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Biliverdin Reductase-A correlates with inducible nitric oxide synthase in atorvastatin treated aged canine brain

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Research Highlights

- (1) Atorvastatin increases inducible nitric oxide synthase protein levels in the parietal cortex whereas decreases inducible nitric oxide synthase protein levels in the liver of aged beagles
- (2) Up-regulation of inducible nitric oxide synthase and heme oxygenase-1 are positively associated with biliverdin reductase-A protein levels and activity.
- (3) Down-regulation of inducible nitric oxide synthase and heme oxygenase-1 are negatively associated with biliverdin reductase-A oxidation

Abstract

Alzheimer's disease is a neurodegenerative disorder characterized by progressive cognitive impairment and neuropathology. Recent preclinical and epidemiological studies proposed statins as a possible therapeutic drug for Alzheimer's disease, but the exact mechanisms of action are still unknown. Biliverdin reductase-A is a pleiotropic enzyme involved in cellular stress responses. It not only transforms biliverdin-IX alpha into the antioxidant bilirubin-IX alpha but its serine/threonine/tyrosine kinase activity is able to modulate cell signaling networks. We previously reported the beneficial effects of atorvastatin treatment on biliverdin reductase-A and heme oxygenase-1 in the brains of a well characterized pre-clinical model of Alzheimer's disease, aged beagles, together with observed improvement in cognition. Here we extend our knowledge of the effects of atorvastatin on inducible nitric oxide synthase in parietal cortex, cerebellum and liver of the same animals. We demonstrated that atorvastatin treatment (80 mg/day for 14.5 months) to aged beagles selectively increased inducible nitric oxide synthase in the parietal cortex but not in the cerebellum. In contrast, inducible nitric oxide synthase protein levels were significantly decreased in the liver. Significant positive correlations were found between biliverdin reductase-A and inducible nitric oxide synthase as well as heme oxygenase-1 protein levels in the parietal cortex. The opposite was observed in the liver. Inducible nitric oxide synthase up-regulation in the parietal cortex was positively associated with improved biliverdin reductase-A functions, whereas the oxidative-induced impairment of biliverdin reductase-A in the liver negatively affected inducible nitric oxide synthase expression, thus suggesting a role for biliverdin reductase-A in atorvastatin-dependent inducible nitric oxide synthase changes. Interestingly, increased inducible nitric oxide synthase levels in the parietal cortex were not associated with higher oxidative/nitrosative stress levels. We hypothesize that biliverdin reductase-A-dependent inducible nitric oxide synthase regulation strongly contributes to the cognitive improvement observed following atorvastatin treatment.

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Author contribution:

Barone E conceived and designed the experiments. Barone E and Domenico FD performed the experiments. Barone E, Domenico FD and Perluigi M analyzed the data and wrote the paper. All authors approved the final version of the paper.

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Key Words: neural regeneration; age; Alzheimer's disease; atorvastatin; biliverdin reductase-A; cell stress-response; cognitive function; 4-hydroxy-2-nonenal; heme oxygenase-1; inducible nitric oxide synthase; oxidative stress; neuroregeneration.

INTRODUCTION

Alzheimer's disease is the most common form of dementia among the elderly and is characterized by progressive loss of memory and cognition. Amyloid- β -peptide plaques and neurofibrillary tangles composed of hyperphosphorylated tau, are two major hallmarks of Alzheimer's disease neuropathology. Both Amyloid- β -peptide and tau promote the formation of reactive oxygen and nitrogen species, induce calcium-dependent excitotoxicity and impairment of cellular respiration^[1]. Alzheimer's disease neuropathology is observed in the hippocampus and underlying cortex, as well as in neocortex but with the cerebellum remaining relatively spared until late in the disease^[2-3]. Treatments currently available primarily target the symptoms of dementia but do not appear to modify disease processes. Thus, identifying new approaches to preventing or slowing disease progression in patients with Alzheimer's disease is critically important.

Statins, a class of hypolipidemic drugs, have been proposed as potential agents for the treatment or prevention of Alzheimer's disease^[4-6]. Data from animal models studies suggest possible mechanisms underlying the beneficial role of atorvastatin in preventing Alzheimer's disease, including the reduction of amyloid- β -peptide^[7], β -secretase protein levels^[8] and oxidative stress^[9]. In addition, atorvastatin induces the activation of the heme oxygenase-1/biliverdin reductase-A system, which was proposed to have neuroprotective effects in the brain^[10-11]. Interestingly, previous studies showed that statins enhance neurogenesis, synaptogenesis, and angiogenesis, and significantly improves neurological outcome after stroke^[12] as well as after traumatic brain injuries^[13]. However, the importance of statin treatment in Alzheimer's disease is still under debate,

given that randomized clinical trials did not show any significant benefit on cognition^[6, 14]. In previous studies from our group, atorvastatin promoted beneficial effects in the brain of aged beagles, potentially through the modulation of the heme oxygenase-1/biliverdin reductase-A system^[9-11] (Table 1).

Aged beagles naturally develop learning and memory impairments in association with the accumulation of amyloid- β -peptide of the same amino acid sequence as humans representing a valuable model of early Alzheimer's disease pathology^[15].

We hypothesized that the observed increase of biliverdin reductase-A protein levels and activity^[10] could trigger a cell stress response improving cognition by the following mechanisms: (i) Interaction with members of the MAPK family, such as ERK1/2-Mek-Elk1, through which biliverdin reductase-A regulates important metabolic pathway as well as the expression of oxidative-stress-responsive genes such as heme oxygenase-1 or inducible nitric oxide synthase^[16-19]; (ii) activation of both conventional and atypical protein kinase C isoforms^[16] whose involvement in memory function is now well established^[20]; (iii) production of the powerful antioxidant BR as result of its reductase activity. In this context, since the phosphorylation of biliverdin reductase-A on Tyr residues is required to interact with ERK-Mek-Elk1^[17], the increase of phospho-tyrosine on biliverdin reductase-A in the parietal cortex following atorvastatin treatment^[10], coupled with the negative correlation between phospho-tyrosine on biliverdin reductase-A and size discrimination error scores^[10], could suggest an activation of the MAPK-related signal transduction pathways that in turn promote a robust cell stress response^[16].

