) [4,6–13].AD is an age-related neurodegenerative disorder characterized histopathologically by the presence of senile plaques (SP), neurofibrillary tangles (NFT), and synapse loss in selected brain regions such as medial temporal lobe [14–16]. The main component of senile plaques is amyloid beta-peptide (Aβ), a 40–42 amino acid peptide derived by the proteolytic cleavage of amyloid precursor protein (APP) through the activity of beta- and gamma-secretases, and SP are extracellular in localization. A number of *in vitro* and *in vivo* studies have shown that Aβ(1–42) is a neurotoxic peptide which exists in both soluble (monomers, oligomers, and protofibrils) and insoluble (fibrils) forms. Recent studies have suggested that the small oligomers, rather than Aβ fibrils, are the actual toxic species of this peptide [17–20] and they generate oxidative/nitrosative damage in the brain [4,6,21–25] that may be responsible for the clinical aspects of the disease, including memory loss and dementia.

Cholesterol is a major lipid constituent of cellular membrane, and regulates cell signaling pathways, gene transcription, as well as the availability of bioactive steroids [26–28]. The cholesterol content of the CNS is largely independent of dietary uptake or hepatic synthesis, as circulating cholesterol does not cross the blood–brain

Abbreviations: PC, protein carbonyls; HNE, 4-hydroxy-2-nonenal; 3-NT, 3-nitrotyrosine; Aβ, amyloid beta peptide; 7-K, 7-ketocholesterol; 25-OH, 25-hydroxy cholesterol.

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barrier. Brain cholesterol turnover is extremely slow, with a half-life estimated in years in humans [26,29]. Cholesterol can undergo oxidative modifications at least by two mechanism: a direct radical attack involving ROS or RNS (non-enzymatic way), or by the activity of a specific enzymes (enzymatic way). This leads to the formation of cholesterol oxidation products, called oxysterols. These latter are major regulators of cholesterol homeostasis in the central nervous system [27]. Among oxysterols, 7-ketocholesterol (7-K) and 25-hydroxycholesterol (25-OH) have been shown to cause apoptotic neuronal death by inducing mitochondrial dysfunction [30], Ca^{2+} influx and perturbation of intracellular ionic homeostasis [31,32].

There is accumulating evidence that cholesterol and its products may be implicated in the pathogenesis of dementia, and this has led investigators to assess the possible role of lipid lowering agents in the treatment of dementia. Several cross-sectional or case–control epidemiological studies have revealed a striking link between cholesterol-lowering drugs (statins or others) and up to 70% reduction in AD prevalence in the general population [33–40]. Furthermore, in preliminary AD clinical trials with simvastatin [41] and atorvastatin [42,43] modest cognitive benefits have been reported. In particular, mild-to-moderate AD subjects treated with atorvastatin (80 mg/day) exhibited a significant improvement in cognitive function (evaluated by the Alzheimer Disease Assessment Scale-Cognitive, ADAS-cog) at 6 months with smaller non significant benefits at 12 months [43]. The LEADe study is also ongoing and involves testing atorvastatin (80 mg/day) in combination with donepezil in patients with AD, but given previous studies this approach may provide similar modest benefits [44].

The purpose of this study was to evaluate if a long-term administration of high dose (80 mg/day) of atorvastatin in aged beagles, that represent a good pre-clinical model of AD [45], is associated with more benefits due to its ability to modulate oxidative and nitrosative stress-induced changes in protein and lipid profiles.

2. Materials and methods

2.1. Animals

Twelve beagles ranging in age from 8.9 to 13.2 yrs were obtained from the Lovelace Respiratory Research Institute and Harlan (Indianapolis, IN). Based on our previous work, dogs of this age show cognitive decline and significant amounts of brain Aβ [45,46]. All animals had documented dates of birth, comprehensive medical histories and a veterinary examination ensuring that the animal was in good health prior to the start of the study. At the end of the study, all but one control animal had received treatment for 14.5 months and they ranged in age from 10.1 to 14.6 yrs. All research was conducted in accordance with approved IACUC protocols. Animals were ranked by cognitive test scores and placed into equivalent groups with 2 males and 4 females per group. These groups were randomly designated as either the placebo-treated control group or the atorvastatin-treated group.

2.2. Cognitive testing

Animals were given a series of cognitive tests while on treatment as described previously [47]. For the current study, scores from the size discrimination learning problem was used as they were obtained after 6 months of treatment and was sensitive to treatment effects.

2.3. Drug treatment

Atorvastatin calcium (also known as Lipitor®,40 mg tablets) and placebo tablets were kindly provided by Pfizer Inc. (New York, NY). Atorvastatin-treated animals received 2 × 40 mg tablets per day for a daily dose of 80 mg, and control animals received 2 placebo tablets per day. Atorvastatin was chosen for this study because long term studies using an 80 mg/day dose in dogs did not report adverse events such as cataracts [48]. In addition, the administration of 80 mg/day atorvastatin in hypercholesterolemic people produces plasma drug concentrations in the range 187–252 ng/ml [49–51], which is comparable to that achieved in dogs treated with 6 mg/kg atorvastatin (about 90 mg/dog, i.e., about 500 ng/ml [52]).

2.4. Serum samples

Serum samples were collected as previously described [47]. Briefly, blood samples were collected in 10 cm^3 tubes without anti-coagulant EDTA at regular intervals prior to and during the treatment study. Serum was aliquoted and frozen at −80 °C. For the current study, serum collected 62 days prior to euthanasia was analyzed.

2.5. Tissue collection

Twenty minutes before induction of general anesthesia, animals were sedated by subcutaneous injection with 0.2-mg/kg acepromazine. General anesthesia was induced by inhalation with 5% isoflurane. While maintained under anesthesia, dogs were exsanguinated by cardiac puncture. Within 15 min, the brain was removed from the skull and sectioned midsagitally. The intact left hemisphere was immediately placed in 4% paraformaldehyde for 48–72 h at 4 °C prior to long term storage in phosphate buffered saline containing 0.02% sodium azide at 4 °C. The right hemisphere was coronally sectioned (∼1 cm) and flash frozen at −80 °C. The dissection procedure was completed within 20 min yielding a 35–45 min postmortem interval.

2.6. Measurement of serum and brain cholesterol

A detailed description of each measurement for the current study has been described previously [47]. Briefly, for serum samples, fresh samples were immediately provided to a commercial laboratory for measures of basic biochemistry (e.g., liver function), cholesterol, triglycerides, low density lipoproteins (LDL), and high density lipoproteins (HDL). For brain samples, frozen tissues were weighed and homogenized in methanol containing the following internal standards: heptadecanoic acid (Nu-Chek Prep, Elysian, MN) and cholesterol-D7 (Avanti Polar Lipids, Alabaster, AL). Lipids were extracted with 2 volumes of chloroform and washed with 1 volume of water. Organic phases were collected and dried under liquid N2. Lipids were reconstituted in chloroform/methanol (1:4, vol/vol, 0.1 ml) for liquid chromatography/mass spectrometry (LC/MS) analyses [47].

2.7. Sample preparation

Serum samples from control and atorvastatin-treated dogs were diluted 10-fold with Media I lysis buffer (pH 7.4) containing 320 mM sucrose, 1% of 990 mM Tris–HCl (pH 8.8), 0.098 mM $MgCl_2$, 0.076 mM EDTA, proteinase inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), aprotinin (0.5 mg/ml) and PMSF (40 μg/ml) and phosphatase inhibitor cocktail (Sigma–Aldrich). The brain tissues (parietal cortex) from control and atorvastatin-treated dogs were thawed and placed in Media I buffer. The brains were homogenized by 20 passes of a Wheaton tissue homogenizer, and the resulting homogenate was centrifuged at 14,000 × *g* for 10 min to remove debris. The supernatant was extracted to determine the total protein concentration by BCA method (Pierce, Rockford, IL, USA).

