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ORIGINAL ARTICLE

Biliverdin reductase-A: a novel drug target for atorvastatin in a dog pre-clinical model of Alzheimer disease

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Abstract

Biliverdin reductase-A (BVR-A) is a pleiotropic enzyme involved in cellular stress responses. It not only transforms biliverdin-IX alpha into the antioxidant bilirubin-IX alpha but through its serine/threonine/tyrosine kinase activity is able to modulate cell signaling networks. BVR-A's involvement in neurodegenerative disorders such as Alzheimer disease (AD) and amnestic mild cognitive impairment was previously described. Statins have been proposed to reduce risk of AD. In this study we evaluated the effect of atorvastatin treatment (80 mg/day for 14.5 months) on BVR-A in the parietal cortex, cerebellum and liver of a well characterized pre-clinical model of AD, the aged beagle. We found that atorvastatin significantly increased BVR-A protein levels, phosphorylation and activity only in parietal cortex. Additionally, we found significant negative correlations between BVR-A and oxidative stress indices, as well as discrimination learning error scores. Furthermore, BVR-A up-regulation and post-translational modifications significantly correlated with β-secretase protein levels in the brain, suggesting a possible role for BVR-A in Aβ formation.

Keywords: Alzheimer disease, atorvastatin, biliverdin reductase, cognitive function, oxidative stress.

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Alzheimer disease (AD) is the most common form of dementia among the elderly and is characterized by progressive loss of memory and cognition. Amyloid-β-peptide (Aβ) forms senile plaques, which, together with hyperphosphorylated tau-based neurofibrillary tangles, are the hallmarks of AD neuropathology. Both Aβ and tau promote the formation of reactive oxygen and nitrogen species, and induce calcium-dependent excitotoxicity and impairment of cellular respiration (Querfurth and LaFerla 2010).

The up-regulation of the heme oxygenase-1/biliverdin reductase-A (HO-1/BVR-A) system is one of the earlier events in the adaptive response to stress. HO-1/BVR-A reduces the intracellular levels of pro-oxidant heme and generates equimolar amounts of the free radical scavengers biliverdin-IX alpha (BV)/bilirubin-IX alpha (BR) as well as the pleiotropic gaseous neuromodulator carbon monoxide (CO) and iron (II) (Poon *et al.* 2004; Stocker 2004; Mancuso

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Abbreviations used: AD, Alzheimer disease; BACE1, β-secretase; BR, bilirubin-IX alpha; BV, biliverdin-IX alpha; BVR-A, biliverdin reductase-A; DNP, dinitrophenylhydrazone; ERK1/2, extracellular signal-regulated kinases 1/2; HNE, 4-hydroxy-2-nonenal; HO-1, heme oxygenase-1; MCI, mild cognitive impairment; 3-NT, nitrotyrosine.

et al. 2006, 2010; Mancuso and Barone 2009). Specifically, the up-regulation of the HO-1/BVR-A system was proposed as a useful mechanism to counteract AD-induced oxidative/nitrosative damage (Markesbery 1997; Butterfield and Lauderback 2002; Smith *et al.* 2002; Calabrese *et al.* 2006, 2007; Mancuso *et al.* 2007). However, BVR-A not only reduces BV into BR, but is also a serine/threonine/tyrosine kinase involved in various cellular functions (Maines 2005; Kapitulnik and Maines 2009). Interestingly, BVR-A stimulates its own reductase activity through the autophosphorylation of specific serine/threonine residues (Lerner-Marmarosh *et al.* 2005). In addition, phosphorylated BVR-A interacts with members of the mitogen activated protein kinase family, in particular, the extracellular signal-regulated kinases 1/2 (ERK1/2), and regulates the expression of oxidative-stress-responsive genes such as HO-1 or inducible nitric oxide synthase (iNOS) (Maines 2007; Lerner-Marmarosh *et al.* 2008; Tudor *et al.* 2008; Kapitulnik and Maines 2009).

Statins, a class of hypolipidemic drugs, have been proposed as potential agents for the treatment or prevention of AD (Jick *et al.* 2000; Kandiah and Feldman 2009). Data from animal models studies suggest possible mechanisms underlying the beneficial role of atorvastatin in preventing AD, including the reduction of Aβ (Kurata *et al.* 2011), β-secretase (BACE1) protein levels (Murphy *et al.* 2010) and oxidative stress (Barone *et al.* 2011b). However, the importance of statin treatment in AD is still under debate, given that some randomized clinical trials did not show any significant benefit on cognition as reviewed by (McGuinness *et al.* 2010; Butterfield *et al.* 2011a).

Although a possible link between statins administration and HO-1 up-regulation was demonstrated *in vivo* (Hsu *et al.* 2006; Lai *et al.* 2008; Butterfield *et al.* 2011b), no observations on the statins-BVR-A interaction have been reported. The aim of this work was to evaluate in a pre-clinical model of AD, the aged canine (which naturally develops learning and memory impairments in association with the accumulation of human sequence Aβ) (Cotman and Head 2008), whether chronic administration of atorvastatin has neuroprotective effects through the up-regulation of BVR-A. We also predicted that BVR-A would be associated with reduced oxidative damage, previously reported reduced BACE1 protein levels and impaired/improved cognition (Murphy *et al.* 2010).

Materials and methods

Animals

Twelve beagles ranging in age from 8.9 to 13.2 years were obtained from the Lovelace Respiratory Research Institute (Albuquerque, NM, USA) and Harlan (Indianapolis, IN, USA). Based on our previous work, dogs of this age show cognitive decline and significant amounts of brain Aβ (Head *et al.* 2000; Studzinski *et al.* 2006). 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 ranged in age from 10.1 to 14.6 years. All research was conducted in accordance with approved IACUC protocols. Animals were ranked by cognitive test scores and placed into equivalent groups with two males and four females per group. 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 task were used.

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 × 40 mg tablets per day for a daily dose of 80 mg/day and control animals received two 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 (Walsh *et al.* 1996). As previously demonstrated, in beagles treated with 6 mg/kg atorvastatin (approximately 90 mg/dog), plasma concentrations of atorvastatin were approximately 500 ng/mL (Shen *et al.* 2006). 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) (Cilla *et al.* 1996; Stern *et al.* 2000). This differs from rodent studies that have reported reduced brain Aβ in response to statin treatment. In these studies, doses are typically between 200- and 400-times higher than those used in humans (Petanceska *et al.* 2002), which leads to some concern regarding the translation of these outcomes to AD clinical trials.