Table 1 Main effects observed during previous studies by our group about oxidative stress levels and heme oxygenase-1/biliverdin reductase-A system in the brain and liver of aged beagles following atorvastatin treatment

	Parietal cortex	Cerebellum	Liver	Ref
Protein carbonyls total levels	↓ 11%, $P < 0.05$	≠	≠	[9, 11]
4-hydroxy-2-nonenal total levels	↓ 32%, $P < 0.05$	≠	≠	[9, 11]
3-nitrotyrosine total levels	↓ 26%, $P < 0.05$	≠	≠	[9, 11]
Heme oxygenase-1 protein levels	↑ 75%, $p < 0.01$	≠	≠	[11]
Biliverdin reductase-A protein levels	↑ 21%, $P < 0.05$	≠	60%, $**P < 0.01$	[10]
Phospho-tyrosine on biliverdin reductase-A	↑ 54%, $P < 0.05$	≠	≠	[10]
Phosphor-Serine/Threonine on biliverdin reductase-A	↑ 17%	≠	≠	[10]
Biliverdin reductase activity	↑ 35%, $P < 0.05$	≠	≠	[10]
Protein carbonyls-biliverdin reductase-A	≠	≠	60%	[10]
4-hydroxy-2-nonenal on biliverdin reductase-A	≠	≠	60%, $**P < 0.05$	[10]
3-nitrotyrosine on biliverdin reductase-A	↓ 18%	≠	≠	[10]

↑ Increase; ↓ decrease; ≠ no changes. Ref: Reference.

At the same time, the significant correlations found between biliverdin reductase activity and decreased total protein carbonyls and 3-nitrotyrosine levels^[10] suggest, consistently with prior studies^[21-24], a crucial antioxidant role for BR. Considering the broad functions of biliverdin reductase-A in the cell, increased biliverdin reductase-A phosphorylation likely would have consequence beyond immediate changes in its scaffolding and reductase activities. In order to analyze this aspect in depth, this study aimed to measure the expression levels of inducible nitric oxide synthase and the possible correlations with biliverdin reductase-A levels and activities following of atorvastatin treatment in aged beagles. We hypothesized that the improved function of biliverdin reductase-A is associated with increased inducible nitric oxide synthase levels and that biliverdin reductase-A could contribute to the maintenance of physiological levels of nitric oxide in order to avoid its neurotoxic effects. In addition, we provide new results about the association between biliverdin reductase-A and heme oxygenase-1, the latter another main target

of biliverdin reductase-A activity during cell stress-response, as noted above.

RESULTS

Biliverdin reductase-A protein levels increased with age in the parietal cortex of aged beagles

Age-associated changes of biliverdin reductase-A expression were previously reported in rat brain^[25]. Here we provide novel data of an age-dependent modulation of biliverdin reductase-A protein in dogs. We observed a significant 2.4-fold increase of biliverdin reductase-A protein levels in 12 year old beagles ($t(6) = 2.68$, $P < 0.05$) compared to 4 years old (Figure 1).

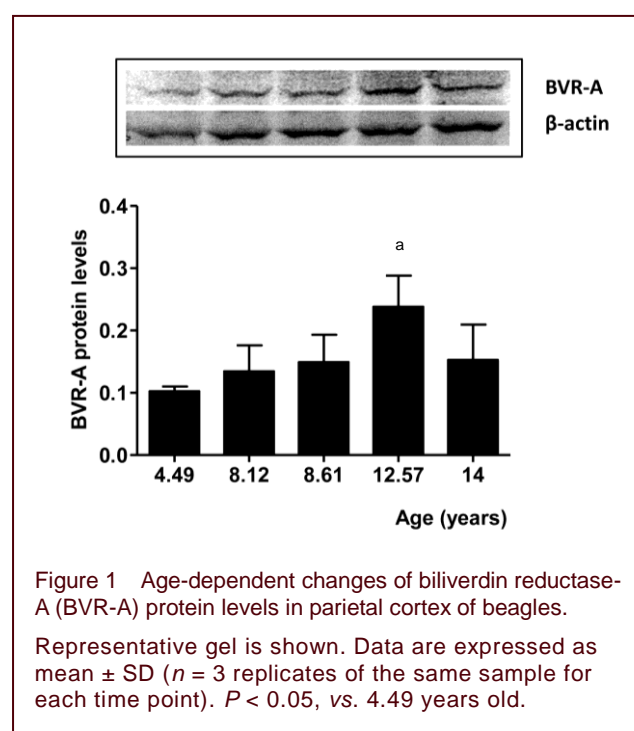


Figure 1 Age-dependent changes of biliverdin reductase-A (BVR-A) protein levels in parietal cortex of beagles.

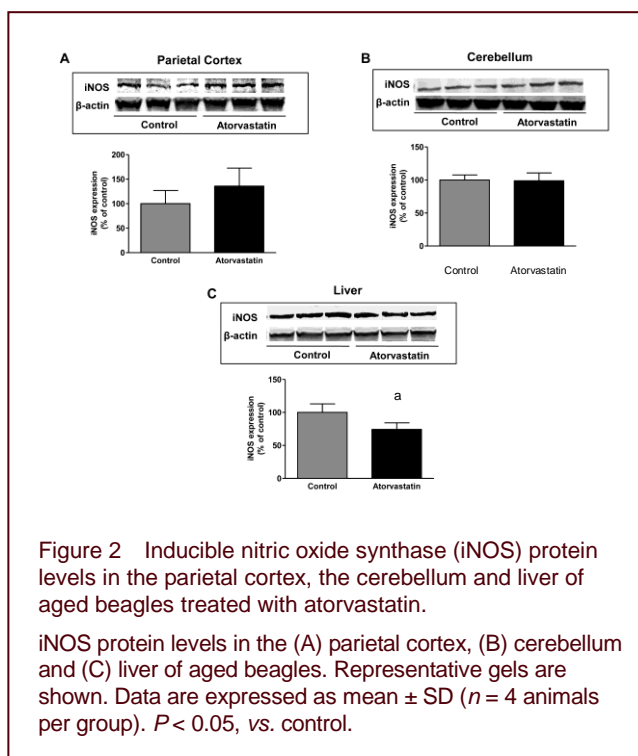
Representative gel is shown. Data are expressed as mean ± SD ($n = 3$ replicates of the same sample for each time point). $P < 0.05$, vs. 4.49 years old.

Furthermore, a 1.6-fold decrease was observed in the parietal cortex of 14-year-old dogs, compared to 12 years old, although this value did not reach statistical significance.