2.8. Protein carbonyls

Samples (5 μl) of parietal cortex homogenate or diluted serum, 12% sodium dodecyl sulfate (SDS; 5 μl), and 10 μl of 10 times diluted 2,4-dinitrophenylhydrazine (DNPH) from 200 mM stock were incubated at room temperature for 20 min, followed by neutralization with 7.5 μl neutralization solution (2 M Tris in 30% glycerol). Protein (250 ng) was loaded in each well on a nitrocellulose membrane under vacuum using a slot blot apparatus. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 h and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 h. The membrane was washed in PBS following primary antibody incubation three times at intervals of 5 min each. The membrane was incubated after washing with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS in a 1:8000 ratio for 1 h. The membrane was washed three times in PBS for 5 min each and developed with Sigma fast tablets (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate [BCIP/NBT substrate]). Blots were dried, scanned in Adobe Photoshop, and quantified in Scion Image (PC version of Macintosh-compatible NIH image). No nonspecific binding of antibody to the membrane was observed.

2.9. Protein-bound HNE and 3-NT

Samples (5 μl) of parietal cortex homogenate or diluted serum, 12% SDS (5 μl), and 5 μl modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature and were loaded (250 ng) in each well on a nitrocellulose membrane in a slot blot apparatus under vacuum. The membrane was treated as described above and incubated with a 1:5000 dilution of anti protein-bound HNE polyclonal antibody or 1:2000 3-NT antibody in PBS for 1 h 30 min. The membranes were further developed and quantified as described above. A faint background staining resulting from the antibody alone was observed, but, because each sample had a control, this minor effect was controlled.

2.10. Extraction procedures

7-Ketocholesterol and 25-OH were extracted from both serum and brain samples as previously described [53] with modifications. Briefly, for serum samples, 1 ml of serum from control and atorvastatin-treated dogs, was mixed with 1 ml of ethanol containing 0.1 mM butylated hydroxytoluene and extracted with 3 ml of hexane. For brain samples, 1 ml of homogenate was mixed with 1 ml of methanol and extracted with 3 ml of hexane. Each sample was then centrifuged at 4000 × *g* for 10 min to separate the hexane layer from the solution. The hexane phase was then evaporated to dryness under nitrogen stream and the residues re-dissolved in methanol. Twenty microliters was analyzed by HPLC.

2.11. HPLC equipment

The HPLC system consisted of a Waters 616 quaternary pump, equipped with a Waters 996 Diode array detector that was used for the analysis. The samples were eluted through a Thermo Scientific Hypersil GOLD column (C18, 4.6 cm × 25 cm, 5 μm particle size), with a guard column (10 mm) of the same material matrix.

2.12. Measurement of serum and brain 7-ketocholesterol and 25-hydroxycholesterol levels

The HPLC evaluation of 7-K and 25-OH was performed as previously described by Chen and Chen [54] with modifications. Briefly, the samples were analyzed by using a mobile phase of acetonitrile:methanol (55:45, v/v) and UV detector. The flow-rate was maintained at 0.5 ml/min for 30 min. The wavelength for UV detection was set at 234 nm for 7-K, and at 212 nm for 25-OH. 7-K and 25-OH concentrations were calculated by reference to a standard curve of 7-K and 25-OH (0.39–50 μM) in methanol. By this method, a linear fitting ($r^2 = 0.99$) has been obtained.

2.13. Reduced (GSH) and oxidized (GSSG) glutathione assay

Determination of GSH and GSSG was performed by the method of Hissin and Hilf [55]. Briefly, tissue homogenate was deproteinated with 10% meta-phosphoric acid, and after a centrifugation at 100,000 × *g* for 30 min at 4 °C, the deproteinated supernatant was used for GSH and GSSG assays. Reduced glutathione was measured by adding deproteinated sample (10 μl) to a mixture of *o*-phthalaldehyde (1.0 mg/ml in reagent grade methanol) and 0.1 M phosphate-buffered saline (pH 8) with 5 mM EDTA. After incubation for 15 min at room temperature, flourescence at emission 420 nm was recorded following excitation at 350 nm. Oxidized glutathione was measured by adding 10 μl of deproteinated sampled to 0.04 M N-ethylmaleimide for 30 min to interact with GSH present in the sample. This mixture was added to a mixture containing *o*-phthalaldehyde (1.0 mg/ml), 0.1 N NaOH. After incubation for 15 min at room temperature, flourescence at emission 420 nm was recorded by excitation at 350 nm.

2.14. Statistical analysis

Data are expressed as mean ± SD of *N* independent samples. All statistical analysis was performed using a two-tailed Student's *t*-test. $p < 0.05$ was considered significantly different from control. Pearson correlations were calculated to test the linear association between cognitive test scores and markers of oxidative damage.

3. Results

3.1. Effect of atorvastatin treatment on the levels of oxidative and nitrosative stress markers in both parietal cortex and serum

In the current study, a comparative analysis of the levels of oxidative and nitrosative stress markers was carried out in both parietal cortex and serum obtained after a chronic administration of atorvastatin (80 mg/day for 14.5 months) in aged beagles. As shown in Fig. 1A, C, and E, atorvastatin treatment produced a significant decrease of protein carbonyls (PC) ($t = 2.325$, df = 10, $p = 0.042$), 4-hydroxy-2-nonenal (HNE) ($t = 3.115$, df = 9, $p = 0.012$) and 3-nitrotyrosine (3-NT) ($t = 2.331$, df = 10, $p = 0.042$) levels of ~10.8%, 31.6% and 25.6%, respectively, in the parietal cortex compared to the control group. Conversely, as shown in Fig. 1B, 1D and 1F, in serum samples obtained from atorvastatin treated dogs a non-statistically significant trend towards increased levels of each oxidative stress marker was observed with respect to the control group (PC, $t = 1.375$, df = 8, $p = 0.21$; HNE, $t = 0.97$, df = 9, $p = 0.36$; 3-NT, $t = 1.54$, df = 9, $p = 0.16$).

3.2. Atorvastatin effects on brain and serum levels of 7-ketocholesterol and 25-hydroxycholesterol

In order to address the hypothesis whether the differences observed in the expression of oxidative stress markers in serum

