International Journal of Neuropsychopharmacology (2012), **15**, 981–987. © CINP 2011
doi:10.1017/S1461145711001118

RAPID COMMUNICATION

CINP

Atorvastatin treatment in a dog preclinical model of Alzheimer's disease leads to up-regulation of haem oxygenase-1 and is associated with reduced oxidative stress in brain

D. Allan Butterfield[1], Eugenio Barone[1,2], Fabio Di Domenico[1,3], Giovanna Cenini[1,4], Rukhsana Sultana[1], Michael P. Murphy[4], Cesare Mancuso[2] and Elizabeth Head[5]

[1] *Department of Chemistry, Center of Membrane Sciences, Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY, USA*

[2] *Institute of Pharmacology, Catholic University School of Medicine, Rome, Italy*

[3] *Department of Biochemical Sciences, Sapienza University of Rome, Rome, Italy*

[4] *Department of Molecular and Cellular Biochemistry, Sanders–Brown Center on Aging, University of Kentucky, Lexington, KY, USA*

[5] *Department of Molecular and Biomedical Pharmacology, Sanders–Brown Center on Aging, University of Kentucky, Lexington, KY, USA*

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive impairment and neuropathology. Only acetylcholinesterase inhibitors and the NMDA antagonist memantine are approved for AD treatment. Recent preclinical and epidemiological studies proposed statins as novel therapeutics for AD, but the mechanisms of action are still unknown. Here, we demonstrate that atorvastatin (80 mg/d for 14.5 months) treatment resulted in an up-regulation of the inducible isoform of haem oxygenase (HO-1), an enzyme with significant neuroprotective activity. Atorvastatin selectively increased HO-1 in the parietal cortex but not cerebellum. In contrast, HO-2 was increased in cerebellum but not parietal cortex. No changes were observed in HO-1 or HO-2 in the liver. Significant negative correlations between HO-1 and oxidative stress indices and positive correlations with glutathione levels in parietal cortex were found. HO-1 up-regulation significantly correlated with lower discrimination learning error scores in aged beagles. Reference to therapeutic applications of atorvastatin in AD is discussed.

Received 6 May 2011; Reviewed 6 June 2011; Revised 14 June 2011; Accepted 14 June 2011; First published online 18 July 2011

Key words: Alzheimer's disease, atorvastatin, haem oxygenase-1, oxidative stress.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss, inability to perform activities of daily living and personality changes. The main pathological features of AD are senile plaques (SP), characterized primarily by the deposition of fibrillar β-amyloid, and neurofibrillary tangles (NFT) formed by hyperphosphorylated tau protein (Mirra, 1997). Both SP and NFT have been linked to generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and damage to lipid, proteins and nucleic acids that may ultimately lead to neuronal cell death (Butterfield & Lauderback, 2002). Unfortunately, only a few drugs are approved for the treatment of AD, which include the acetylcholinesterase inhibitors donepezil, galantamine, rivastigmine and the NMDA receptor antagonist memantine. These drugs provide modest relief from symptoms such as those related to memory loss and inability to perform activities of daily living and this beneficial effect is lost within 1–2 yr from the beginning of treatment (Chopra *et al.* 2011).

Address for correspondence: Professor D. A. Butterfield, Department of Chemistry, Center of Membrane Sciences, Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506-0055, USA.
Tel.: 859-257-3184 *Fax*: 859-259-5876
Email: dabcns@uky.edu