Effect of atorvastatin treatment on inducible nitric oxide synthase protein levels in the parietal cortex, the cerebellum and liver of aged beagle

We previously reported differential effects of atorvastatin on biliverdin reductase-A protein levels and activities in the parietal cortex, cerebellum and liver of aged beagles^[10] (Table 1). The current aging study shows that biliverdin reductase-A decreases as animals move from 12–14 years of age. In the canine atorvastatin study, beagles ranging in age from 8.9–13.2 received treatment

for 14.5 months and a significant increase of biliverdin reductase-A protein levels in the parietal cortex following atorvastatin treatment was observed^[10], perhaps suggesting it protects against further age-associated decreases of biliverdin reductase-A. Given tissue-specific changes in the levels of biliverdin reductase-A (Table 1,^[10]), and due to its role in the regulation of inducible nitric oxide synthase^[16, 26-27], we predicted that inducible nitric oxide synthase would be modified in response to atorvastatin treatment. Consistent with this hypothesis, atorvastatin (80 mg/kg per day for 14.5 months) increased inducible nitric oxide synthase protein levels by approximately 35% ($t(6) = 1.57$, $P = 0.16$) in the parietal cortex (Figure 2A). However, the increased inducible nitric oxide synthase expression was not statistically significant compared to controls. There was no effect of treatment on inducible nitric oxide synthase protein levels in the cerebellum ($t(6) = 0.14$, $P = 0.88$) (Figure 2B). Conversely, a significant down-regulation of inducible nitric oxide synthase by 30% ($t(6) = 3.17$, $P < 0.05$) was observed in the liver (Figure 2C).



Atorvastatin-induced changes on biliverdin reductase-A protein levels, phosphorylation and oxidation were associated with changes of inducible nitric oxide synthase and heme oxygenase-1 protein levels

Changes observed for inducible nitric oxide synthase protein levels were hypothesized to be associated with atorvastatin-induced alteration of biliverdin reductase-A. As our group previously reported, atorvastatin adminis-

tration to aged beagles not only increased biliverdin reductase-A protein levels in the parietal cortex, but also promoted positive changes on the post-translational modifications affecting the protein (Table 1,^[10]).

In particular, we found an increase of biliverdin reductase-A phosphorylation (phospho-tyrosine on biliverdin reductase-A, phosphor-Serine/Threonine on biliverdin reductase-A) and a decrease of biliverdin reductase-A nitration (3-nitrotyrosine on biliverdin reductase) which led to an increased activation of biliverdin reductase-A in the parietal cortex (Table 1^[10]). Furthermore, an opposite situation characterized by an increase of biliverdin reductase-A protein levels together with an increase of its oxidation (4-hydroxy-2-nonenal on biliverdin reductase-A) was found in the liver (Table 1^[10]). These latter results, obtained on samples coming from the same animals to which we refer in the current study, were used to analyze any possible association between inducible nitric oxide synthase protein levels and (i) biliverdin reductase-A protein levels, (ii) phospho-tyrosine on biliverdin reductase-A levels and (iii) phosphor-Serine/Threonine on biliverdin reductase-A levels in the parietal cortex (Figure 3A–C) and liver (Figure 3D–F).

Due to the fact that no changes occurred in cerebellum, a further analysis of this brain region was not addressed. Biliverdin reductase-A protein levels positively and significantly correlated with inducible nitric oxide synthase protein levels (Pearson $r = 0.78$, $P < 0.05$; Figure 3A) only in the parietal cortex. In addition, the extent of biliverdin reductase-A phosphorylation positively correlated with inducible nitric oxide synthase protein levels in parietal cortex [phospho-tyrosine on biliverdin reductase-A (Pearson $r = 0.53$) and phosphor-Serine/Threonine on biliverdin reductase-A (Pearson $r = 0.77$; $P < 0.05$)] (Figure 3B, C, respectively), thus supporting the idea that an increase of biliverdin reductase-A protein levels together with its activation (increased phosphorylation) could play a main role in the regulation of inducible nitric oxide synthase expression in the brain. Very interestingly, we found the opposite in the liver. Indeed, after atorvastatin treatment, inducible nitric oxide synthase protein levels were significantly decreased in treated animals compared to controls despite a significant increase of biliverdin reductase-A protein levels^[10] (Table 1). Furthermore, in the liver, correlation analysis demonstrated, negative associations between inducible nitric oxide synthase protein levels and (i) biliverdin reductase-A protein levels (Pearson $r = -0.60$, $P = 0.11$; Figure 3D) or (ii) phosphorylation [phospho-tyrosine on

biliverdin reductase-A, (Spearman $r = -0.47$, $P = 0.24$) and phosphor-Serine/Threonine on biliverdin reductase-A (Spearman $r = -0.31$, $p = 0.46$) (Figure 3E, F, respectively). In order to strengthen the hypothesis with regard to the role of biliverdin reductase-A on the effects produced by atorvastatin treatment in aged beagles, a correlation analysis between biliverdin reductase-A and heme oxygenase-1 protein levels was performed, since heme oxygenase-1 is another protein tightly regulated by the activation of biliverdin reductase-A^[16, 18, 28-30]. We previously demonstrated a selec-

tive increase of heme oxygenase-1 in the parietal cortex of the same animals used for this study, following atorvastatin administration, whereas no changes were observed in the liver^[11] (Table 1). Here a positive correlation between biliverdin reductase-A and heme oxygenase-1 in parietal cortex, was found.

In particular, biliverdin reductase-A and heme oxygenase-1 protein levels positively correlated in the parietal cortex (Spearman $r = 0.69$), and this association is close to statistical significance ($P = 0.06$) (Figure 4A).