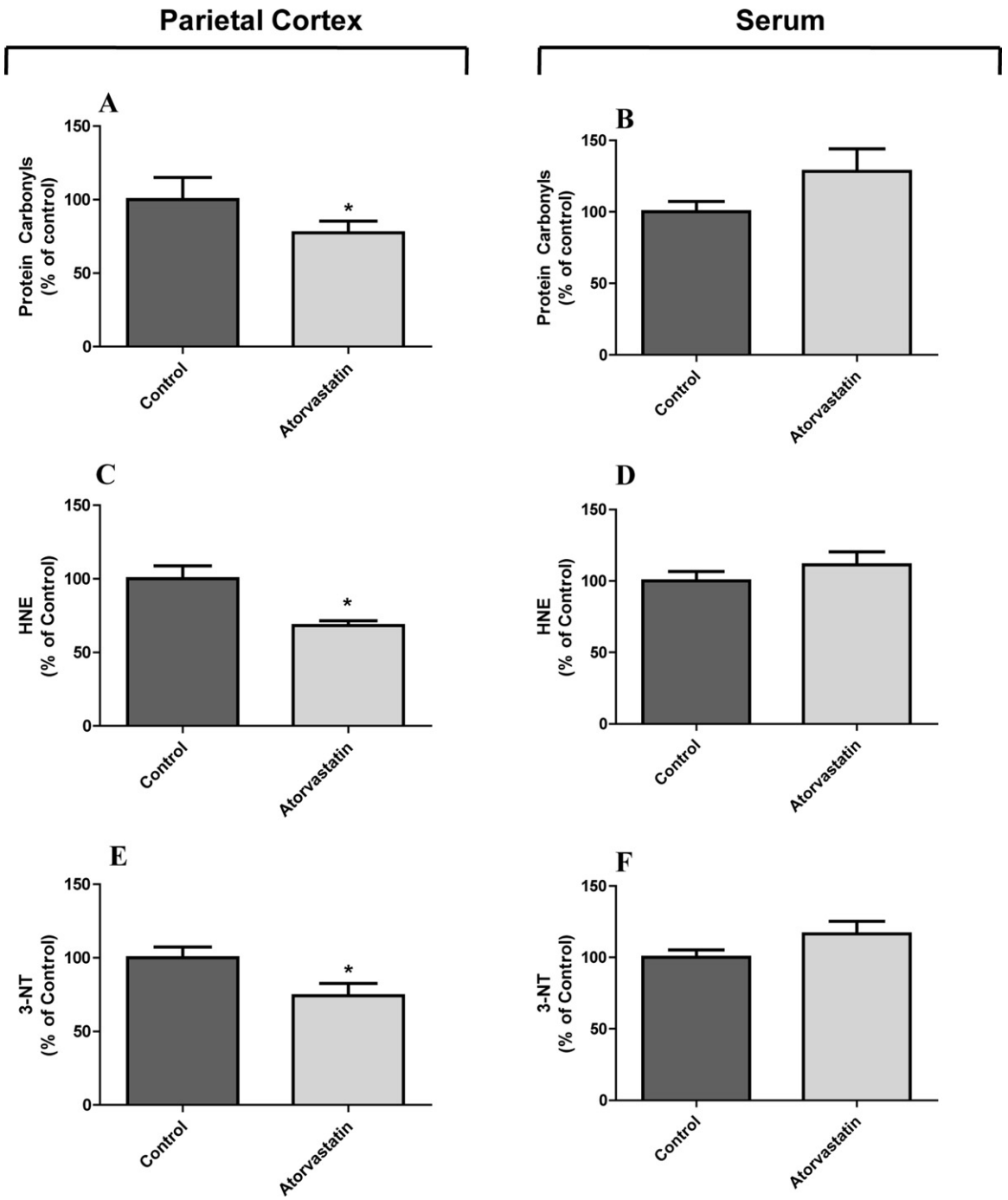

Fig. 1. *In vivo* oxidative and nitrosative modifications observed in brain (parietal cortex) (Panels A, C and E) and serum (Panels B, D, and F) of aged beagles during lipid-lowering therapy with atorvastatin (80 mg/day). (A and B) Protein carbonyls (PC), (C and D) protein-bound HNE and (E and F) 3-NT levels. Brain samples of parietal cortex, or serum samples were probed with anti-DNP protein adducts polyclonal antibody (Panel A and B), anti-HNE polyclonal antibody (Panels C and D) and anti-nitrotyrosine polyclonal antibody (Panels E and F) as described under Materials and Methods. Densitometric values shown are given as percentage of the control group, set as 100%. Data are expressed as mean ± SD of three replicates for each of individual control and atorvastatin treated beagle, per group. $^{*}p < 0.05$ versus control (Student's *t*-test).

and parietal cortex could be linked, at least in part, to a formation of well known pro-oxidant products of cholesterol oxidation, the levels of 7-K and 25-OH cholesterol both in serum and parietal cortex were measured. There were no significant atorvastatin-mediated changes in the absolute levels of 7-K (−28%) ($t = 1.45$, df = 9, $p = 0.18$) (Fig. 2, Panel A) and 25-OH (−18%) ($t = 1.19$, df = 9, $p = 0.26$) (Fig. 2, Panel C) in parietal cortex. Conversely, as shown in Fig. 3, atorvastatin slightly increased the absolute levels of 7-K (+20%) ($t = 1.94$, df = 9, $p = 0.08$) in serum (Panel A) while no change were observed for 25-OH ($t = 0.40$, df = 9, $p = 0.70$) (Panel C). 7-K and 25-OH also vary as a function of total cholesterol, and as shown in Fig. 2, the ratio of both 7-K/total cholesterol and 25-OH/total cholesterol were decreased, in parietal cortex, by 48% (Panel B) and 43% (Panel D), respectively, although no significant differences were observed with respect to the control group (7-K/total cholesterol, $t = 1.81$, df = 9, $p = 0.10$; 25-OH/total cholesterol, $t = 2.12$, df = 9, $p = 0.06$). In contrast, a significant increase was observed for the serum ratio of 7-K/total cholesterol (+49%) ($t = 3.6$, df = 9, $p = 0.006$) (Fig. 3, Panel B), while no change in the blood ratio of 25-OH/total cholesterol ($t = 0.11$, df = 9,