Over the last few years, statins, a class of drugs which act primarily by reducing blood cholesterol and triglyceride levels, have been proposed as potential therapeutic agents for the treatment of AD (Kandiah & Feldman, 2009). Data from preclinical studies corroborated the beneficial role for atorvastatin in reducing β-secretase protein level (Murphy *et al.* 2010) and oxidative stress biomarkers in the parietal cortex of aged beagles (Barone *et al.* 2011*a*). In the same experimental model, atorvastatin potentiated antioxidant defence by increasing reduced glutathione concentration and reduced/oxidized glutathione ratio (Barone *et al.* 2011*a*). The potential therapeutic role for statins in AD and other forms of dementia was also proposed on the basis of epidemiological studies showing that individuals taking statins are at reduced risk for developing AD (Jick *et al.* 2000). However, the importance of statin treatment in AD is still under debate since some randomized clinical trials did not show any significant benefit on cognition (McGuinness *et al.* 2010). These results suggest that statins may protect against AD but not be useful as a therapeutic. The mechanism(s) through which statins may protect against AD are not completely understood. Statins were shown to (i) interfere with isoprenoid synthesis and further modulation of members of the Rho/Ras family, (ii) prevent the overexpression of pro-inflammatory cytokines, (iii) stimulate pro-survival intracellular systems such as the PI3K/Akt pathway and (iv) reduce oxidative stress biomarkers in the brain (Barone *et al.* 2011*a*; Blum & Shamburek, 2009). In addition to these pleiotropic effects, statins may counteract free radical-induced damage by increasing the expression of key proteins involved in the cell stress response. Particularly interesting is the ability of statins to modulate the haem oxygenase/biliverdin reductase (HO/BVR) axis, which plays a pivotal role in the maintenance of cellular homeostasis by reducing pro-inflammatory haem and generating cytoprotective molecules including carbon monoxide and bilirubin (Mancuso & Barone, 2009). A single dose of simvastatin, lovastatin, atorvastatin, and rosuvastatin increased HO activity in rat heart, lung, and liver tissues (Hsu *et al.* 2006; Lai *et al.* 2008). This effect appears to be protective since the administration of Zn-PP-IX increases simvastatin toxicity, as manifested by elevated alanine transaminase levels (Hsu *et al.* 2006; Lai *et al.* 2008).

The aim of this work was to evaluate, in a preclinical model of AD (the aged canine), whether chronic administration of atorvastatin could have neuroprotective effects through the up-regulation of both the inducible and constitutive isoforms of HO, named HO-1 and HO-2, respectively. In addition, the possible correlations between HO-1 or HO-2 expression, levels of oxidative damage and cognitive were evaluated.

Materials and methods

Animals

Eight beagles ranging in age from 8.9 to 13.2 yr were obtained from the Lovelace Respiratory Research Institute and Harlan (USA). Based on our previous work, dogs of this age show cognitive decline and significant amounts of brain Aβ (Head *et al.* 2000). 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 years. All research was conducted in accordance with approved IACUC protocols following NIH guidelines. Animals were ranked by cognitive test scores and placed into equivalent groups. These groups were randomly designated as either the placebo-treated control group or the atorvastatin-treated group.

Cognitive testing

Animals were given a series of cognitive tests while on treatment as described previously (Murphy *et al.* 2010). For the current study, scores from the size discrimination learning problem were used as they were obtained after 6 months of treatment and were sensitive to treatment effects.

Drug treatment

Atorvastatin calcium (also known as Lipitor®, 40 mg tablets) and placebo tablets were kindly provided by Pfizer Inc. (USA). Atorvastatin-treated animals received 2×40 mg tablets per day for a daily dose of 80 mg, and control animals received two placebo tablets per day. Atorvastatin was chosen for this study because long-term studies using an 80 mg/d dose in dogs did not report adverse events such as cataracts (Walsh *et al.* 1996). This dose is still acceptable and allows a translational approach of the study. As previously demonstrated, in beagle dogs treated with 6 mg/kg atorvastatin (about 90 mg/dog) drug plasma concentration was about 500 ng/ml (Shen *et al.* 2006) and this figure is in the same order of magnitude as those achieved in hypercholesterolaemic people treated with atorvastatin 80 mg/d (187–252 ng/ml) (Cilla *et al.* 1996; Stern *et al.* 2000). On the contrary, in

rodent studies reporting a reduction of brain Aβ in response to statin treatment, doses are typically between 200 and 400 times higher than those used in humans, which leads to some concern regarding the translation of these outcomes to AD clinical trials (see below).

Tissue collection and sample preparation

Brain tissues (parietal cortex and cerebellum) and liver samples were collected, frozen and prepared for Western blot analysis as described previously (Barone *et al.* 2011*a*; Murphy *et al.* 2010).

Western blot analysis

Western Blot analysis was performed as described previously (Barone *et al.* 2011*b*). The following primary antibodies were used: polyclonal anti-rabbit HO-1 (Assay design – Stressgen, USA, dilution 1:1000); polyclonal anti-rabbit HO-2 (Assay design – Stressgen, dilution 1:1000); and polyclonal anti-rabbit β-actin (Sigma-Aldrich, USA, dilution 1:2000).