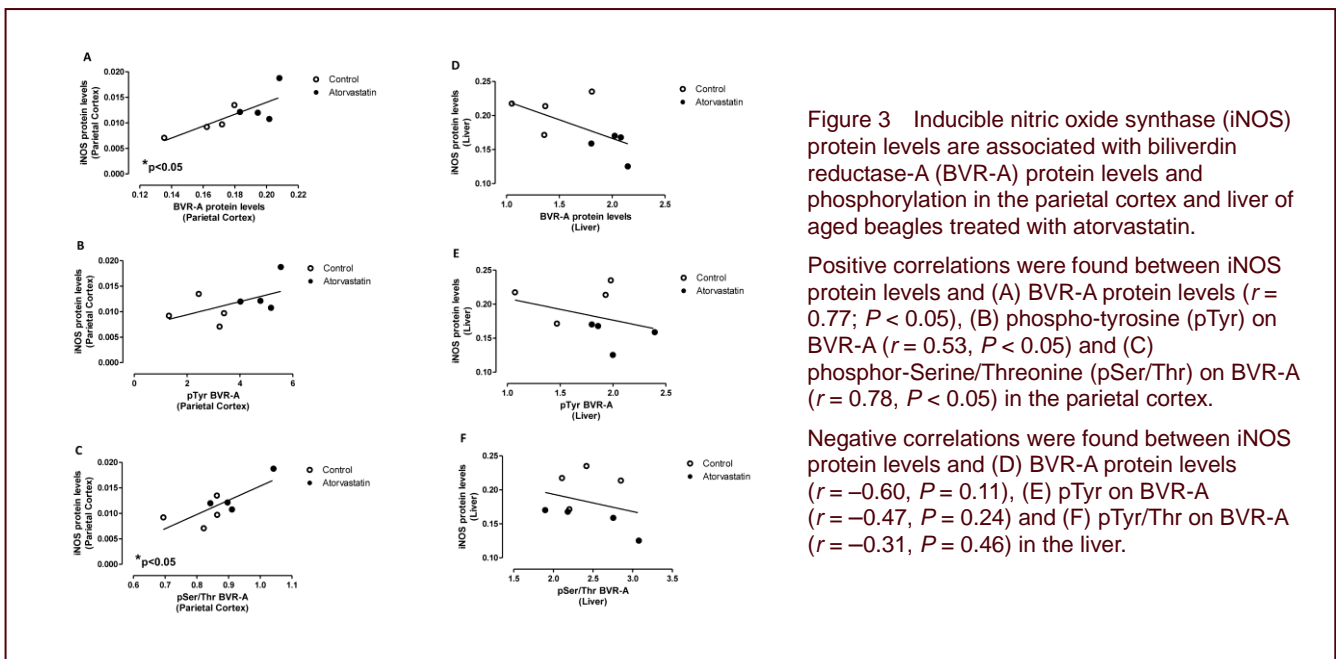


Figure 3 Inducible nitric oxide synthase (iNOS) protein levels are associated with biliverdin reductase-A (BVR-A) protein levels and phosphorylation in the parietal cortex and liver of aged beagles treated with atorvastatin.

Positive correlations were found between iNOS protein levels and (A) BVR-A protein levels ($r = 0.77$; $P < 0.05$), (B) phospho-tyrosine (pTyr) on BVR-A ($r = 0.53$, $P < 0.05$) and (C) phosphor-Serine/Threonine (pSer/Thr) on BVR-A ($r = 0.78$, $P < 0.05$) in the parietal cortex.

Negative correlations were found between iNOS protein levels and (D) BVR-A protein levels ($r = -0.60$, $P = 0.11$), (E) pTyr on BVR-A ($r = -0.47$, $P = 0.24$) and (F) pTyr/Thr on BVR-A ($r = -0.31$, $P = 0.46$) in the liver.

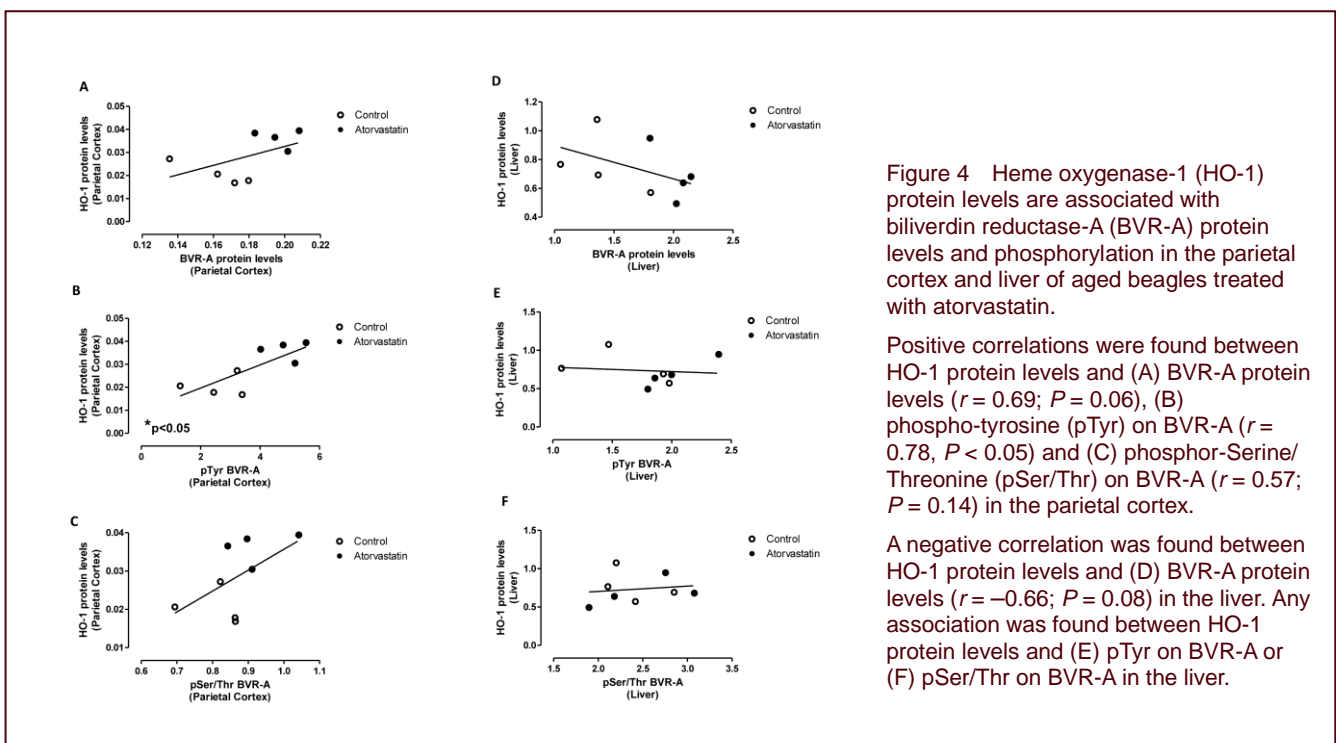


Figure 4 Heme oxygenase-1 (HO-1) protein levels are associated with biliverdin reductase-A (BVR-A) protein levels and phosphorylation in the parietal cortex and liver of aged beagles treated with atorvastatin.

Positive correlations were found between HO-1 protein levels and (A) BVR-A protein levels ($r = 0.69$; $P = 0.06$), (B) phospho-tyrosine (pTyr) on BVR-A ($r = 0.78$, $P < 0.05$) and (C) phosphor-Serine/Threonine (pSer/Thr) on BVR-A ($r = 0.57$; $P = 0.14$) in the parietal cortex.

A negative correlation was found between HO-1 protein levels and (D) BVR-A protein levels ($r = -0.66$; $P = 0.08$) in the liver. Any association was found between HO-1 protein levels and (E) pTyr on BVR-A or (F) pSer/Thr on BVR-A in the liver.