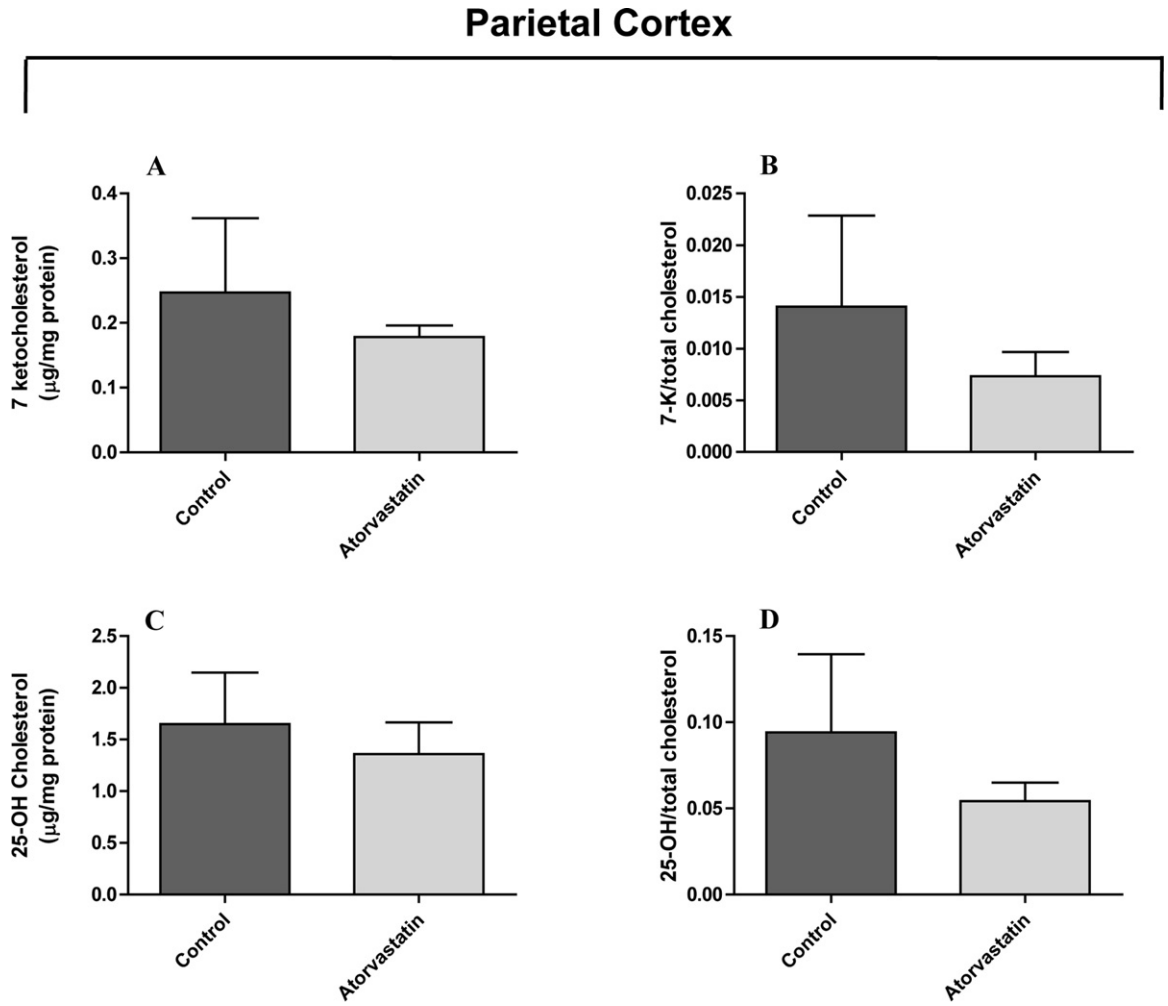

Fig. 2. Changes in brain (parietal cortex) concentrations of 7-ketocholesterol (7-K) and 25-hydroxycholesterol (25-OH) in aged beagles during lipid-lowering therapy with atorvastatin (80 mg/day). Parietal cortex levels of (A) 7-K (absolute levels), (B) 7-K/total cholesterol ratio (C) 25-OH (absolute levels), (D) 25-OH/total cholesterol ratio. Data are expressed as mean ± SD of $n = 5$ (controls) and $n = 6$ (atorvastatin-treated) individual samples per group.

$p = 0.92$) (Fig. 3, Panel D) with respect to the control group was observed.

3.3. Atorvastatin and the glutathione system in dog parietal cortex

A major mechanism involved in the brain adaptive response to oxidative stress is the modulation of the glutathione system. As shown in Fig. 4, atorvastatin significantly increased the GSH concentration in the parietal cortex of treated dogs ($t = 2.38$, df = 10, $p = 0.03$), whereas no significant change was found in GSSG levels ($t = 0.718$, df = 10, $p = 0.489$). As result, the GSH/GSSG ratio significantly increased in the brain of atorvastatin-treated animals ($t = 2.42$, df = 9, $p = 0.03$).

3.4. Atorvastatin-induced changes in oxidative stress levels are correlated with learning

We next hypothesized that the reduced brain oxidative damage in response to atorvastatin would be associated with learning error scores. We analyzed the association between each marker's (PC, HNE, 3-NT, 7-K/total cholesterol and 25-OH/total cholesterol) concentrations and size discrimination learning error scores across treatment and control groups. Interestingly, discrimination learning error scores were positively correlated with parietal cortex PC (Pearson $r = 0.702$, $p = 0.0236$), HNE (Pearson $r = 0.826$, $p = 0.006$), and 3-NT (Pearson $r = 0.588$, $p = 0.073$) (Fig. 5, Panel A–C). These results suggest that poorer learning was associated with higher levels of oxidative damage. Although correlations were significant also for 7-K/total cholesterol (Pearson $r = 0.751$, $p = 0.012$) and 25-OH/total cholesterol (Pearson $r = 0.759$, $p = 0.017$) this was primarily due to one animal showing a high error score and very high 7-K/total cholesterol and 25-OH/total cholesterol ratios. No correlations were found with serum levels of each marker (data not shown).

4. Discussion

Aged beagles represent a good pre-clinical model of AD because they deposit endogenous levels of Aβ of identical sequence to human Aβ [56] as they age and thus are a natural higher mammalian model of aging. The canine β-amyloid precursor protein (APP) is virtually identical to human APP (~98% homology). Most of the deposits in the canine brain are of the diffuse subtype, but are fibrillar at the ultrastructural level and at an advanced stage, which models early plaque formation in humans [57–59]. Moreover in terms of the pattern and severity of cognitive decline, the aged canine parallels age-associated memory impairment in humans [60]. The current study represents the first evidence that in aged dogs, chronic treatment with atorvastatin, may exert anti-oxidant

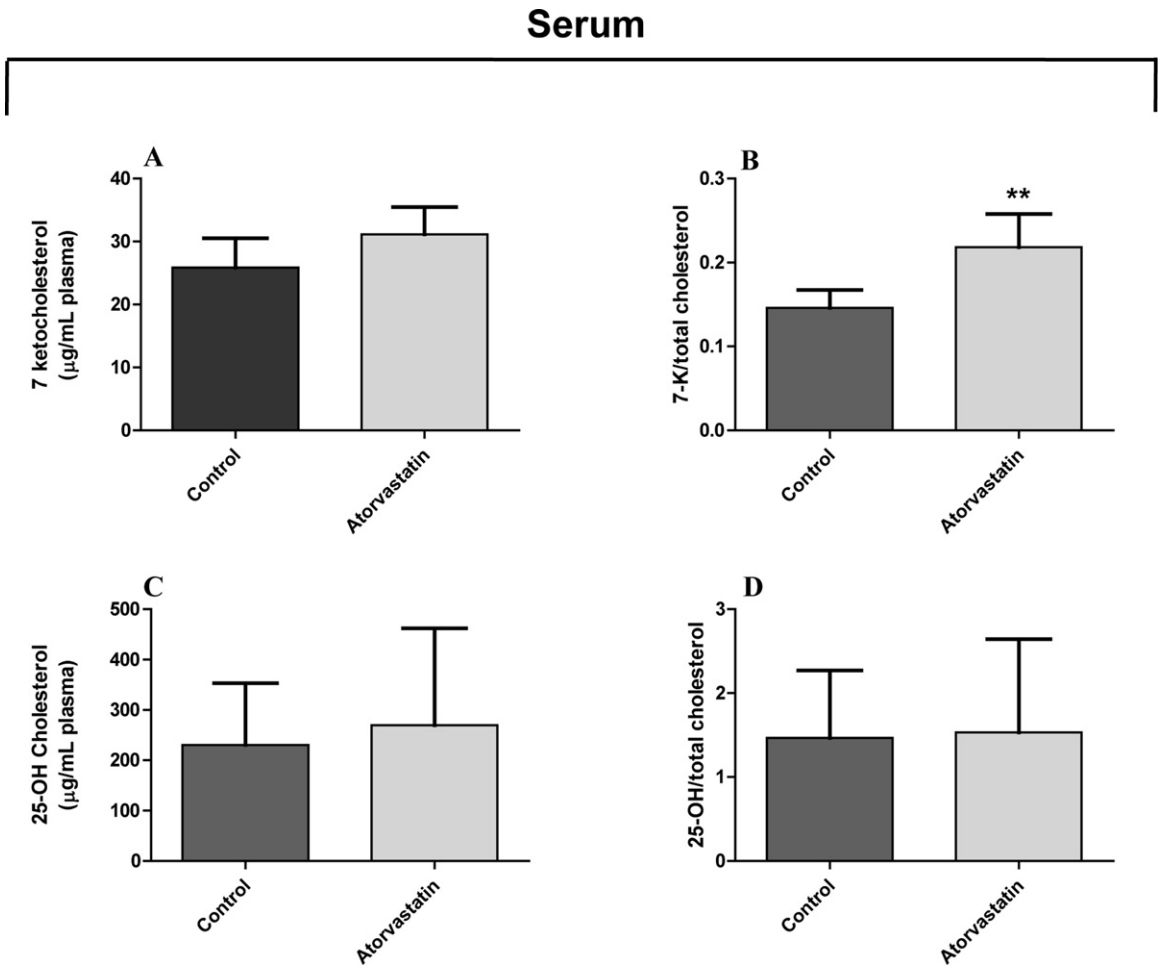

Fig. 3. Changes in serum concentrations of 7-ketocholesterol (7-K) and 25-hydroxycholesterol (25-OH) in aged beagles during lipid-lowering therapy with atorvastatin (80 mg/day). Serum levels of (A) 7-K (absolute levels), (B) 7-K/total cholesterol ratio (C) 25-OH (absolute levels), (D) 25-OH/total cholesterol ratio. Data are expressed as mean ± SD of $n = 5$ (controls) and $n = 6$ (atorvastatin-treated) individual samples per group. $^{**}p < 0.01$ versus control (Student's t-test).