Tissue collection

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

Sample preparation

Brain (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 M sucrose, 0.10 mM

Tris-HCl (pH = 8.8), 0.10 mM $MgCl_2$, 0.08 mM EDTA, proteinase inhibitors leupeptin (0.5 mg/mL), pepstatin (0.7 μg/mL), aprotinin (0.5 mg/mL) and phenylmethylsulfonyl fluoride (40 μg/mL) and phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). As the phosphorylation of specific Ser/Thr/Tyr residues on BVR-A by itself or other kinases (e.g. the insulin receptor kinase) are involved in both the reductase and metabolic activities of BVR-A (Lerner-Marmarosh *et al.* 2005; Kapitulnik and Maines 2009), kinase inhibitors could interfere with such activities. For this reason, kinase inhibitors where not included in Media 1 lysis buffer. Samples 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 cellular debris. Total protein concentration of the supernatant was determined by BCA method (Pierce, Rockford, IL, USA).

Western blot analysis

Western blot analyses were performed as previously described by Barone *et al.* (2011c). The following primary antibodies were used as separate experiments: anti-BVR-A (Sigma-Aldrich; dilution 1 : 1000), anti-phosphoserine and anti-phosphothreonine (Invitrogen, Camarillo, CA, USA; dilution 1 : 250), anti-phosphotyrosine, (Invitrogen; dilution 1 : 1000), anti-nitrotyrosine (3-NT) (Sigma-Aldrich; dilution 1 : 100), anti-dinitrophenylhydrazone (DNP) protein adducts (Millipore, Billerica, MA, USA; dilution 1 : 100), anti-4-hydroxy-2-nonenal (HNE) (Alpha Diagnostic International, San Antonio, TX, USA; dilution 1 : 100) or anti-β-actin (Sigma-Aldrich; dilution 1 : 2000).

Immunoprecipitation

The immunoprecipitation procedure was performed as previously described (Cenini *et al.* 2008), with minor modifications. Briefly, 150 μg proteins were dissolved in 500 μL RIPA buffer (10 mM Tris, pH 7.6; 140 mM NaCl; 0.5% NP40 including protease inhibitors) and incubated with 1 μg anti-BVR-A polyclonal antibody at 4°C overnight. Immunocomplexes were collected using protein A/G suspension for 2 h at 4°C and washed five times with immunoprecipitation buffer. Immunoprecipitated BVR-A was recovered by resuspending the pellets in reducing sodium dodecyl sulfate buffers and electrophoresing them on 12% gels, followed by western blot analysis. Membrane were then stripped and re-probed using an anti-BVR-A antibody as described above. Total BVR-A was used as a loading control as previously described (Salim *et al.* 2001; Lerner-Marmarosh *et al.* 2008).

BVR-A Phosphoserine/threonine and phosphotyrosine detection

BVR-A specific phosphorylation on serine/threonine or tyrosine residues were detected as previously described by using a mix of antibodes directed against either phosphoserine and phosphothreonine or tyrosine residues (Salim *et al.* 2001; Lerner-Marmarosh *et al.* 2008). Membranes were then stripped and re-probed using and anti-BVR-A antibody as described above. Total BVR-A was used as a loading control (Salim *et al.* 2001; Lerner-Marmarosh *et al.* 2008).

Post-derivatization of protein

Samples were post-derivatized with dinitrophenylhydrazine on the membrane and probed with anti-DNP antibody to identify the carbonylated proteins. The nitrocellulose membranes where equilibrated in solution A [20% (v/v) methanol and 80% (v/v) wash blot buffer (phosphate-buffered saline solution containing 0.04% (v/v) Tween 20 and 0.10 M NaCl)] for 5 min, followed by incubation of membranes in 2 N HCl for 5 min. The proteins on blots were then derivatized in solution B (0.5 mM dinitrophenylhydrazine in 2 N HCl) for 10 min as described by (Conrad *et al.* 2000). The membranes were successively washed 5 min per time in 2N HCl for three times, wash blot buffer/methanol (50/50) for five times and finally wash blot buffer for two times. The DNP adducts were detected immunochemically as described above.

Biliverdin reductase activity assay

Two isoforms of BVR, BVR-A and BVR-B, have been previously described (Maines 2005; Franklin *et al.* 2009; Kapitulnik and Maines 2009). Both enzymes generate BR, but only BVR-A reduces BV-IX alpha into the powerful antioxidant molecule BR-IX-alpha, whereas BVR-B prefers the other BV isoforms, such as BV-β, BV-γ and BV-δ (Maines 2005; Franklin *et al.* 2009; Kapitulnik and Maines 2009). However, it is not possible to separate the differential contribution of BVR-A and BVR-B to the generation of BR (Franklin *et al.* 2009). For this reason, here we refer to BVR activity. We determined BVR activity in parietal cortex, cerebellum and liver samples from aged beagles using a BVR assay kit (Sigma-Aldrich) as per manufacturer's instructions with minor modification. Briefly, 150 μg proteins were prepared for the assay and loaded in the 96 well plate. BVR positive control solution (2.5, 5, 10, 15 20 μL) was included in the assay for generation of a standard curve. Fifty-microliter assay buffer and 150 μL working solution (containing NAPDH, substrate solution and assay buffer) were added to each standard and sample on the plate. The plate was placed on the UV-VIS plate reader at 37°C and read every minute for ten min. The reading at 5 min had a linear reaction rate, and was chosen for BVR activity calculations.

Oxidative stress biomarkers assays

Assays for oxidative stress biomarkers for protein oxidation (protein carbonyls; 3-nitrotyrosine) and lipid peroxidation (protein-bound HNE) were performed as described previously (Barone *et al.* 2011b).

β-Secretase 1 (BACE1) analysis

Western blot assays for BACE1 protein levels were performed as described previously (Murphy *et al.* 2010).

Statistical analysis

For western blot analyses, the intensity of each band from control samples normalized to β-actin was considered as 100% and the atorvastatin-treated beagles values were expressed as percentage of controls. Data are expressed as mean ± SD of N independent samples per group. 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 among BVR-A protein levels, post-translational modifications, and activity, as well as cognitive test scores, markers of oxidative damage and BACE1 levels.