Similarly, phospho-tyrosine on biliverdin reductase-A (Pearson $r = 0.78$; $P < 0.05$) and phospho-serine/threonine on biliverdin reductase-A (Pearson $r = 0.57$; $P = 0.14$) positively correlated with heme oxygenase-1 protein levels in the parietal cortex (Figures 4B, C, respectively). On the contrary, in the liver, biliverdin reductase-A protein levels negatively correlated with heme oxygenase-1 protein levels (Pearson $r = -0.66$; $P = 0.08$, Figure 4D) and an association was found between the extent of biliverdin reductase-A phosphorylation and heme oxygenase-1 protein levels (Figure 4E, F). Thus, higher levels and activation of biliverdin reductase-A were associated with increased heme oxygenase-1 protein levels only in the parietal cortex, and these data resemble those obtained for inducible nitric oxide synthase.

However, there is a discrepancy between the results of inducible nitric oxide synthase and heme oxygenase-1 changes in response to atorvastatin treatment in the parietal cortex and liver, despite a significant and consistent increase of biliverdin reductase-A protein^[10]. Although it is well known that several and distinct mechanisms could participate in the regulation of inducible nitric oxide synthase and heme oxygenase-1 expression, negative associations between the oxidation of biliverdin reductase-A (4-hydroxy-2-nonenal on biliverdin reductase-A) and (i) inducible nitric oxide synthase (Spearman $r = -0.76$, $P < 0.05$; Figure 5A) or (ii) heme oxygenase-1 protein levels (Spearman $r = -0.45$, $P = 0.32$; Figure 5B) in the liver, were observed. These data support the hypothesis that an oxidative impairment of biliverdin reductase-A in the liver could be responsible, at least in part, for the loss of the effect on inducible nitric oxide synthase and heme oxygenase-1 following atorvastatin treatment.

Atorvastatin-induced changes of inducible nitric oxide synthase protein levels were associated with 3-nitrotyrosine levels only in the liver

An evaluation of the association between inducible nitric oxide synthase protein levels and a well-known biomarker of nitrosative stress, *i.e.*, 3-nitrotyrosine, was performed. As we previously reported, atorvastatin produced a significant decrease of 3-nitrotyrosine in the parietal cortex^[9] (Table 1), whereas no changes were observed in the liver^[11] of the animals used for this study (Table 1). Since inducible nitric oxide synthase is one of the main sources of nitric oxide during conditions responsible for an increase of oxidative/nitrosative stress levels (*e.g.*, inflammation), this study aimed to understand if changes produced by atorvastatin also are associated with nitrosative stress levels.

As expected, no significant association between inducible nitric oxide synthase and 3-nitrotyrosine in the parietal cortex (Pearson $r = -0.29$, $P = 0.42$; Figure 5C), was found. On the contrary, inducible nitric oxide synthase protein levels were negatively associated with 3-nitrotyrosine levels in the liver (Pearson $r = 0.69$, $P < 0.05$, Figure 5D). Thus, lower levels of inducible nitric oxide synthase protein were associated with reduced nitrosative damage in the liver.

DISCUSSION

Aging is one of the greatest risk factors for cognitive decline and Alzheimer's disease. Among the elderly, Alzheimer's disease represents one of the main causes of severe cognitive decline and dementia^[1].

Increased oxidative and nitrosative stress levels with age and in the pathogenesis of neurodegenerative disorders could be a significant contributor to cognitive impairment and dementia^[31-40]. Among the defenses that the human brain possesses, a major role is played by the heme oxygenase-1/biliverdin reductase-A system, whose up-regulation (i) is one of the earlier events in the adaptive response to stress^[41], and (ii) was proposed as a useful pathway to manipulate to counteract Alzheimer's disease-induced oxidative/nitrosative damage^[39, 42-46].

We previously described phosphorylation and oxidative modifications to the levels and activity of the heme oxygenase-1/biliverdin reductase-A system in the brain and plasma of Alzheimer's disease or mild cognitive impairment subjects^[47-50], opening a debate on its possible pathophysiological and clinical significance. Indeed, we found that biliverdin reductase-A protein levels were significantly increased in the brain of Alzheimer's disease and or mild cognitive impairment subjects, whereas the activity of the protein was significantly decreased^[47]. The explanation we provided to clarify this apparent paradox was that biliverdin reductase-A undergoes oxidative/nitrosative post-translational modifications which affects its structure and thus, its activity. Very interestingly, by using a well characterized method to identify protein oxidatively modified^[10, 47, 50-51], we found that the levels of 3-nitrotyrosine on biliverdin reductase-A were significantly increased in the hippocampi of both Alzheimer's disease or or mild cognitive impairment subjects whereas the levels of phosphorylation of serine/threonine/tyrosine residues were reduced, making biliverdin reductase-A dysfunctional^[47, 50].

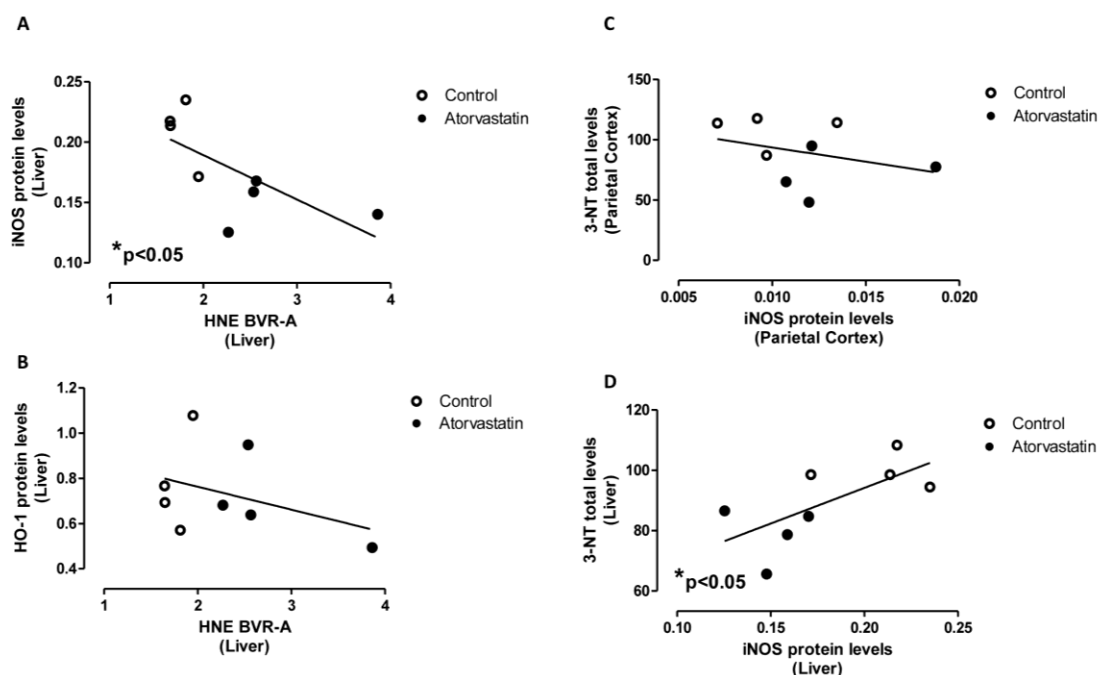


Figure 5 Association between biliverdin reductase-A (BVR-A) oxidation and (A) inducible nitric oxide synthase (iNOS), (B) heme oxygenase-1 (HO-1) protein levels in the liver of aged beagles treated with atorvastatin.