effects on the brain. Although the sample size was relatively small, consistent effects with multiple oxidative stress outcome measures suggest this may be a robust effect.

It is well known that statins are the most prescribed drugs worldwide for the treatment of hypercholesterolemia [61] and due to their ability to reduce cardiovascular events [62]. The main mechanism of action of statins is to lower cholesterol by acting on hydroxyl-methylglutaryl (HMG)-CoA reductase, a key enzyme responsible for the synthesis of cholesterol. Moreover, there are many downstream modifications to other molecular pathways leading to pleiotropic effects that may be both beneficial and adverse [63,64]. Interestingly, many pathways modified by statins could have direct effects on AD pathogenesis and Aβ associated neuropathology. Among these effects is the antioxidant effect exerted by atorvastatin [65–69].

Interestingly, statins are also associated with the reduced risk to develop AD [36,61]. The mechanism by which statins may reduce the risk of incident AD may be through the reduction of Aβ [70]. High dietary cholesterol in transgenic mouse models of AD leads to increases in brain Aβ [71]. In contrast, reducing cholesterol [72] or treatment with statins can reduce Aβ [73]. However, rodents respond to statin treatment by up-regulating HMG-CoA reductase levels after suppression by statins in the liver, the net effect of which is to prevent any stable, long term reduction in cholesterol levels [74]. This leads to difficulties in conducting long term studies in rodents with extensive behavioral testing but additionally leads to doses of statins that are physiologically excessive relative to human clinical trials. Thus, translating outcomes from rodent studies to humans is limited. In contrast, aged beagles, are a good model of human aging and disease and show cognitive and neurological changes with age that are consistent with human [75].

Although epidemiological studies show that statins are associated with reduced risk of AD, typically these observational studies are from individuals who are hypercholesterolemic and require statin treatment. However, several clinical studies and a meta-analysis of a pooled set of clinical studies [76] were completed in AD patients who were normocholesterolemic as were our animals. Thus, the work in dogs is comparable to humans particularly if statins were to be used as a means to improve cognition in AD patients who may or may not have high cholesterol. Our study suggests that statins may improve or maintain cognition through mechanisms independent of cholesterol reduction, particularly in the brain. The latter would have important implications for using statins to treat AD as not all patients with AD have high cholesterol levels and a concern is that reducing cholesterol below optimal levels may lead to adverse events.

This study suggests that an additional benefit of atorvastatin is possible based on its antioxidant properties. The significant correlations found between decreased levels of oxidative stress markers and decrease in size discrimination error score (reflecting improved cognition), observed in aged dogs after treatment with atorvastatin (Fig. 4) led us to speculate that the effect on cognition could be due to the reduced oxidative stress instead of the ability of atorvastatin to reduce cholesterol levels. Indeed, our previous studies [47]

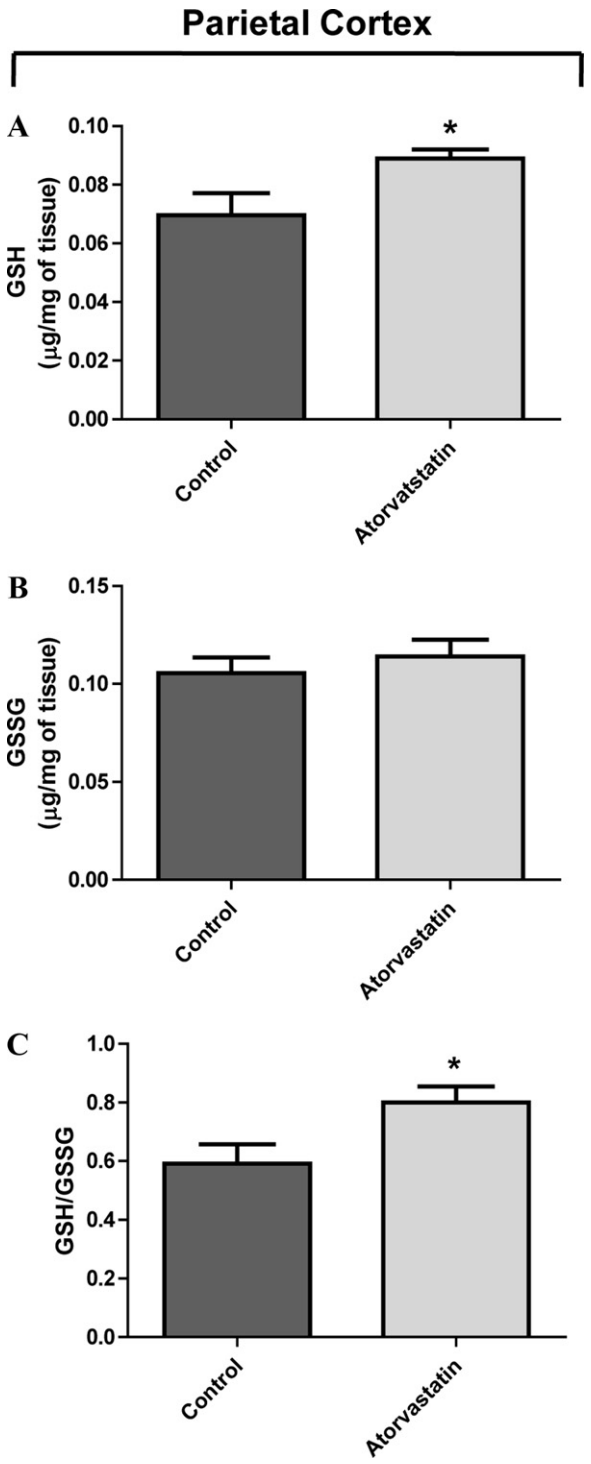

Fig. 4. Changes in brain (parietal cortex) concentrations of reduced glutathione (GSH),oxidized glutathione (GSSG), and in the reduced/oxidized ratio (GSH/GSSG) in aged beagles during lipid-lowering therapy with atorvastatin (80 mg/day). Parietal cortex levels of (A) GSH, (B) GSSG (C) GSH/GSSG. Data are expressed as mean ± SD of $n = 6$ (controls) and $n = 6$ (atorvastatin-treated) individual samples per group.

showed that atorvastatin did not significantly reduce brain cholesterol or Aβ levels in these aged dogs, despite a significant reduction in plasma cholesterol levels [47]. A lack of significant reduction of brain cholesterol and Aβ may reflect the lower blood–brain barrier penetrance of atorvastatin compared to other statins such as simvastatin [77]. Thus, it is conceivable that the reduced oxidative stress exerted in parietal cortex (Figs. 1 and 2) could be attributable to a modulation of other systems. Increased GSH concentration and elevated GSH/GSSG ratio in parietal cortex secondary to atorvastatin treatment also supports the hypothesis that atorvastatin could exert its pleiotropic actions through multiple pathways.