Assays for oxidative stress biomarkers

For a detailed description of oxidative stress biomarker (protein carbonyls, 4-hydroxy-nonenal, 3-nitrotyrosine, and 7-ketocholesterol) assays, see Barone *et al.* (2011*a*).

Reduced glutathione (GSH) assay

Determination of GSH was performed as described previously (Barone *et al.* 2011*a*).

Statistical analysis

Data are expressed as mean $\pm$ S.D. of N individual samples per group. All statistical analysis were 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 HO-1 and (i) markers of oxidative damage, (ii) GSH concentration and (iii) cognitive test scores.

Results

Atorvastatin effects on HO-1 and HO-2 protein levels

Atorvastatin (80 mg/kg.d for 14.5 months) significantly increased HO-1 protein levels by approximately 75% in the parietal cortex of aged beagles (Fig. 1*a*). There was no effect on the level of constitutive HO-2 in the same brain area (Fig. 1*b*). Conversely, a significant up-regulation of HO-2 ($\sim$57%) with no significant increases in HO-1 was observed in the cerebellum of atorvastatin-treated beagles (Fig. 1*c*, *d*). No changes were found in liver between atorvastatin-treated and control group (data not shown).

Effect of atorvastatin treatment on the levels of oxidative and nitrosative stress markers in cerebellum and liver

Chronic administration of atorvastatin (80 mg/d for 14.5 months) was followed by a significant reduction of oxidative and nitrosative stress biomarkers in parietal cortex of aged beagles (Barone *et al.* 2011*a*). In order to evaluate whether this antioxidant effect was restricted only to the parietal cortex, in the current study oxidative stress biomarkers were extended to include the cerebellum. Atorvastatin had no effect on protein carbonyls (PC), 4-hydroxy-2-nonenal (HNE) and 3-nitrotyrosine (3-NT) levels in the cerebellum of aged beagles compared to the control group (data not shown). In addition, atorvastatin did not significantly modify oxidative stress biomarkers in the liver, the main target of statins' activity (data not shown).

Atorvastatin-induced changes in HO-1 protein levels in parietal cortex are correlated with oxidative stress levels and learning scores

We next hypothesized that reduced parietal cortex oxidative damage in response to atorvastatin would be associated with the expression of HO-1. Higher levels of HO-1 were associated with lower HNE-bound protein (Pearson's $r=-0.75$, $p=0.04$) and 7-ketocholesterol [absolute levels (Pearson's $r=-0.85$, $p=0.02$); ratio with total cholesterol (Pearson's $r=-0.76$, $p<0.05$)], two well-known markers of oxidative stress (Fig. 2*a–c*). In addition, a significant positive association between higher HO-1 levels and GSH concentration (Pearson's $r=0.857$, $p=0.023$) was observed in the same brain region (Fig. 2*d*).

The association between HO-1 protein levels and size discrimination learning error scores across treatment and control groups was analysed. Interestingly, size discrimination learning error scores were negatively correlated with parietal cortex HO-1 (Pearson's $r=-0.74$, $p=0.04$) (Fig. 2*e*), suggesting that lower HO-1 is associated with poorer learning. A significant correlation also exists between HO-2 and size discrimination learning error scores (Pearson's $r=-0.75$, $p=0.03$) but this was primarily due to one animal showing a high error score and very low levels of HO-2 (Fig. 2*f*).