Results

Effect of atorvastatin treatment on BVR-A protein levels and post-translational modifications in parietal cortex, cerebellum and liver of aged beagles

In aged beagles, atorvastatin significantly increased BVR-A protein levels by ~21% ($t = 3.06$, df = 10, $p < 0.05$) in the parietal cortex (Fig. 1a) and by 60% ($t = 7.90$, df = 10, $p < 0.01$) in the liver (Fig. 1c). No differences were found in the cerebellum (Fig. 1b).

As shown in previous studies, post-translational modifications (phosphorylation, oxidation and nitration) of BVR-A structure significantly affect its biological activity in the hippocampus of AD and mild cognitive impairment (MCI) subjects (Barone *et al.* 2011a; c). For this reason, we investigated whether the up-regulation of BVR-A protein levels were paralleled by changes in the phosphorylation of serine/threonine (Ser/Thr) and tyrosine (Tyr) residues. Atorvastatin significantly increased pTyr-BVR-A by ~54% ($t = 2.99$, df = 9, $p < 0.05$) (Fig. 2a) in the parietal cortex of aged beagles with respect to the control group. Additionally, pSer/Thr-BVR-A was increased by ~17% ($t = 2.06$, df = 9), although this increase was not statistically significant (Fig. 2b). No significant differences were found in cerebellum and liver (data not shown).

With regard to the oxidative/nitrosative post-translational modifications on BVR-A, increased 3-NT-BVR-A in the hippocampus of AD and MCI subjects was previously reported by our group (Barone *et al.* 2011a). This effect was paralleled by the high levels of oxidative stress observed in this brain area with respect to the matched controls (Barone *et al.* 2011a). Because atorvastatin significantly decreased total levels of oxidative stress biomarkers (PC, HNE, and 3-NT) in the parietal cortex of these aged beagles (Barone *et al.* 2011b), we hypothesized that BVR-A oxidation or nitration would also be reduced in the brain of atorvastatin-treated dogs. Immunoprecipitation with specific anti-BVR-A antibody was used to measure the levels of PC, HNE and 3-NT modifications on BVR-A. The levels of PC-BVR-A (Fig. 3a) and HNE-BVR-A (Fig. 3b) did not change in the parietal cortex after atorvastatin administration. In contrast, atorvastatin-treated aged beagles showed an ~18% decrease ($t = 1.98$, df = 10) in the levels of 3-NT-BVR-A (Fig. 3c) in parietal cortex, although this effect was not statistically significant. No differences were observed in the cerebellum (data not shown). Conversely, HNE-BVR-A was significantly increased by ~60% in liver samples (Fig. 4b, $t = 3.19$, df = 9, $p < 0.05$). Similarly, a trend towards increased levels of PC-BVR-A (~45%) as compared with controls ($t = 2.18$, df = 10) was observed (Fig. 4a). No changes were found in the liver for 3-NT modified BVR-A (Fig. 4c).

BVR activity

To evaluate if the post-translational modifications on BVR-A structure affect its enzymatic activity, reductase activity was measured. BVR activity, evaluated as nmol of BR formed from 1 mg of protein, was significantly increased by ~35% ($t = 2.55$, df = 10, $p < 0.05$) in the parietal cortex of atorvastatin-treated aged beagles with respect to the control group (Fig. 5). No treatment effects were observed in either the cerebellum or liver (Fig. 5). Furthermore, we found that

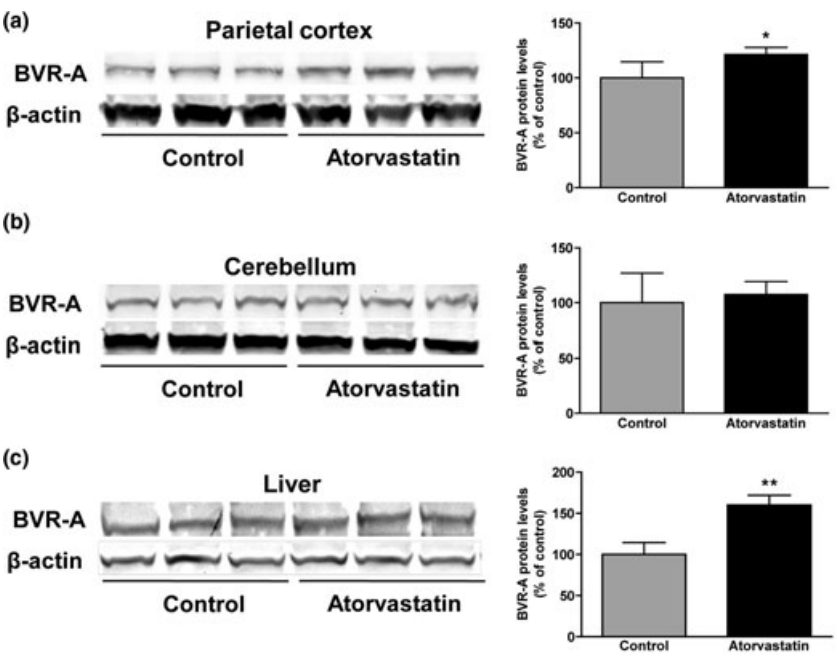

Fig. 1 Biliverdin reductase-A (BVR-A) protein levels in parietal cortex, cerebellum and liver of aged beagles treated with atorvastatin. BVR-A protein levels in (a) parietal cortex, (b) cerebellum and (c) liver of aged beagles. Representative gels are shown. Data are expressed as mean ± SD ($n = 6$ animals per group). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control.

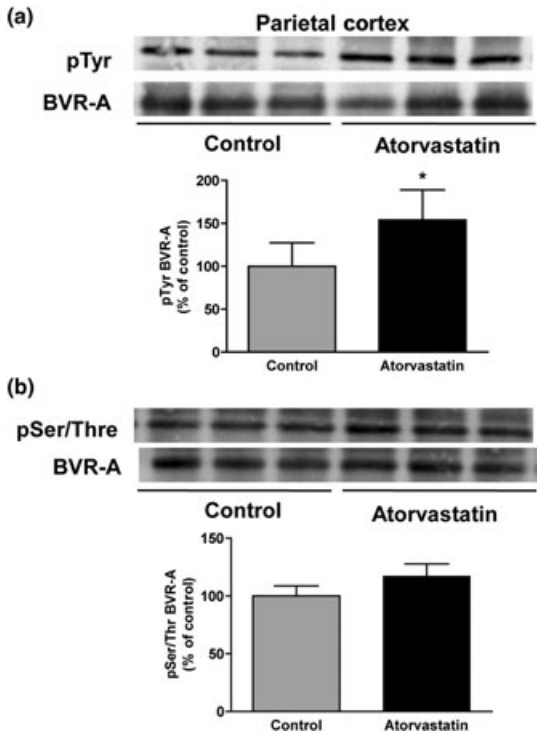

Fig. 2 Biliverdin reductase-A (BVR-A) phosphorylation on Serine/Threonine (pSer/Thr) and Tyrosine (pTyr) residues in parietal cortex of aged beagles treated with atorvastatin. (a) pTyr-BVR-A and (b) pSer/Thr-BVR-A levels in the parietal cortex of aged beagles. Representative gels are shown. Data are expressed as mean ± SD ($n = 6$ animals per group). *$p < 0.05$ versus control.