This study provides novel information regarding the levels of cholesterol oxidation products following chronic atorvastatin administration. Although some evidence suggests the importance of cholesterol oxidation products both as *in vivo* markers of oxidative stress [78–80], as well as for their pro-oxidant features [30–32,81], few studies exist regarding the effect of statins on cholesterol oxidation products *in vivo* [78,82,83]. Moreover, differ-

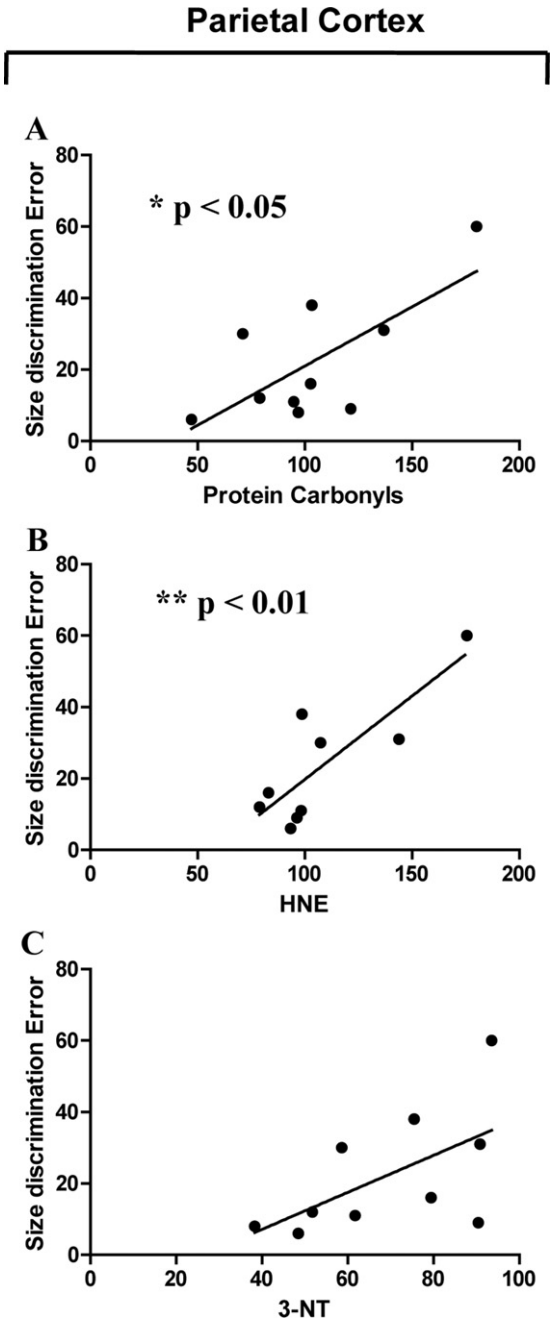

Fig. 5. Correlation between individual oxidative/nitrosative stress markers measured in the parietal cortex and size discrimination learning error scores in aged dogs during lipid-lowering therapy with atorvastatin (80 mg/day). A positive correlations were found between size discrimination error scores and (A) PC (Pearson $r = 0.702$, $p = 0.0236$), (B) protein-bound HNE (Pearson $r = 0.826$, $p = 0.006$). (C) 3-NT (Pearson $r = 0.588$, $p = 0.073$) showed a similar trend but was not statistically significant.

ent effects were observed for cholesterol reduction in brain and plasma of aged dogs treated with atorvastatin [47]. Surprisingly, a reduction of cholesterol was not associated with a reduction of 7-K or 25-OH and *vice versa*. This result suggests that might be two independent effects of atorvastatin. In fact, the absolute levels of both 7-K and 25-OH were reduced in brain, while 7-K absolute levels were increased in serum without change in 25-OH in dogs receiving atorvastatin. Further, after correction for total cholesterol [65,78], we observed consistent effects.

Taken together, these observations suggest a novel mechanism of action for statins that may contribute to reports of a reduced risk of developing AD. It would be interesting to test other statins, with higher blood–brain barrier penetrance, to determine if this is a significant contributor to the current findings. Indeed, the results of the canine study are consistent with a previous study conducted by Garjani et al., who showed that atorvastatin could have both anti- or pro-inflammatory features, which were independent of HMG-CoA reductase inhibition and can be mediated directly by atorvastatin [84].

In conclusion, the results of this study suggest that atorvastatin can exert antioxidant effects in brain independent of its ability to reduce cholesterol and through the activation of the GSH system, which may mediate cognitive benefits.