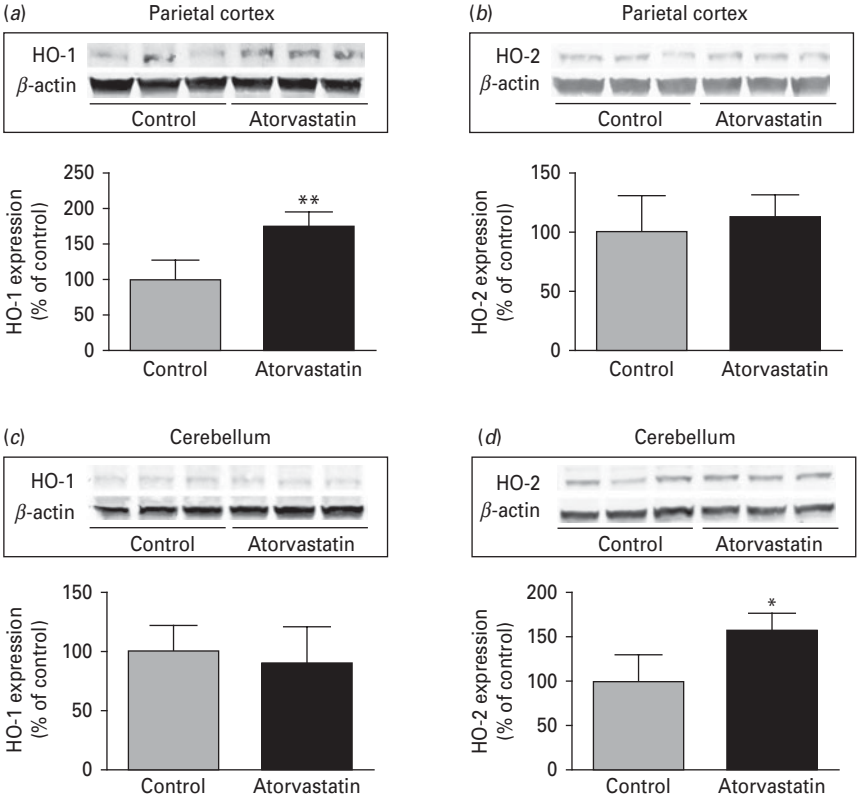

Fig. 1. HO-1 and HO-2 protein levels in the parietal cortex and cerebellum of aged beagles treated with atorvastatin (80 mg/d). Brain samples of parietal cortex and cerebellum from aged beagles treated with atorvastatin (80 mg/d) were assayed for (*a*, *c*) HO-1 or (*b*, *d*) HO-2 by Western blot as described in the Materials and methods section. Densitometric values shown by the bars are given as percentage of control, set as 100%, and are the product of the band value of the levels of each protein normalized per β-actin as loading control. In panels (*a*)–(*d*) representative gels are shown. Data are expressed as mean ± S.D. of four individual samples per group. * $p < 0.05$ and ** $p < 0.01$ *vs.* control (Student's *t* test). HNE, 4-hydroxy-2-nonenal; 7-K, 7-7-ketocholesterol.

Discussion

Epidemiological studies suggest that statin use is associated with reduced risk of developing AD (Feldman *et al.* 2010). However, the mechanisms through which these drugs may protect the brain has not been clearly established based upon research using rodent models. Rodents respond to statin treatment in a bi-phasic way: initially HMG-CoA reductase is inhibited but after chronic treatment the enzyme undergoes up-regulation. The net effect is to prevent any stable, long-term reduction in cholesterol levels (Thelen *et al.* 2006). This leads to difficulties in conducting long-term studies in rodents with extensive behavioural 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 ageing and disease and show cognitive and neurological changes with age that are consistent with humans (Head, in press). In particular, by using this pre-clinical model of AD long-term studies with statins can be conducted since HMG-CoA reductase is not up-regulated.

Previous studies from our laboratories showed that atorvastatin exerts antioxidant effects in the brain independently of its ability to reduce cholesterol and through the activation of the GSH system, which are linked to lower error scores on a size discrimination learning problem (Barone *et al.* 2011*a*).

On this basis, statins can be considered as 'pathogenetic' drugs since they could interfere with the free radical-induced oxidative damage.

In this study, we extend the neurobiological benefits of atorvastatin to include (i) potentiation of the cell stress response and (ii) observed the selectivity of the atorvastatin-mediated reduction of oxidative stress biomarkers in the brain to an area involved in cognitive function, the parietal cortex, but not the cerebellum.