atorvastatin leads to a significant increases in BR production in parietal cortex of about 30% ($t = 3.03$, df = 10, $p < 0.05$) with respect to cerebellum, and ~24% ($t = 3.05$, df = 10, $p < 0.05$) with respect to liver (Fig. 5).

BVR-A protein levels and activity are associated with (i) changes in oxidative stress levels in parietal cortex and (ii) learning scores

We first examined the correlations between BVR-A protein levels and BVR-A post-translational modifications and activity in the parietal cortex. BVR-A protein levels were positively correlated with pTyr-BVR-A ($r = 0.87$, $p < 0.01$; Fig. 6a), pSer/Thr-BVR-A ($r = 0.83$, $p < 0.05$; Fig. 6b), and BVR activity ($r = 0.78$, $p < 0.05$; Fig. 6d) whereas these oxidative stress-related post-translational modifications and activity of BVR-A in the parietal cortex were negatively correlated with 3-NT-BVR-A ($r = -0.48$, $p = 0.26$; Fig. 6c) Thus, an increase of BVR-A protein levels was associated with an increase in both pTyr-BVR-A and pSer/Thr-BVR-A, as well as BVR activity.

We next hypothesized that the previously reported reduction in oxidative stress damage in the parietal cortex in response to atorvastatin (Barone *et al.* 2011b) would be associated with increased BVR-A protein levels and/or activity. As shown in Fig. 7, BVR-A protein levels negatively correlated with HNE ($r = -0.74$, $p < 0.01$; Fig. 7a) and 3-NT ($r = -0.73$, $p < 0.05$; Fig. 7b) total levels in parietal cortex. Similarly, BVR activity negatively correlated with PC ($r = -0.75$, $p < 0.01$; Fig. 7c) and 3-NT ($r = -0.61$,

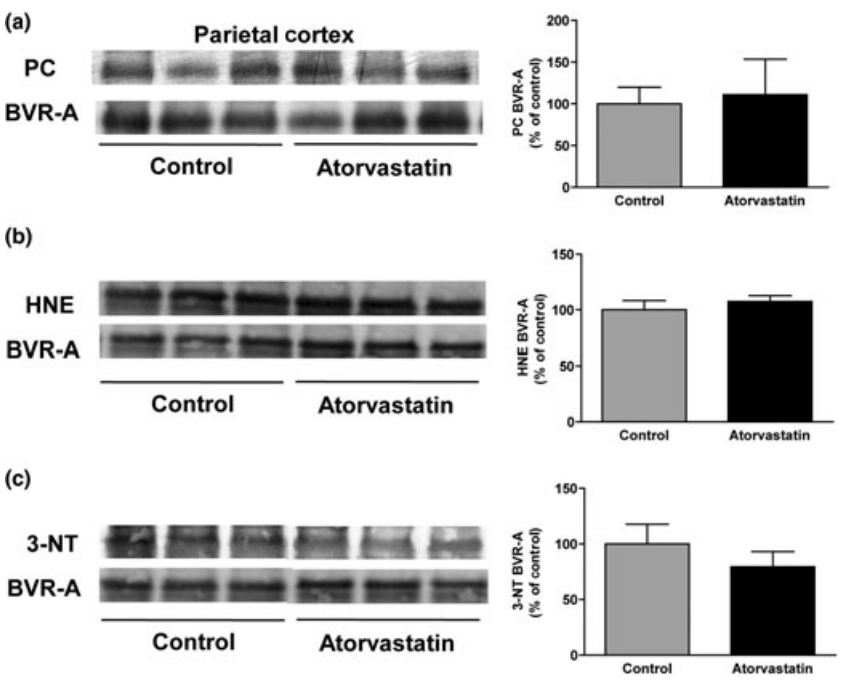

Fig. 3 Biliverdin reductase-A (BVR-A) oxidative and nitrosative post-translational modifications in the parietal cortex of aged beagles treated with atorvastatin. (a) Protein carbonyls (PC), (b) 4-hydroxy-2nonenal (HNE) and (c) 3-nitrotyrosine (3-NT) levels on BVR-A in the parietal cortex of aged beagles. Representative gels are shown. Data are expressed as mean ± SD ($n = 6$ animals per group).