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References

[1] Halliwell B. Oxidative stress and neurodegeneration: where are we now? J Neurochem 2006;97:1634–58.
[2] Lovell MA, Xie C, Markesbery WR. Acrolein is increased in alzheimer's disease brain and is toxic to primary hippocampal cultures. Neurobiol Aging 2001;22:187–94.
[3] Mark RJ, Lovell MA, Markesbery WR, Uchida K, Mattson MP. A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid beta-peptide. J Neurochem 1997;68:255–64.
[4] Markesbery WR. Oxidative stress hypothesis in alzheimer's disease. Free Radic Biol Med 1997;23:134–47.
[5] Smith MA, Richey PL, Taneda S, Kutty RK, Sayre LM, Monnier VM, et al. Advanced maillard reaction end products, free radicals, and protein oxidation in alzheimer's disease. Ann N Y Acad Sci 1994;738:447–54.
[6] Butterfield DA, Galvan V, Lange MB, Tang H, Sowell RA, Spilman P, et al. In vivo oxidative stress in brain of alzheimer disease transgenic mice: requirement for methionine 35 in amyloid beta-peptide of app. Free Radic Biol Med 2010;48:136–44.
[7] Butterfield DA. Beta-amyloid-associated free radical oxidative stress and neurotoxicity: implications for alzheimer's disease. Chem Res Toxicol 1997;10:495–506.
[8] Butterfield DA, Poon HF, St Clair D, Keller JN, Pierce WM, Klein JB, et al. Redox proteomics identification of oxidatively modified hippocampal proteins in mild cognitive impairment: insights into the development of alzheimer's disease. Neurobiol Dis 2006;22:223–32.
[9] Butterfield DA, Reed T, Newman SF, Sultana R. Roles of amyloid beta-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of alzheimer's disease and mild cognitive impairment. Free Radic Biol Med 2007;43:658–77.
[10] Butterfield DA, Reed T, Perluigi M, De Marco C, Coccia R, Cini C, et al. Elevated protein-bound levels of the lipid peroxidation product, 4-hydroxy-2-nonenal, in brain from persons with mild cognitive impairment. Neurosci Lett 2006;397:170–3.
[11] Hensley K, Hall N, Subramaniam R, Cole P, Harris M, Aksenov M, et al. Brain regional correspondence between alzheimer's disease histopathology and biomarkers of protein oxidation. J Neurochem 1995;65:2146–56.
[12] Lyras L, Cairns NJ, Jenner A, Jenner P, Halliwell B. An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with alzheimer's disease. J Neurochem 1997;68:2061–9.
[13] Varadarajan S, Yatin S, Aksenova M, Butterfield DA. Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. J Struct Biol 2000;130:184–208.
[14] Jicha GA, Parisi JE, Dickson DW, Johnson K, Cha R, Ivnik RJ, et al. Neuropathologic outcome of mild cognitive impairment following progression to clinical dementia. Arch Neurol 2006;63:674–81.
[15] Petersen RC. Mild cognitive impairment clinical trials. Nat Rev Drug Discov 2003;2:646–53.
[16] Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 2001;81:741–66.
[17] Drake J, Link CD, Butterfield DA. Oxidative stress precedes fibrillar deposition of alzheimer's disease amyloid beta-peptide (1–42) in a transgenic caenorhabditis elegans model. Neurobiol Aging 2003;24:415–20.
[18] Lambert JC, Mann DM, Harris JM, Chartier-Harlin MC, Cumming A, Coates J, et al. The −48 c/t polymorphism in the presenilin 1 promoter is associated with an increased risk of developing alzheimer's disease and an increased abeta load in brain. J Med Genet 2001;38:353–5.
[19] Oda T, Wals P, Osterburg HH, Johnson SA, Pasinetti GM, Morgan TE, et al. Clusterin (apoj) alters the aggregation of amyloid beta-peptide (a beta 1–42) and forms slowly sedimenting a beta complexes that cause oxidative stress. Exp Neurol 1995;136:22–31.
[20] Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, et al. Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 1999;274:25945–52.
[21] Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat Rev Neurosci 2007;8:766–75.
[22] Mancuso C, Scapagini G, Curro D, Giuffrida Stella AM, De Marco C, Butterfield DA, et al. Mitochondrial dysfunction, free radical generation and cellular stress response in neurodegenerative disorders. Front Biosci 2007;12:1107–23.
[23] Calabrese V, Sultana R, Scapagnini G, Guagliano E, Sapienza M, Bella R, et al. Nitrosative stress, cellular stress response, and thiol homeostasis in patients with alzheimer's disease. Antioxid Redox Signal 2006;8:1975–86.
[24] Butterfield DA, Lauderback CM. Lipid peroxidation and protein oxidation in alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. Free Radic Biol Med 2002;32:1050–60.
[25] Smith MA, Perry G, Pryor WA. Causes and consequences of oxidative stress in alzheimer's disease. Free Radic Biol Med 2002;32:1049.
[26] Pfrieger FW. Cholesterol homeostasis and function in neurons of the central nervous system. Cell Mol Life Sci 2003;60:1158–71.
[27] Vaya J, Schipper HM. Oxysterols, cholesterol homeostasis, and alzheimer disease. J Neurochem 2007;102:1727–37.
[28] Canevari L, Clark JB. Alzheimer's disease and cholesterol: the fat connection. Neurochem Res 2007;32:739–50.
[29] Benarroch EE. Brain cholesterol metabolism and neurologic disease. Neurology 2008;71:1368–73.
[30] Han JH, Kim YJ, Han ES, Lee CS. Prevention of 7-ketocholesterol-induced mitochondrial damage and cell death by calmodulin inhibition. Brain Res 2007;1137:11–9.
[31] Panini SR, Yang L, Rusinol AE, Sinensky MS, Bonventre JV, Leslie CC. Arachidonate metabolism and the signaling pathway of induction of apoptosis by oxidized LDL/oxysterol. J Lipid Res 2001;42:1678–86.
[32] Trousson A, Bernard S, Petit PX, Liere P, Pianos A, El Hadri K, et al. 25-hydroxycholesterol provokes oligodendrocyte cell line apoptosis and stimulates the secreted phospholipase a2 type iia via lxr beta and pxr. J Neurochem 2009;109:945–58.
[33] Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA. Statins and the risk of dementia. Lancet 2000;356:1627–31.
[34] Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G. Decreased prevalence of alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme a reductase inhibitors. Arch Neurol 2000;57:1439–43.
[35] Rockwood K, Kirkland S, Hogan DB, MacKnight C, Merry H, Verreault R, et al. Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. Arch Neurol 2002;59:223–7.
[36] Hajjar I, Schumpert J, Hirth V, Wieland D, Eleazer GP. The impact of the use of statins on the prevalence of dementia and the progression of cognitive impairment. J Gerontol A Biol Sci Med Sci 2002;57:M414–418.
[37] Rodriguez EG, Dodge HH, Birzescu MA, Stoehr GP, Ganguli M. Use of lipid-lowering drugs in older adults with and without dementia: a community-based epidemiological study. J Am Geriatr Soc 2002;50:1852–6.
[38] Dufouil C, Richard F, Fievet N, Dartigues JF, Ritchie K, Tzourio C, et al. Apoe genotype, cholesterol level, lipid-lowering treatment, and dementia: the three-city study. Neurology 2005;64:1531–8.
[39] Zamrini E, McGwin G, Roseman JM. Association between statin use and alzheimer's disease. Neuroepidemiology 2004;23:94–8.
[40] Wolozin B, Wang SW, Li NC, Lee A, Lee TA, Kazis LE. Simvastatin is associated with a reduced incidence of dementia and parkinson's disease. BMC Med 2007;5:20.
[41] Simons M, Schwarzler F, Lutjohann D, von Bergmann K, Beyreuther K, Dichgans J, et al. Treatment with simvastatin in normocholesterolemic patients with alzheimer's disease: a 26-week randomized, placebo-controlled, double-blind trial. Ann Neurol 2002;52:346–50.
[42] Sparks DL, Connor DJ, Sabbagh MN, Petersen RB, Lopez J, Browne P. Circulating cholesterol levels, apolipoprotein e genotype and dementia severity influence the benefit of atorvastatin treatment in alzheimer's disease: results of the alzheimer's disease cholesterol-lowering treatment (adclt) trial. Acta Neurol Scand Suppl 2006;185:3–7.