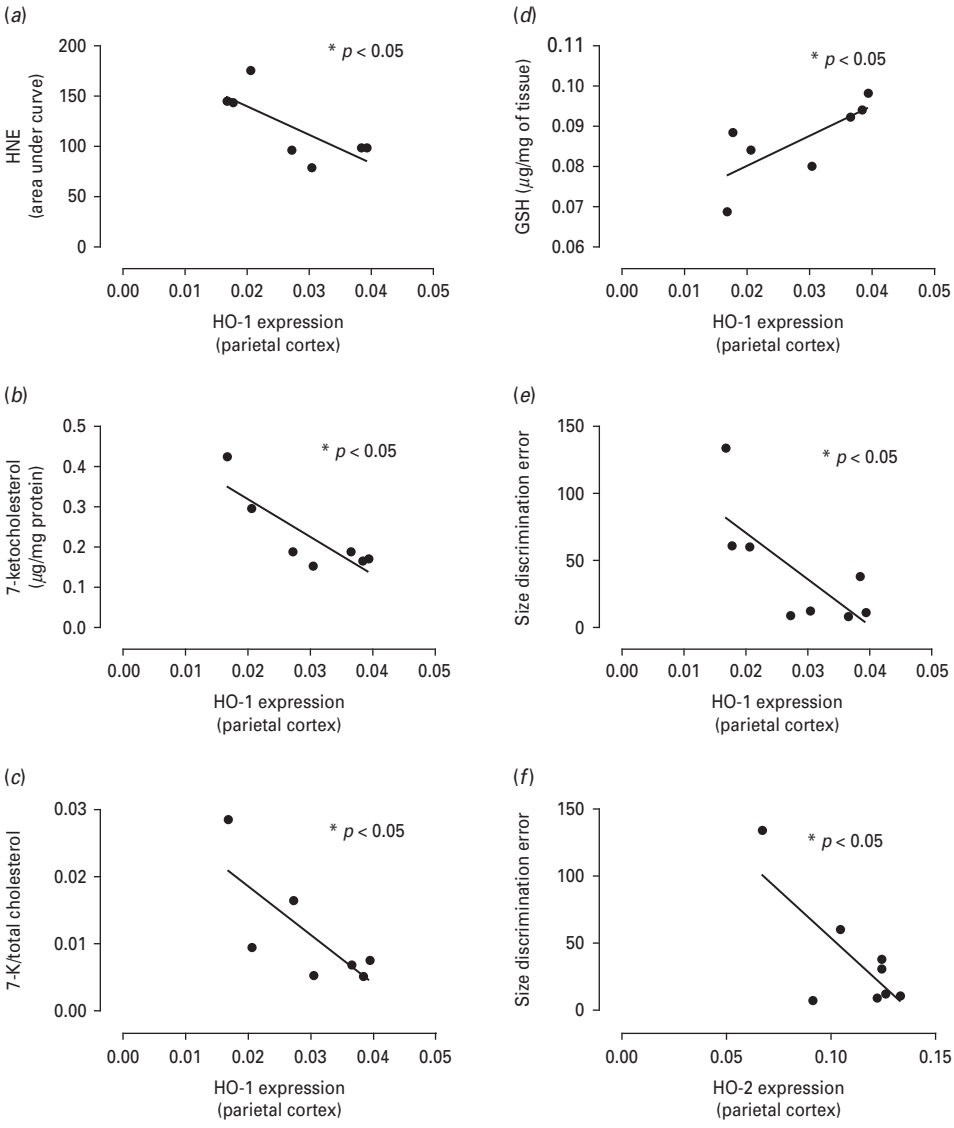

Fig. 2. Correlation between HO-1 or HO-2 protein levels and (i) oxidative stress biomarkers, (ii) reduced glutathione (GSH) concentration, and (iii) size discrimination learning error scores in the parietal cortex from aged beagles treated with atorvastatin (80 mg/d). Negative correlations were found between HO-1 protein levels and oxidative stress biomarkers such as (*a*) total 4-hydroxy-2-nonenal (HNE)-bound proteins; (*b*) absolute levels of 7-ketocholesterol and (*c*) the levels of 7-ketocholesterol normalized for total cholesterol levels measured in parietal cortex. A positive correlation was found between (*d*) HO-1 protein levels and increased GSH levels. Finally a negative correlation was found between (*e*) HO-1 protein levels or (*f*) HO-2 protein levels and size discrimination error scores.