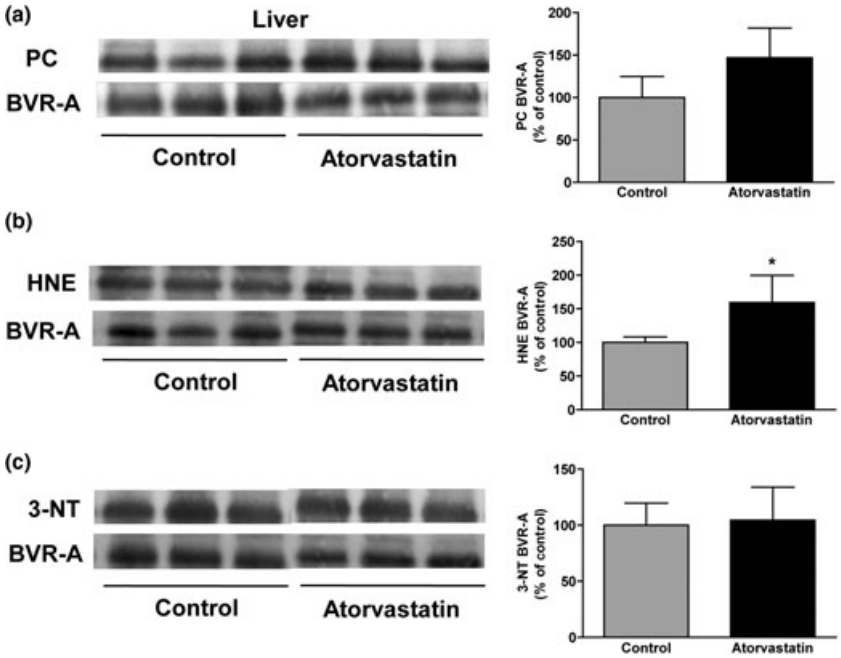

Fig. 4 Biliverdin reductase-A (BVR-A) oxidative and nitrosative post-translational modifications in the liver of aged beagles treated with atorvastatin. (a) Protein carbonyls (PC), (b) 4-hydroxy-2nonenals (HNE) and (c) 3-nitrotyrosine (3-NT) levels on BVR-A in the parietal cortex of aged beagles. Representative gels are shown. Data are expressed as mean ± SD ($n = 6$ animals per group). *$p < 0.05$ versus control.

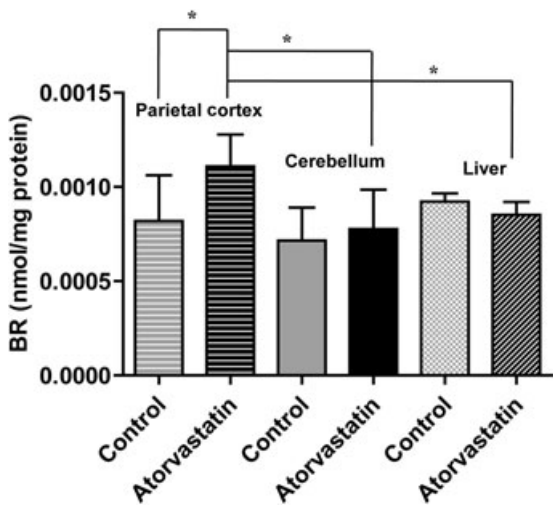

Fig. 5 Biliverdin reductase activity in parietal cortex, cerebellum and liver of aged beagles treated with atorvastatin (80 mg/day for 14.5 months). Samples of parietal cortex, cerebellum and liver of aged beagles treated with atorvastatin (80 mg/day for 14.5 months) were assayed for BVR activity. Values are given as nmol of bilirubin (BR) formed per 1 mg of protein. Data are expressed as mean ± SD ($n = 6$ animals per group). *$p < 0.05$ versus matched group.

$p < 0.05$; Fig. 7d) total levels in parietal cortex. Thus, higher levels of BVR-A protein and activity were associated with reduced oxidative damage.

To determine if BVR-A protein levels or activity were associated with cognition, correlations were calculated based on size discrimination learning scores. Size discrimination learning error scores were negatively correlated with BVR-A protein levels ($r = -0.82$, $p < 0.05$; Fig. 8a), pTyr-BVR-A ($r = -0.78$, $p < 0.05$; Fig. 8b) and BVR activity ($r = -0.76$, $p < 0.01$; Fig. 8c), suggesting that reduced error scores associated with improved cognition in discrimination learning were associated with activated BVR-A.

BVR-A protein levels, pTyr-BVR-A and pSer/Thr-BVR-A are associated with BACE1 protein levels in parietal cortex

BACE1 protein levels were significantly decreased in parietal cortex of aged beagles treated with atorvastatin (Murphy *et al.* 2010). Here, we report a significant inverse association between BVR-A and BACE1 protein levels. BVR-A protein levels ($r = -0.73$, $p < 0.05$; Fig. 9a), pTyr-BVR-A ($r = -0.93$, $p < 0.0001$; Fig. 9b) and pSer/Thr-BVR-A ($r = -0.67$, $p < 0.05$; Fig. 9c) were significantly negatively correlated with BACE1 protein levels in the parietal cortex of aged beagles.

Discussion

Epidemiological studies suggest that statins use is associated with reduced risk of developing AD (Feldman *et al.* 2010). However, the mechanism through which these drugs may protect the brain has not been clearly established. We previously reported decreased BACE1 protein levels (Murphy *et al.* 2010), decreased oxidative stress markers along with a concomitant increase of HO-1 protein levels and reduced glutathione in the parietal cortex of aged beagles treated with atorvastatin (80 mg/day for 14.5 months) (Barone *et al.* 2011b; Butterfield *et al.* 2011b). In this study, we extend the neurobiological benefits of atorvastatin in the brain of aged beagles to include (i) potentiation of the cell stress response through

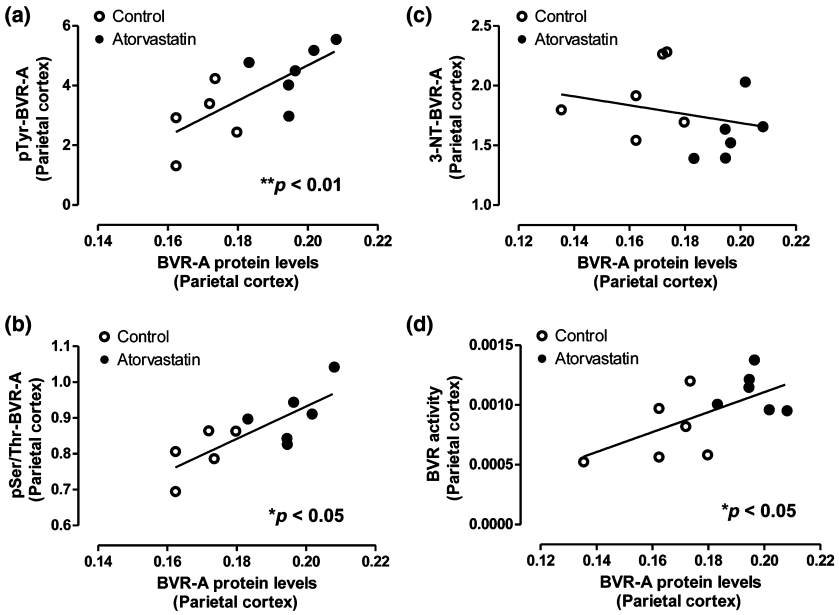

Fig. 6 Biliverdin reductase-A (BVR-A) protein levels are associated with BVR-A post-translational modifications in the parietal cortex of aged beagles treated with atorvastatin. Positive correlations were found between BVR-A protein levels and (a) pTyr-BVR-A ($r = 0.87$, $p < 0.01$), (b) pSer/Thr-BVR-A ($r = 0.83$, $p < 0.05$) and (d) BVR activity ($r = 0.78$, $p < 0.05$) in the parietal cortex. A negative correlation was found between (c) BVR-A protein levels and 3-NT-BVR-A ($r = -0.48$, $p = 0.26$) in the parietal cortex.

