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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.

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Keywords

Alzheimer disease; Atorvastatin; Cholesterol oxidation products; Oxidative stress; Cognitive function

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 (A β), 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 A β (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 A β 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 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], Ca²⁺ 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 8.9–13.2 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 A β [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 10.1–14.6 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 $\times$ 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 10cc 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 minutes, the brain was removed from the skull and sectioned midsagittally. The intact left hemisphere was immediately placed in 4% paraformaldehyde for 48–72 hr 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 minute 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 MgCl₂, 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 hr 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 hr. 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 1:8,000 ratio for 1 hr. 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 1:5,000 dilution of anti protein- bound

HNE polyclonal antibody or 1:2,000 3-NT antibody in PBS for 1 hr 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 $4000 \times g$ for 10 minutes 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 $\times$ 25 cm, 5 μ m 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 minutes. 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 μ M) in methanol. By this method, a linear fitting ($r^2 = 0.99$) has been obtained.

2.12 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 deproteinized with 10% meta-phosphoric acid, and after a centrifugation at $100,000 \times g$ for 30 min at 4°C, the deproteinized supernatant was used for GSH and GSSG assays. Reduced glutathione was measured by adding deproteinized sample (10 μ l) 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, fluorescence at emission 420 nm was recorded following excitation at 350 nm. Oxidized glutathione was measured by adding 10 μ l of deproteinized sample 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, fluorescence at emission 420 nm was recorded by excitation at 350 nm.

2.13 Statistical analysis

Data are expressed as mean $\pm$ 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 Figures 1A, 1C, and 1E, 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 Figure 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 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%) ($t=1.45$, $df=9$, $p=0.18$) (Figure 2, Panel A) and 25-OH (− 18%) ($t=1.19$, $df=9$, $p=0.26$) (Figure 2, Panel C) in parietal cortex. Conversely, as shown in Figure 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 Figure 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%) ($t=3.6$, $df=9$, $p=0.006$) (Figure 3, Panel B), while no change in the blood ratio of 25-OH/total cholesterol ($t=0.11$, $df=9$, $p=0.92$) (Figure 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 Figure 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 $r = 0.588$, $p = 0.073$) (Figure 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 A β of identical sequence to human A β [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 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 A β 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 A β [70]. High dietary cholesterol in transgenic mouse models of AD leads to increases in brain A β [71]. In contrast, reducing cholesterol [72] or treatment with statins can reduce A β [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 (Figure 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] showed that atorvastatin did not significantly reduce brain cholesterol or A β levels in these aged dogs, despite a significant reduction in plasma cholesterol levels [47]. A lack of significant reduction of brain cholesterol and A β 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 (Figure 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, different 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.

Non standard abbreviation

PC	protein carbonyls
HNE	4-hydroxy-2-nonenal
3-NT	3-nitrotyrosine
A β	amyloid beta peptide
7-K	7-ketocholesterol

25-OH 25-hydroxy cholesterol

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Figure 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 $\pm$ SD of three replicates for each of individual control and atorvastatin treated beagle, per group. * $P < 0.05$ versus control (Student's t-test).

Figure 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 $\pm$ SD of n=5 (controls) and n=6 (atorvastatin-treated) individual samples per group.

Figure 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 $\pm$ SD of n=5 (controls) and n=6 (atorvastatin-treated) individual samples per group. **P < 0.01 versus control (Student's t-test).

Figure 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 $\pm$ SD of $n=6$ (controls) and $n=6$ (atorvastatin-treated) individual samples per group.

Figure 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.