[43] Sparks DL, Sabbagh MN, Connor DJ, Lopez J, Launer LJ, Petanceska S, et al. Atorvastatin therapy lowers circulating cholesterol but not free radical activity in advance of identifiable clinical benefit in the treatment of mild-to-moderate ad. Curr Alzheimer Res 2005;2:343–53.
[44] Jones RW, Kivipelto M, Feldman H, Sparks L, Doody R, Waters DD, et al. The atorvastatin/donepezil in alzheimer's disease study (leade): design and baseline characteristics. Alzheimers Dement 2008;4:145–53.
[45] Head E, McCleary R, Hahn FF, Milgram NW, Cotman CW. Region-specific age at onset of beta-amyloid in dogs. Neurobiol Aging 2000;21:89–96.
[46] Studzinski CM, Christie LA, Araujo JA, Burnham WM, Head E, Cotman CW, et al. Visuospatial function in the beagle dog: an early marker of cognitive decline in a model of human aging and dementia. Neurobiol Learn Mem 2006;86:197–204.
[47] Murphy MP, Morales J, Beckett TL, Astarita G, Piomelli D, Weidner A, et al. Changes in cognition and amyloid-beta processing with long term cholesterol reduction using atorvastatin in aged dogs. J Alzheimers Dis 2010.
[48] Walsh KM, Albassam MA, Clarke DE. Subchronic toxicity of atorvastatin, a hydroxymethylglutaryl-coenzyme a reductase inhibitor, in beagle dogs. Toxicol Pathol 1996;24:468–76.
[49] Cilla Jr DD, Whitfield LR, Gibson DM, Sedman AJ, Posvar EL. Multiple-dose pharmacokinetics, pharmacodynamics, and safety of atorvastatin, an inhibitor of hmg-coa reductase, in healthy subjects. Clin Pharmacol Ther 1996;60:687–95.
[50] Lea AP, McTavish D, Atorvastatin. A review of its pharmacology and therapeutic potential in the management of hyperlipidaemias. Drugs 1997;53:828–47.
[51] Stern RH, Yang BB, Hounslow NJ, MacMahon M, Abel RB, Olson SC. Pharmacodynamics and pharmacokinetic-pharmacodynamic relationships of atorvastatin, an hmg-coa reductase inhibitor. J Clin Pharmacol 2000;40:616–23.
[52] Shen HR, Li ZD, Zhong MK. Hplc assay and pharmacokinetic study of atorvastatin in beagle dogs after oral administration of atorvastatin self-microemulsifying drug delivery system. Pharmazie 2006;61:18–20.
[53] Palozza P, Barone E, Mancuso C, Picci N. The protective role of carotenoids against 7-keto-cholesterol formation in solution. Mol Cell Biochem 2008;309:61–8.
[54] Chen BH, Chen YC. Evaluation of the analysis of cholesterol oxides by liquid-chromatography. J Chromatogr A 1994;661:127–36.
[55] Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976;74:214–26.
[56] Johnstone EM, Chaney MO, Norris FH, Pascual R, Little SP. Conservation of the sequence of the alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis. Brain Res Mol Brain Res 1991;10:299–305.
[57] Torp R, Head E, Cotman CW. Ultrastructural analyses of beta-amyloid in the aged dog brain: neuronal beta-amyloid is localized to the plasma membrane. Prog Neuro-Psychopharmacol Biol Psychiatry 2000;24:801–10.
[58] Torp R, Head E, Milgram NW, Hahn F, Ottersen OP, Cotman CW. Ultrastructural evidence of fibrillar β-amyloid associated with neuronal membranes in behaviorally characterized aged dog brains. Neuroscience 2000;93:495–506.
[59] Torp R, Ottersen OP, Cotman CW, Head E. Identification of neuronal plasma membrane microdomains that colocalize beta-amyloid and presenilin: implications for beta-amyloid precursor protein processing. Neuroscience 2003;120:291–300.
[60] Cotman CW, Head E. The canine (dog) model of human aging and disease: dietary, environmental and immunotherapy approaches. J Alzheimers Dis 2008;15:685–707.
[61] Hamelin BA, Turgeon J. Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of hmg-coa reductase inhibitors. Trends Pharmacol Sci 1998;19:26–37.
[62] Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005;366:1267–78.
[63] Wang CY, Liu PY, Liao JK. Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. Trends Mol Med 2008;14:37–44.
[64] Liao JK, Laufs U. Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 2005;45:89–118.
[65] Liu CS, Lii CK, Chang LL, Kuo CL, Cheng WL, Su SL, et al. Atorvastatin increases blood ratios of vitamin e/low-density lipoprotein cholesterol and coenzyme q10/low-density lipoprotein cholesterol in hypercholesterolemic patients. Nutr Res 2010;30:118–24.
[66] Li J, Sun YM, Wang LF, Li ZQ, Pan W, Cao HY. Comparison of effects of simvastatin versus atorvastatin on oxidative stress in patients with coronary heart disease. Clin Cardiol 2010;33:222–7.
[67] Wang ZH, Liu XL, Zhong M, Zhang LP, Shang YY, Hu XY, et al. Pleiotropic effects of atorvastatin on monocytes in atherosclerotic patients. J Clin Pharmacol 2010;50:311–9.
[68] Bolayirli IM, Aslan M, Balci H, Altug T, Hacibekiroglu M, Seven A. Effects of atorvastatin therapy on hypercholesterolemic rabbits with respect to oxidative stress, nitric oxide pathway and homocysteine. Life Sci 2007;81:121–7.
[69] Haendeler J, Hoffmann J, Zeiher AM, Dimmeler S. Antioxidant effects of statins via s-nitrosylation and activation of thioredoxin in endothelial cells: a novel vasculoprotective function of statins. Circulation 2004;110:856–61.
[70] Simons M, Keller P, Strooper BD, Beyreuther K, Dotti CG, Simons K. Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proc Natl Acad Sci USA 1998;95:6460–4.
[71] Refolo LM, Malester B, LaFrancois J, Bryant-Thomas T, Wang R, Tint GS, et al. Hypercholesterolemia accelerates the alzheimer's amyloid pathology in a transgenic mouse model. Neurobiol Dis 2000;7:321–31.
[72] Refolo LM, Pappolla MA, LaFrancois J, Malester B, Schmidt SD, Thomas-Bryant T, et al. A cholesterol-lowering drug reduces beta-amyloid pathology in a transgenic mouse model of alzheimer's disease. Neurobiol Dis 2001;8:890–9.
[73] Petanceska SS, DeRosa S, Olm V, Diaz N, Sharma A, Thomas-Bryant T, et al. Statin therapy for alzheimer's disease: will it work? J Mol Neurosci 2002;19:155–61.
[74] Thelen KM, Rentsch KM, Gutteck U, Heverin M, Olin M, Andersson U, et al. Brain cholesterol synthesis in mice is affected by high dose of simvastatin but not of pravastatin. J Pharmacol Exp Ther 2006;316:1146–52.
[75] Head E. Neurobiology of the aging dog. Age (Dordr) 2010.
[76] McGuinness B, O'Hare J, Craig D, Bullock R, Malouf R, Passmore P. Statins for the treatment of dementia. Cochrane Database Syst Rev 2010. CD007514.
[77] Saheki A, Terasaki T, Tamai I, Tsuji A. In vivo and in vitro blood–brain barrier transport of 3-hydroxy-3-methylglutaryl coenzyme a (hmg-coa) reductase inhibitors. Pharm Res 1994;11:305–11.
[78] Arca M, Natoli S, Micheletta F, Riggi S, Di Angelantonio E, Montali A, et al. Increased plasma levels of oxysterols, in vivo markers of oxidative stress, in patients with familial combined hyperlipidemia: reduction during atorvastatin and fenofibrate therapy. Free Radic Biol Med 2007;42:698–705.
[79] Kathir K, Dennis JM, Croft KD, Mori TA, Lau AK, Adams MR, et al. Equivalent lipid oxidation profiles in advanced atherosclerotic lesions of carotid endarterectomy plaques obtained from symptomatic type 2 diabetic and nondiabetic subjects. Free Radic Biol Med 2010;49:481–6.
[80] Larsson H, Bottiger Y, Iuliano L, Diczfalusy U. In vivo interconversion of 7beta-hydroxycholesterol and 7-ketocholesterol, potential surrogate markers for oxidative stress. Free Radic Biol Med 2007;43:695–701.
[81] Berthier A, Lemaire-Ewing S, Prunet C, Monier S, Athias A, Bessede G, et al. Involvement of a calcium-dependent dephosphorylation of bad associated with the localization of trpc-1 within lipid rafts in 7-ketocholesterol-induced thp-1 cell apoptosis. Cell Death Differ 2004;11:897–905.
[82] Kumar BS, Chung BC, Lee YJ, Yi HJ, Lee BH, Jung BH. A gc/ms-based simultaneous quantitative analytical method for urinary oxysterols and bile acids in rats. Anal Biochem 2010.
[83] Thelen KM, Laaksonen R, Paiva H, Lehtimaki T, Lutjohann D. High-dose statin treatment does not alter plasma marker for brain cholesterol metabolism in patients with moderately elevated plasma cholesterol levels. J Clin Pharmacol 2006;46:812–6.
[84] Garjani A, Andalib S, Ziaee M, Maleki-Dizaji N. Biphasic effects of atorvastatin on inflammation. Pak J Pharm Sci 2008;21:125–30.