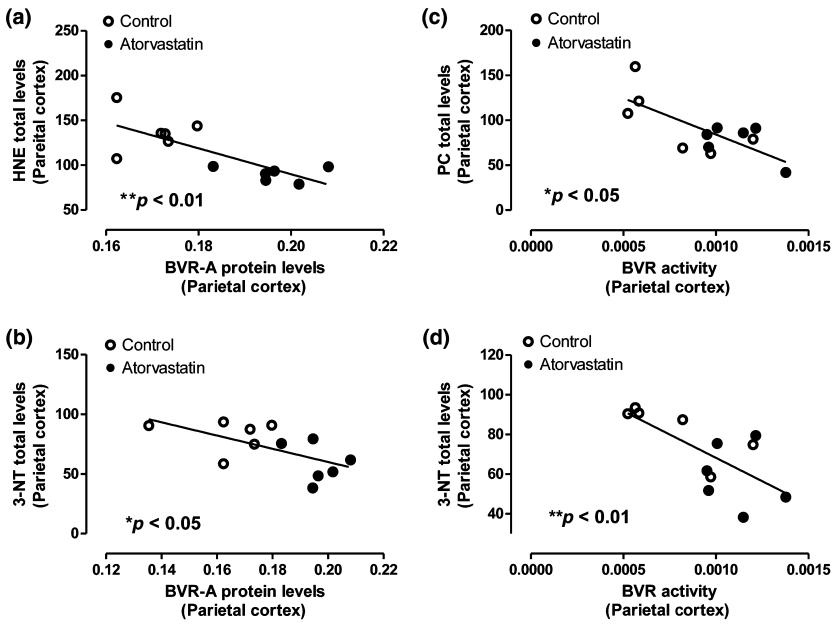

Fig. 7 Biliverdin reductase-A (BVR-A) protein levels as well as BVR activity are associated with reduced oxidative stress levels in the parietal cortex of aged beagles treated with atorvastatin. Negative correlations were found between BVR-A protein levels and (a) HNE total levels ($r = -0.74$, $p < 0.01$) and (b) 3-NT total levels ($r = -0.73$, $p < 0.05$) in the parietal cortex of aged beagles. Negative correlation were also found between BVR activity (measured as bilirubin produced for 1 mg of protein) and (c) PC total levels ($r = -0.75$, $p < 0.01$) and (d) 3-NT total levels ($r = -0.61$, $p < 0.05$) in the parietal cortex of aged beagles.

the up-regulation of BVR-A; (ii) observation that the selectivity of BVR-A-associated reduction of oxidative stress biomarkers in the brain to an area involved in cognitive function, the parietal cortex, but not to cerebellum; (iii) improvement of cognitive functions related to the modulation of BVR-A post-translational modifications in the parietal cortex; and (iv) proposed a possible interaction between BVR-A and BACE1.

Although the sample size was relatively small, consistent effects with BVR-A protein levels, post-translational modifications and activity outcome measures suggest this may be a robust effect.

Aged beagles, represent a good pre-clinical model to study AD because they deposit endogenous levels of Aβ of identical sequence to human Aβ (Johnstone *et al.* 1991) 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 which models early plaque formation in humans (Torp *et al.* 2000a,b, 2003). Moreover, in terms of the pattern and severity of cognitive decline, the aged canine parallels mild cognitive impairment in humans (Cotman and Head 2008).

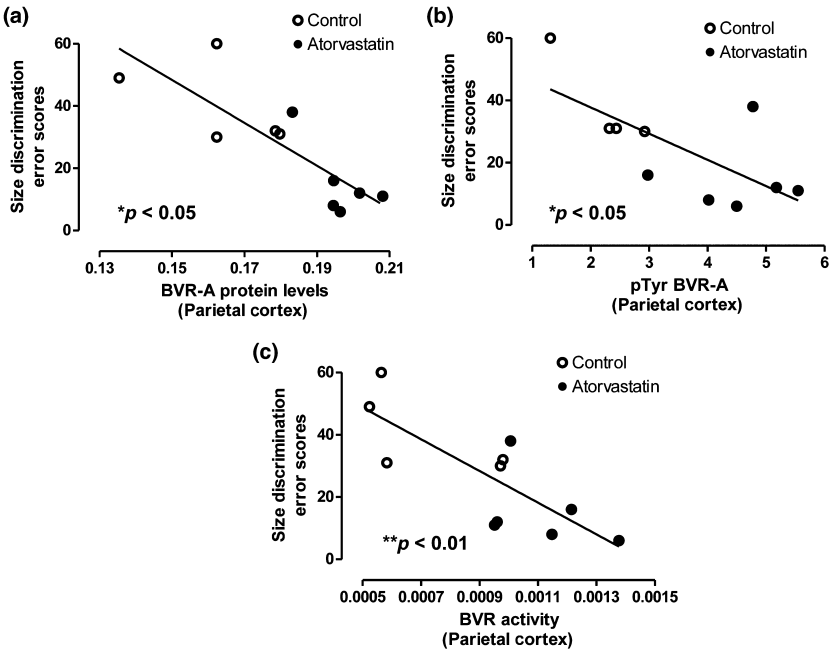

Fig. 8 Biliverdin reductase-A (BVR-A) protein levels, phosphorylation on tyrosine residues (pTyr-BVR-A) and activity measured in parietal cortex are associated with improvement in size discrimination learning in aged beagles treated with atorvastatin. Negative correlations were found between size discrimination error scores and (a) BVR-A protein levels ($r = -0.82$, $p < 0.05$), (b) pTyr-BVR-A ($r = -0.78$, $p < 0.05$) and (c) BVR activity ($r = -0.76$, $p < 0.01$) in the parietal cortex of aged beagles.