Association between 3-nitrotyrosine (3-NT) levels and iNOS protein levels in the (C) parietal cortex and (D) liver of aged beagles treated with atorvastatin. Negative correlations were found between BVR-A protein levels and (A) iNOS protein levels ($r = -0.76$, $P < 0.05$), (B) HO-1 protein levels ($r = -0.45$, $P = 0.32$) in the liver. Any correlation was found between iNOS levels and (C) 3-NT levels in the parietal cortex. A negative correlation was found between iNOS protein levels and (D) 3-NT levels ($r = 0.69$, $P < 0.05$) in the liver.

Since it is well known that the oxidative/nitrosative post-translational modifications alter proteins structure^[52-53] and result in a marked decrease of their function^[45, 51, 54], it is plausible to argue that the formation of biliverdin reductase's 3-nitrotyrosine-adducts are responsible for the the significant reduction of biliverdin reductase activity in Alzheimer's disease and MCI hippocampi.

Furthermore, by using a well characterized pre-clinical model of Alzheimer's disease, such as aged beagles^[15], we showed that parietal-resident biliverdin reductase-A may be a target for atorvastatin, which was able to increase biliverdin reductase-A protein levels as well as to improve its activities with positive effects on cognition^[10]. In this study, we extend the neurobiological benefits of atorvastatin in the brain of aged beagles by showing that inducible nitric oxide synthase protein levels are associated with changes of biliverdin reductase-A levels and activities.

Novel data are provided in this study of age-dependent changes in biliverdin reductase-A protein levels in the

parietal cortex of beagles. These results, are in good agreement with those found previously in rat brain^[25], and strengthen the concept that, in the brain, biliverdin reductase-A is tightly regulated across the lifespan. One possible explanation, is that, since biliverdin reductase-A is an oxidative stress-induced protein^[16], its levels rise together with those of oxidative stress as also observed in Alzheimer's disease and or mild cognitive impairment hippocampus^[47, 50]. Based on these findings, our studies with aging dogs^[10] suggest that atorvastatin could promote biliverdin reductase-A up-regulation in the parietal cortex even at an old age (11–13 years old) when levels naturally decrease as shown in Figure 1. Thus, despite the observed decrease of biliverdin reductase-A protein levels at 14 years old, which may be due to progressive neurodegeneration, it may still be possible to improve biliverdin reductase-A functions with atorvastatin. The mechanisms through which biliverdin reductase-A could exert its neuroprotective effects are those noted above, and it is conceivable to think that a therapeutic approach able to recover biliverdin reductase-A activities could represent a good strategy aimed to improve cognitive function.

Consistent with this hypothesis, there appears to be a very interesting link between biliverdin reductase-A and inducible nitric oxide synthase^[16, 26-27]. Nitric oxide, the end-product of Nitric Oxide Synthase(s) activity, has an important role in the regulation of synaptic plasticity, which in turn is critically involved in cognition, including memory^[42, 55-56]. Inducible nitric oxide synthase levels in the central nervous system are generally fairly low; however, an increased expression of inducible nitric oxide synthase in astrocytes and microglia occurs due to pro-inflammatory conditions^[42, 57]. Thus, increased production of nitric oxide becomes harmful involving the formation of RNS, such as peroxynitrite, which in turn leads to the formation of 3-nitrotyrosine adducts^[53, 58-59]. Our results clearly showed that following atorvastatin treatment, the levels of inducible nitric oxide synthase protein are differentially affected in the brain, and between brain and periphery (Figure 2A–C). Despite an increase of inducible nitric oxide synthase in parietal cortex (Figure 2A), the levels of 3-nitrotyrosine in this area were significantly lower than those observed in the control dogs^[9] (Table 1). This result is surprising given the patho-physiological role of inducible nitric oxide synthase, but it may be conceivable that the improvement of biliverdin reductase-A activities^[10] together with the concomitant increased production of the antioxidant and anti-nitrosative molecule BR^[10, 24, 60] are able to maintain nitric oxide levels under the pathological threshold, providing a direct neuroprotective effect. Furthermore, these findings are in good agreement with those found in Alzheimer's disease brain where an increase of 3-nitrotyrosine levels together with an impairment of biliverdin reductase-A activities were observed^[50]. In this scenario, the biliverdin reductase-A-dependent modulation of inducible nitric oxide synthase expression becomes fascinating. In fact, as recently demonstrated in an elegant paper by Maines and colleagues^[26], biliverdin reductase, as an intracellular scaffold/bridge/anchor protein, is required for placing ERK1/2 in proximity to its kinases, MEK1/2, in the cytoplasm and bringing Elk1 in contact with the activated ERK1/2 in the nucleus^[26]. Through this mechanism it regulates the activation of an estimated 50 nuclear factors and proteins that influence cell differentiation, proliferation, stress response, and promote tumor growth including inducible nitric oxide synthase and heme oxygenase-1^[16, 26]. That said, the positive correlations found in the parietal cortex with regard to biliverdin reductase-A protein levels or phosphorylation and inducible nitric oxide synthase protein levels seem to support in an *in vivo* model the mechanism proposed above. To strengthen this hypothesis further, we analyzed the association between biliverdin reduc-

tase-A and another target of its activity, heme oxygenase-1. Similarly to the results obtained for inducible nitric oxide synthase, heme oxygenase-1 protein levels were positively associated with biliverdin reductase-A protein levels and phosphorylation in the parietal cortex, although a significant association was found only for phospho-tyrosine on biliverdin reductase-A. In combination, these observations could suggest that the increase of biliverdin reductase-A (i) protein levels and (ii) phospho-Serine/Threonine/Tyrosine represents an important feature in the signaling pathway stimulated by atorvastatin and involving biliverdin reductase-A-dependent regulation of stress-responsive gene such as heme oxygenase-1 or inducible nitric oxide synthase^[16].