HO is one of the major systems involved in the adaptive cell response to stress (Maines, 1997; Mancuso & Barone, 2009). HO exists in two main isoforms named HO-1 and HO-2. HO-1 is the inducible isoform and is up-regulated in response to oxidative and nitrosative stress or some pharmacological treatments. Conversely, HO-2 is the constitutive isoform and is involved in the physiological turnover of haem. Both isoforms catalyse the same reaction, the oxidation of haem, to generate equimolar amounts of iron, carbon monoxide and biliverdin, the latter being the precursor of a powerful antioxidant, bilirubin (Maines, 1997; Mancuso & Barone, 2009). Due to its abundance in brain tissue and immediate and robust response to oxidative stress, HO-1 has been studied for its possible role in preventing neurodegeneration that occurs in AD patients. The results of the current study provide further evidence in support of this hypothesis as

atorvastatin-induced HO-1 up-regulation is associated with a significant reduction of oxidative stress biomarkers (HNE and 7-ketocholesterol) only in the parietal cortex. Interestingly, the significant correlations found between HO-1 overexpression and lower size discrimination error scores, observed in aged dogs after treatment with atorvastatin (Fig. 2) led us to speculate that the effect on cognition could be due not only to an HO-1-mediated reduction of oxidative stress but also to the generation of carbon monoxide, one of the by-products of HO activity, which plays an important role in the maintenance of synaptic plasticity (Zhuo *et al.* 1993). Another intriguing correlation was between higher HO-1 levels with higher GSH intracellular levels. These results were not surprising as the expression of both HO-1 and γ-glutamylcysteine synthetase, a key enzyme in GSH synthesis, is mediated by the transcription factor Nuclear Factor (erythroid-derived 2)-like 2 (Nrf2) (Lu, 2009). In turn, statins stimulate Nrf2 in several experimental systems, including cultured neurons (Hsieh *et al.* 2008). Thus, the neuroprotective effect of atorvastatin could be attributable to the coordinated increase of both HO-1 and GSH synthesis. Furthermore, Collinson *et al.* (2011) recently provided a new point of view about the mechanism of action of HO-1 which functions more than a catabolic and antioxidant enzyme. In particular, they showed that antioxidant activity of HO-1 is dependent on the up-regulation of several genes encoding for antioxidant enzymes such as γ-glutamylcysteine synthetase, glutathione peroxidase, catalase, and methionine sulfoxide reductase. In light of this, the coordinate increase of HO-1 and GSH observed following statin treatment, could be explained not only by the effect of statins on Nrf-2, but also by the HO-1-induced up-regulation of γ-glutamylcysteine synthetase.

Another novel finding of this study is atorvastatin-induced differential modulation of HO isoforms in the brain. While HO-1 is up-regulated in parietal cortex, HO-2 is increased in the cerebellum. This pattern of expression seems to be specific for brain, since no difference was observed in the liver, the main target organ for statins. However, increased HO-2 in the cerebellum was not paralleled by a concomitant reduction of oxidative stress biomarkers in this brain area. A possible explanation for this discrepancy, resides in the different degree of deposition of the pro-oxidant Aβ (Head *et al.* 2000). At this time, we cannot rule out the ultimate significance of the up-regulation of cerebellar HO-2 and *ad-hoc*-designed experiments are ongoing in our laboratories.

Acknowledgements

This research was supported in part by a NIH grant to D.A.B. [AG-05119] and by the Alzheimer's Association (IIRG 035673 to E.H. and M.P.M.).

Statement of Interest

None.

References

Barone E, Cenini G, Di Domenico F, Martin S, *et al.* (2011*a*). Long-term high-dose atorvastatin decreases brain oxidative and nitrosative stress in a preclinical model of Alzheimer disease: a novel mechanism of action. *Pharmacological Research* **63**, 172–180.

Barone E, Di Domenico F, Cenini G, Sultana R, *et al.* (2011*b*). Biliverdin reductase – a protein levels and activity in the brains of subjects with Alzheimer disease and mild cognitive impairment. *Biochimica et Biophysica Acta* **1812**, 480–487.

Blum A, Shamburek R (2009). The pleiotropic effects of statins on endothelial function, vascular inflammation, immunomodulation and thrombogenesis. *Atherosclerosis* **203**, 325–330.

Butterfield DA, Lauderback CM (2002). Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. *Free Radical Biology and Medicine* **32**, 1050–1060.

Chopra K, Misra S, Kuhad A (2011). Current perspectives on pharmacotherapy of Alzheimer's disease. *Expert Opinion on Pharmacotherapy* **12**, 335–350.

Cilla DD, Whitfield LR, Gibson DM, Sedman AJ, *et al.* (1996). Multiple-dose pharmacokinetics, pharmacodynamics, and safety of atorvastatin, an inhibitor of HMG-CoA reductase, in healthy subjects. *Clinical Pharmacology and Therapeutics* **60**, 687–695.