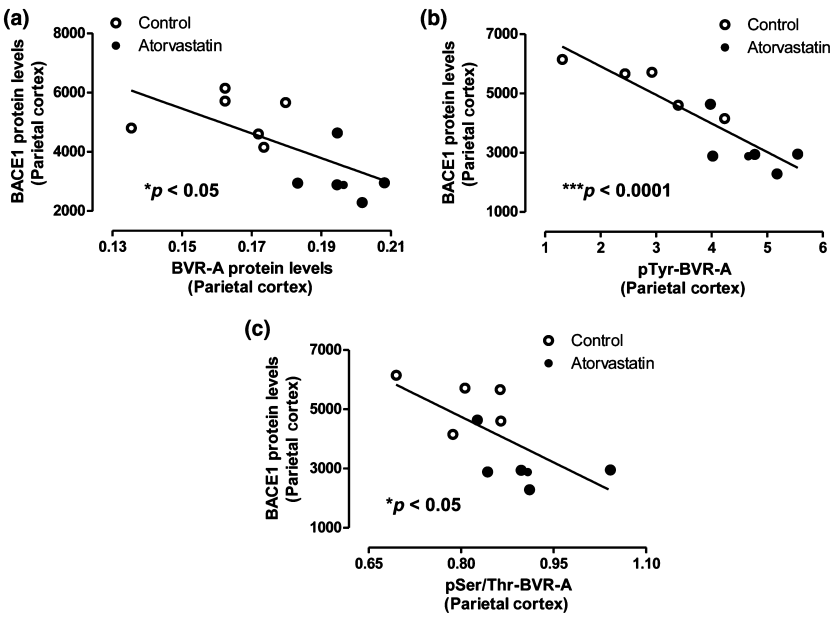

Fig. 9 Biliverdin reductase-A (BVR-A) protein levels and phosphorylation on tyrosine/serine/threonine (pTyr- and pSer/Thr-BVR-A) are associated with reduced levels of β-secretase 1 (BACE1) in the parietal cortex of aged beagles treated with atorvastatin. Negative correlations were found between BACE1 protein levels and (a) BVR-A protein levels ($r = -0.73$, $p < 0.05$), (b) pTyr-BVR-A ($r = -0.93$, $p < 0.0001$) and (c) pSer/Thr-BVR-A ($r = -0.67$, $p < 0.05$) in the parietal cortex of aged beagles.

Aged beagles can be used to conduct long-term studies using statins and extensive behavioral testing, as HMG-CoA reductase is not up-regulated over time in these animals. Conversely, in rodent models, long-term treatment with statins leads to an up-regulation of HMG-CoA reductase, thereby preventing any stable, long-term reduction in cholesterol levels (Thelen *et al.* 2006). Therefore, studies in beagles can be more directly translated to human clinical trials.

Our results clearly show that, in aged beagles, a long-term atorvastatin treatment increased BVR-A protein levels (Fig. 1a) and is associated with increased pTyr-BVR-A (Fig. 2a) as well as pSer/Thr-BVR-A (Fig. 2b) and decreased 3-NT-BVR-A (Fig. 3c) in a brain area involved in cognitive function. As previously reported by Maines and colleagues, Gly148-Ser-Leu-Arg-Phe-Thr-Ala-Ser-Pro is an important motif in the serine/threonine kinase domain of BVR-A because the phosphorylation of Ser149 is essential for BVR

activity (Salim *et al.* 2001). Furthermore, the phosphorylation of critical Ser/Thr/Tyr residues plays a main role in the regulation of the neuroprotective and/or metabolic activities of this enzyme (Kapitulnik and Maines 2009).

The observed increase of BVR-A protein levels in the parietal cortex of aged beagles following atorvastatin treatment adds a new element in the picture of the stimuli able to modulate this enzyme. In fact, although it was initially considered a non-inducible protein, later studies showed that BVR-A can be induced by substances such as lipopolysaccharide and bromobenzene at the post-transcriptional level, while its expression is unaffected by heat shock (Ewing *et al.* 1993; Maines *et al.* 2001). However, the exact mechanism by which atorvastatin increases BVR-A protein levels cannot be elucidated in the current study, and additional *ad hoc* designed experiments are needed. Conversely, the novelty of our results is that for the first time in a higher mammalian *in vivo* model of AD not only the protein levels, but also the post-translational modifications that affect BVR-A's activities have been analyzed, because the analysis of only expression levels is not sufficient to explain BVR-A's contribution to cell signaling networks (Kapitulnik and Maines 2009; Barone *et al.* 2011a,c).

As shown by our results, BVR activity increased by ~35% in the parietal cortex of atorvastatin-treated animals (Fig. 5), and this effect seems to be secondary to the increased phosphorylation of Ser/Thr/Tyr residues on BVR-A observed in this brain area (Figs 2 and 6). At the same time, treatment-related differences were not observed in the cerebellum or liver, suggesting a specific effect of atorvastatin in the parietal cortex. Consonant with these findings are the results about BVR-A post-translational modifications found in cerebellum and in liver samples. In fact, while in the former we did not observed any significant change, in the latter, the marked increase of BVR-A protein levels is associated with a marked increase of HNE-BVR-A. As it is well known that the formation of HNE adducts alters proteins structure (Subramaniam *et al.* 1997) and results in a marked decrease of their function (Lauderback *et al.* 2001; Butterfield and Lauderback 2002; Owen *et al.* 2010), it is plausible to argue that the formation of BVR's HNE-adducts are responsible for the lack of any significant changes in the enzymatic activity in the liver of atorvastatin-treated dogs.

As noted, our group previously demonstrated an impairment of HO-1/BVR-A system in the hippocampus of AD and MCI subjects (Barone *et al.* 2011c), suggesting that the