In paradoxical support of the role of biliverdin reductase-A, are the results observed in the liver where it appears that other mechanisms affect inducible nitric oxide synthase regulation. In fact, an increase of biliverdin reductase-A protein levels and significant reduction of inducible nitric oxide synthase was observed. In our previous study, we showed that no differences exist for 3-nitrotyrosine in the liver between controls and atorvastatin-treated beagles^[11], and we proposed that this effect could be related to the oxidative-induced impairment of biliverdin reductase-A (increased 4-hydroxy-2-nonenal on biliverdin reductase-A, Table 1). This hypothesis is further corroborated by several reports that many proteins involved in the regulation of important cellular functions became dysfunctional in Alzheimer's disease brain due to oxidative and nitrosative modifications^[61-69]. The novelty of our results is that an increase of biliverdin reductase-A oxidation (4-hydroxy-2-nonenal on biliverdin reductase-A) is associated with decreased levels of inducible nitric oxide synthase (Figure 5A), supporting once again the role of biliverdin reductase-A in inducible nitric oxide synthase regulation. Thus, if biliverdin reductase-A does not function properly, this could have consequences on its downstream target (Figure 6).

Atorvastatin treatment promoted two different effects in the brain and liver of aged beagles. In the brain, atorvastatin increased biliverdin reductase-A protein levels and phosphorylation (phospho-Serine/Threonine/Tyrosine), thus promoting its reductase and kinase activities. Through its reductase activity biliverdin reductase-A produces the antioxidant and antinitrosative molecule bilirubin. Through its kinase activity, biliverdin reductase-A is able to activate downstream survival signaling pathways^[16], as well as to promote the transcription of oxidative-induced genes such as heme oxygenase-1 and

inducible nitric oxide synthase^[16]. It is conceivable to hypothesize that the neuroprotective effects mediated by bilirubin and the activated survival signaling pathways, overcome the neurotoxic increase of nitric oxide due to the concomitant up-regulation of inducible nitric oxide synthase. Thus, the final effect is a reduction of oxidative/nitrosative stress levels, e.g. 3-nitrotyrosine as previously demonstrated^[9]. In the liver, atorvastatin increased biliverdin reductase-A protein levels, but also its oxidation (4-hydroxy-2-nonenal on biliverdin reductase-A) leading to an impairment of biliverdin reductase-A activities. Furthermore, biliverdin reductase-A oxidation was associated with a reduction of inducible nitric oxide synthase protein levels. In this case, the lack of biliverdin reductase-A antioxidant power which should favor an increase of the oxidative/nitrosative stress levels, is balanced by the decrease of inducible nitric oxide synthase protein levels (less nitric oxide production) with the final effect that no changes were observed for the oxidative/nitrosative stress levels e.g., 3-nitrotyrosine.

Also for heme oxygenase-1 we observed the same profile (Figure 5B). Increased heme oxygenase-1 protein levels in parietal cortex were positively associated with biliverdin reductase-A protein levels and phosphorylation (Figures 4A–C). On the contrary, the oxidative-induced impairment of biliverdin reductase-A seems to be associated with a decrease of heme oxygenase-1 protein levels (Figure 5B). In addition, in light of these results, the observation that any significant change of 3-nitrotyrosine levels in the liver was found between control and atorvastatin-treated dog (Table 1,^[11]) requires

a novel explanation. This latter, could account not only the lack of effect on biliverdin reductase-A activities as previously postulated^[10-11], but also the observation of a reduction of inducible nitric oxide synthase protein levels following atorvastatin treatment. As shown in Figure 5D, a significant correlation was found between the levels of 3-nitrotyrosine and those of inducible nitric oxide synthase in the liver suggesting that reduced levels of inducible nitric oxide synthase are associated with lower levels of 3-nitrotyrosine. That said, why we did not find any significant difference between controls and atorvastatin treated dogs (Table 1,^[11])? An intuitive explanation could be that the lack of effect of atorvastatin treatment on 3-nitrotyrosine levels in the liver is due to the small sample size used for this study. However, although the sample size was relatively small, consistent effects with (i) other oxidative stress markers^[9]; (ii) biliverdin reductase-A protein levels, post-translational modifications and activity^[10]; (iii) heme oxygenase-1 protein levels^[11] and inducible nitric oxide synthase protein levels were observed. Therefore, it seems much more consistent with the hypothesis, that in the liver, the impairment of biliverdin reductase-A is an important event which maybe affect oxidative/nitrosative stress levels through different mechanisms. Keeping in mind the role of biliverdin reductase-A in the regulation of inducible nitric oxide synthase transcription into the nucleus^[26], the results obtained in the liver could be explained as the follows: despite it should expect an increase of 3-nitrotyrosine due to the decrease of biliverdin reductase-A antioxidant power, this did not happen because the reduction of inducible nitric oxide synthase levels (less nitric oxide production) (Figure 6).