Collinson EJ, Wimmer-Kleikamp S, Gerega SK, Yang YH, *et al.* (2011). The yeast homolog of heme oxygenase-1 affords cellular antioxidant protection via the transcriptional regulation of known antioxidant genes. *Journal of Biological Chemistry* **286**, 2205–2214.

Feldman HH, Doody RS, Kivipelto M, Sparks DL, *et al.* (2010). Randomized controlled trial of atorvastatin in mild to moderate Alzheimer disease: LEADe. *Neurology* **74**, 956–964.

Head E (in press). Neurobiology of the aging dog. *Age* (*Dordrecht*).

Head E, McCleary R, Hahn FF, Milgram NW, *et al.* (2000). Region-specific age at onset of beta-amyloid in dogs. *Neurobiology of Aging* **21**, 89–96.

Hsieh CH, Rau CS, Hsieh MW, Chen YC, *et al.* (2008). Simvastatin-induced heme oxygenase-1 increases apoptosis of Neuro 2A cells in response to glucose deprivation. *Toxicological Sciences* **101**, 112–121.

Hsu M, Muchova L, Morioka I, Wong RJ, *et al.* (2006). Tissue-specific effects of statins on the expression of heme oxygenase-1 *in vivo*. *Biochemical and Biophysical Research Communication* **343**, 738–744.

Jick H, Zornberg GL, Jick SS, Seshadri S, *et al.* (2000). Statins and the risk of dementia. *Lancet* **356**, 1627–1631.

Kandiah N, Feldman HH (2009). Therapeutic potential of statins in Alzheimer's disease. *Journal of Neurological Sciences* **283**, 230–234.

Lai IR, Chang KJ, Tsai HW, Chen CF (2008). Pharmacological preconditioning with simvastatin protects liver from ischemia-reperfusion injury by heme oxygenase-1 induction. *Transplantation* **85**, 732–738.

Lu SC (2009). Regulation of glutathione synthesis. *Molecular Aspects of Medicine* **30**, 42–59.

Maines MD (1997). The heme oxygenase system: a regulator of second messenger gases. *Annual Review in Pharmacology and Toxicology* **37**, 517–554.

Mancuso C, Barone E (2009). The heme oxygenase/biliverdin reductase pathway in drug research and development. *Current Drug Metabolism* **10**, 579–594.

McGuinness B, O'Hare J, Craig D, Bullock R, *et al.* (2010). Statins for the treatment of dementia. *Cochrane Database of Systematic Reviews*. Issue No. 8. Art. No. CD007514.

Mirra SS (1997). The CERAD neuropathology protocol and consensus recommendations for the postmortem diagnosis of Alzheimer's disease: a commentary. *Neurobiology of Aging* **18** (Suppl. 4), S91–S94.

Murphy MP, Morales J, Beckett TL, Astarita G, *et al.* (2010). Changes in cognition and amyloid-beta processing with long term cholesterol reduction using atorvastatin in aged dogs. *Journal of Alzheimer's Disease* **22**, 135–150.

Shen HR, Li ZD, Zhong MK (2006). HPLC assay and pharmacokinetic study of atorvastatin in beagle dogs after oral administration of atorvastatin self-microemulsifying drug delivery system. *Pharmazie* **61**, 18–20.

Stern RH, Yang BB, Hounslow NJ, MacMahon M, *et al.* (2000). Pharmacodynamics and pharmacokinetic-pharmacodynamic relationships of atorvastatin, an HMG-CoA reductase inhibitor. *Journal of Clinical Pharmacology* **40**, 616–623.

Thelen KM, Rentsch KM, Gutteck U, Heverin M, *et al.* (2006). Brain cholesterol synthesis in mice is affected by high dose of simvastatin but not of pravastatin. *Journal of Pharmacology and Experimental Therapeutics* **316**, 1146–1152.

Walsh KM, Albassam MA, Clarke DE (1996). Subchronic toxicity of atorvastatin, a hydroxymethylglutaryl-coenzyme A reductase inhibitor, in beagle dogs. *Toxicologic Pathology* **24**, 468–476.

Zhuo M, Small SA, Kandel ER, Hawkins RD (1993). Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. *Science* **260**, 1946–1950.