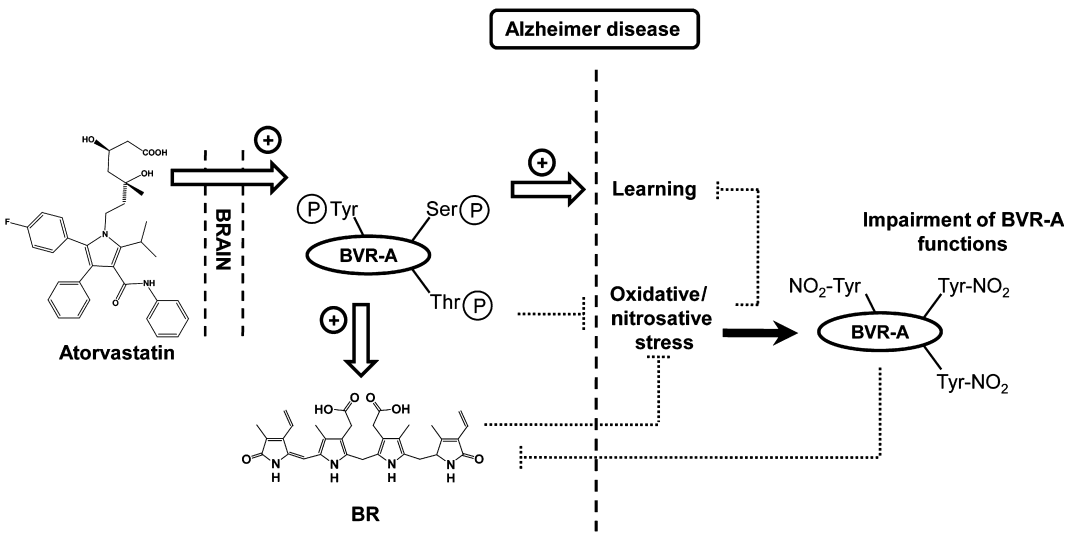

Fig. 10 Schematic representation of atorvastatin-induced BVR-A neuroprotective effects in the parietal cortex of aged beagles. The aged beagles are a good pre-clinical model of Alzheimer disease because they naturally develop learning and memory impairments in association with the accumulation of human sequence Aβ and increased oxidative stress levels (Cotman and Head 2008; Head *et al.* 2008; Opii *et al.* 2008) (right side). Atorvastatin increases both BVR-A protein levels and phosphorylation on Tyr/Ser/Thr residues in parietal cortex of aged beagles (left side). As a consequence, an increase of its reductase activity (increased bilirubin (BR) production) is observed (left side). Both BVR-A and BR posses antioxidant features responsible of the reduction of oxidative stress in the parietal cortex (left side), as demonstrated by the negative correlations found between oxidative stress biomarkers levels and (i) BVR-A protein levels or (ii) BVR activity in the same brain area. Furthermore, BVR-A is associated with an improvement of cognitive functions (learning) following atorvastatin treatment (left side). All these effects contribute to the neuroprotective role of BVR-A in the brain. In contrast, once BVR-A becomes oxidized [e.g. an increase of 3-nitrotyrosine (3-NT) modification on its structure] because of increased oxidative/nitrosative stress levels (right side), an impairment of its functions occurs [e.g. a decrease of its reductase activity, thus a decrease of BR formation (Barone *et al.* 2011a,c)] (right side). This effect contributes to the maintenance of elevated oxidative stress levels due to the lack of antioxidant features of both BVR-A and BR. White arrows, atorvastatin-induced stimulation; filled arrow, stimulation; dotted line, inhibition.

increased levels of oxidative stress observed in this brain area could be related to a defective functioning of this system. The significant correlations found among BVR-A protein levels, activity or phosphorylation in parietal cortex and decreased levels of oxidative stress markers as well as decreased size discrimination error score (reflecting improved cognition) observed in aged dogs after treatment with atorvastatin, led us to speculate that the effects on oxidative stress and cognition could be mediated by the activation of the HO-1/BVR-A system. We believe that an increase of BVR-A protein levels together with its improved functioning could trigger a cell stress response and thus improve cognitive behavior by the following mechanisms: (i) activation of both conventional and atypical protein kinase C isoforms (Kapitulnik and Maines 2009) whose involvement in memory function is now well established (Sacktor 2011); (ii) interaction with members of the MAPK family, such as ERK1/2-Mek-Elk1, through which BVR-A regulates important metabolic pathway as well as the expression of oxidative-stress-responsive genes such as HO-1 or inducible nitric oxide synthase (iNOS) (Maines 2007; Lerner-Marmarosh *et al.* 2008; Tudor *et al.* 2008; Kapitulnik and Maines 2009); (iii) production of the powerful antioxidant BR as result of its reductase activity. In these scenarios, as the phosphorylation of BVR-A on Tyr residues is required to interact with ERK-Mek-Elk1 (Lerner-Marmarosh *et al.* 2008), the increase of pTyr-BVR-A in the parietal cortex following atorvastatin treatment, coupled with the negative correlation between pTyr-BVR-A and size discrimination error scores, could suggest an activation of the MAPK-related signal transduction pathways that in turn promote a robust cell stress response (Kapitulnik and Maines 2009). At the same time, the significant correlations found between BVR activity and decreased total PC and 3-NT levels suggest a main antioxidant role for BR, consistent with prior studies (Stocker *et al.* 1987a,b; Dore *et al.* 1999; Barone *et al.* 2009). Furthermore, as previously demonstrated, BR increased neuronal NOS expression and nitric oxide formation in both primary cultures of cerebellar granule neurons and neurotrophin-sensitive PC12 cells (Mancuso *et al.* 2008), and it was shown that this gaseous neurotransmitter plays a key role in the long-term potentiation and synaptic plasticity (Calabrese *et al.* 2007). In addition, in PC12 cells BR up-regulated cAMP response element-binding (Mancuso *et al.* 2008), which is considered an important transcription factor regulating both short- and long- term memory (Suzuki *et al.* 2011).

We previously reported that atorvastatin treatment decreased BACE1 protein level without affecting levels of Aβ (Murphy *et al.* 2010). An interesting finding was the association between BVR-A and BACE1. BVR-A protein levels and phospshorylation are negatively associated with BACE1 protein levels in the parietal cortex of atorvastatin-treated aged beagles. In particular, pTyr-BVR-A and pSer/Thr-BVR-A were associated with a reduction of BACE1 protein levels (Fig. 9b and c). These correlations may be related to the increased kinase activity of BVR-A, which in turn could either directly or indirectly promote BACE1 degradation in lysosomes (Wahle *et al.* 2005; Tesco *et al.* 2007; Vassar *et al.* 2009). However, at this time we can only speculate about a possible involvement of BVR-A in BACE1 recycling, and *ad hoc* designed experiments to address this idea are ongoing in our laboratories.

In conclusion, though atrovastatin does not cross the blood-brain barrier, our results indicate that parietal-resident BVR-A may be a target for atorvastatin (Fig. 10), thus further demonstrating the pleiotropic effect of statins. In addition, atorvastatin-induced BVR-A post-translational modifications may contribute to the neuroprotective effects (Fig. 10) of this enzyme (decreased oxidative stress levels and improved cognitive functions), thus suggesting a potential therapeutic role of BVR-A in AD.

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