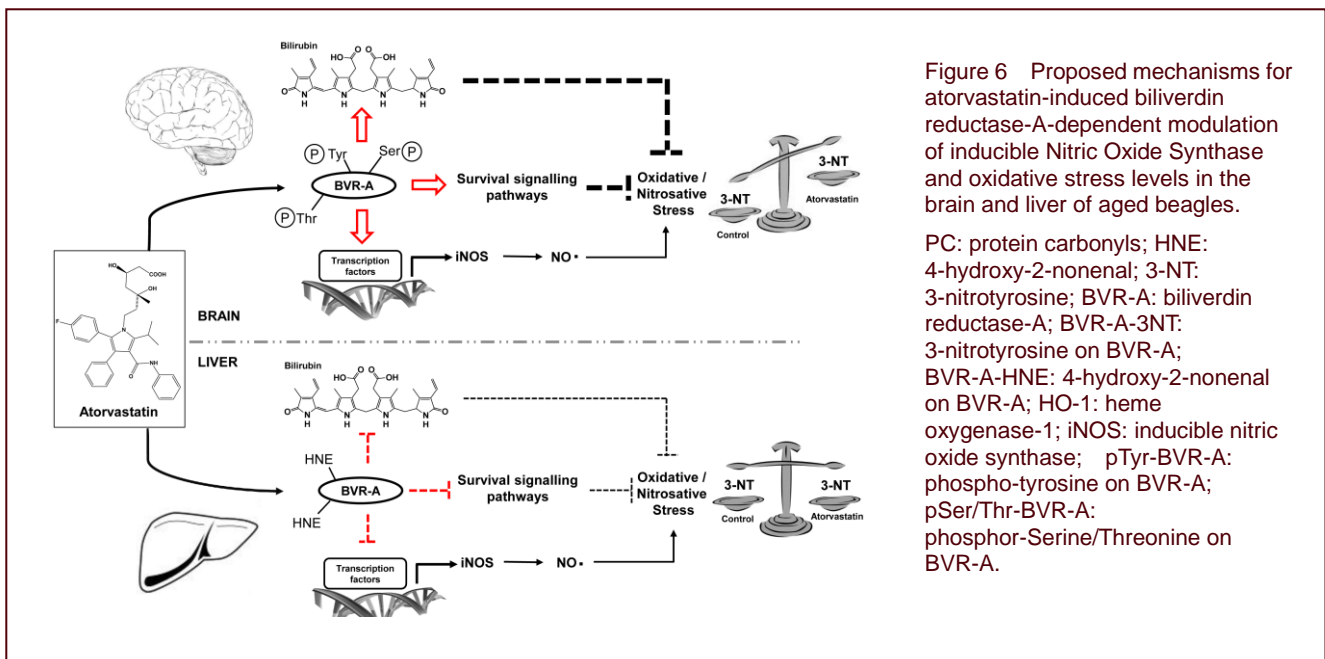


Figure 6 Proposed mechanisms for atorvastatin-induced biliverdin reductase-A-dependent modulation of inducible Nitric Oxide Synthase and oxidative stress levels in the brain and liver of aged beagles.

PC: protein carbonyls; HNE: 4-hydroxy-2-nonenal; 3-NT: 3-nitrotyrosine; BVR-A: biliverdin reductase-A; BVR-A-3NT: 3-nitrotyrosine on BVR-A; BVR-A-HNE: 4-hydroxy-2-nonenal on BVR-A; HO-1: heme oxygenase-1; iNOS: inducible nitric oxide synthase; pTyr-BVR-A: phospho-tyrosine on BVR-A; pSer/Thr-BVR-A: phosphor-Serine/Threonine on BVR-A.

In other words, like in a compensation mechanism, the lack of biliverdin reductase-A antioxidant power which should favor an increase of the oxidative/nitrosative stress levels, is balanced by the decrease of inducible nitric oxide synthase protein levels (Figure 6). Of course, based only on these results, we cannot exclude that other mechanisms participate in atorvastatin-mediated changes of inducible nitric oxide synthase in the brain and liver of aged beagles, and more mechanistic studies are required. *Ad hoc* designed experiments to address this point are ongoing in our laboratories.

In conclusion, this work supports a role for biliverdin reductase-A in the regulation of inducible nitric oxide synthase following atorvastatin treatment in aged beagles. Atorvastatin-induced neuroprotective effects in the brain appear to be mediated by biliverdin reductase-A which, although was associated with an increase of inducible nitric oxide synthase protein levels (Figure 3A–C), because of its antioxidant and antinitrosative features may serve to overcome nitric oxide-induced neurotoxic effects, thus supporting a potential therapeutic role of biliverdin reductase-A in Alzheimer's disease.

MATERIALS AND METHODS

Design

The study design of the atorvastatin treatment of aged beagles has been described previously^[8-11] and is briefly summarized here.

Time and setting

This work was performed at the Department of Chemistry, Center of Membrane Sciences, and Department of Molecular and Biomedical Pharmacology, Sanders-Brown Center on Aging, University of Kentucky, Lexington, Kentucky, USA between 2010 and 2011.

Materials

Beagles were obtained from the Lovelace Respiratory Research Institute and Harlan (Indianapolis, IN, USA). For the age-dependence analysis of biliverdin reductase-A in the parietal cortex, 5 beagles ranging from 4.49 to 14 years were used. For the atorvastatin study, eight beagles ranging in age from 8.9–13.2 years were used. Based on our previous work, dogs of this age show cognitive decline and significant amounts of brain amyloid- β -peptide^[70]. 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

the animals had received treatment for 14.5 months and they ranged in age from 10.1–14.6 years. All research was conducted in accordance with approved Institutional Animal Care and Use Committee protocols following National Institutes of Health 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.

Methods

Drug treatment

Atorvastatin calcium (Lipitor[®], 40 mg/tablet) and placebo tablets were kindly provided by Pfizer Inc (New York, NY, USA). Atorvastatin-treated animals received 2 x 40 mg tablets per day for a daily dose of 80 mg/day 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^[71]. As previously demonstrated, in beagles treated with 6 mg/kg atorvastatin (approximately 90 mg/dog), plasma concentrations of atorvastatin were approximately 500 ng/mL^[72]. This plasma concentration is in the same order of magnitude of those achieved in hypercholesterolemic people treated with 80 mg atorvastatin/day (187–252 ng/mL)^[73-74]. This differs from rodent studies that have reported reduced brain amyloid- β -peptide in response to statin treatment. In these studies, doses are typically between 200- and 400-time higher than those used in humans^[75], which leads to some concern regarding the translation of these outcomes to Alzheimer's disease clinical trials.

Tissue collection

Brain tissues (parietal cortex and cerebellum) and liver samples were collected as previously described^[9, 76].

Sample preparation

Brain (the parietal cortex and cerebellum) and liver samples from control and atorvastatin-treated dogs were thawed and placed in Media I lysis buffer (pH 7.4) containing 0.32 mol/L sucrose, 0.10 mmol/L Tris-HCl (pH = 8.8), 0.10 mmol/L MgCl₂, 0.08 mmol/L EDTA, proteinase inhibitors leupeptin (0.5 mg/mL), pepstatin (0.7 μ g/mL), aprotinin (0.5 mg/mL) and PMSF (40 μ g/mL) and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Samples were homogenized by 20 passes of a Wheaton tissue homogenizer and the resulting homogenate was centrifuged at 14 000 x g for 10 minutes to remove cellular debris. Total protein concentration of the supernatant was determined by BCA method (Pierce, Rockford, IL, USA).

Western blot analysis

Western blot analysis was performed as previously described^[47]. The following primary antibodies were used: polyclonal anti-rabbit biliverdin reductase-A (Sigma-Aldrich, dilution 1:1000); polyclonal anti-rabbit inducible nitric oxide synthase (Lifespan Biosciences, Seattle, WA, USA, dilution 1:500); and polyclonal anti-rabbit β -actin (Sigma-Aldrich; dilution 1:2 000).

Statistical analysis

Data are expressed as mean \pm SD of four individual samples per group. All statistical analyses were performed using a two-tailed Student's *t*-test. *P* < 0.05 was considered significantly different from control. Pearson or Spearman correlations were calculated to test the linear associations between the outcome measures in this study. The values of biliverdin reductase-A, heme oxygenase-1 and 3-nitrotyrosine to which we refer for the correlation analysis were obtained in previous works from our laboratory on the same animals used for this study^[9-11].

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