



Atorvastatin withdrawal elicits oxidative/nitrosative damage in the rat cerebral cortex

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ARTICLE INFO

Article history:

Received 26 October 2012

Received in revised form 1 February 2013

Accepted 11 February 2013

Keywords:

Atorvastatin

Withdrawal

Cerebral cortex

Nitrosative damage

SOD

ABSTRACT

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting step in cholesterol biosynthesis. Statins effectively prevent and reduce the risk of coronary artery disease through lowering serum cholesterol, and also exert anti-thrombotic, anti-inflammatory and antioxidant effects independently of changes in cholesterol levels. On the other hand, clinical and experimental evidence suggests that abrupt cessation of statin treatment (*i.e.* statin withdrawal) is associated with a deleterious rebound phenomenon. In fact, statin withdrawal increases the risk of thrombotic vascular events, causes impairment of endothelium-dependent relaxation and facilitates experimental seizures. However, evidence for statin withdrawal-induced detrimental effects to the brain parenchyma is still lacking. In the present study adult male Wistar rats were treated with atorvastatin for seven days (10 mg/kg/day) and neurochemical assays were performed in the cerebral cortex 30 min (atorvastatin treatment) or 24 h (atorvastatin withdrawal) after the last atorvastatin administration. We found that atorvastatin withdrawal decreased levels of nitric oxide and mitochondrial superoxide dismutase activity, whereas increased NADPH oxidase activity and immunoreactivity for the protein nitration marker 3-nitrotyrosine in the cerebral cortex. Catalase, glutathione-S-transferase and xanthine oxidase activities were not altered by atorvastatin treatment or withdrawal, as well as protein carbonyl and 4-hydroxy-2-nonenal immunoreactivity. Immunoprecipitation of mitochondrial SOD followed by analysis of 3-nitrotyrosine revealed increased levels of nitrated mitochondrial SOD, suggesting the mechanism underlying the atorvastatin withdrawal-induced decrease in enzyme activity. Altogether, our results indicate the atorvastatin withdrawal elicits oxidative/nitrosative damage in the rat cerebral cortex, and that changes in NADPH oxidase activity and mitochondrial superoxide dismutase activities may underlie such harmful effects.

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1. Introduction

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the rate-limiting step in cholesterol biosynthesis [1]. As a consequence, they effectively prevent and reduce the risk of coronary artery disease by lowering serum cholesterol levels [1]. Besides their action on HMG-CoA reductase, it has been shown that statins display anti-thrombotic [2], anti-inflammatory [2] and antioxidant

activity [2–5]. Therefore, it has been proposed that statin treatment would be useful in other conditions than cardiovascular diseases. In fact, the neuroprotective effects of statins have been reported in several clinical and experimental conditions, such as traumatic brain injury [6], stroke [7], ischemia [8], Alzheimer's disease [5], excitotoxic aminoacid exposure [9] and seizures [10,11]. However, compelling clinical and experimental evidence suggests that abrupt cessation of statin treatment (*i.e.* statin withdrawal) is associated with a deleterious rebound phenomenon [10,12–18]. For instance, patients with stable coronary heart disease presented a threefold increase in thrombotic vascular events after simvastatin treatment was stopped and continued with relatively lower doses of fluvastatin [12], and statin discontinuation was associated with poor outcome and higher mortality after intracerebral hemorrhage [19]. Another large epidemiologic study showed that patients who stopped statins on admission for acute myocardial

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infarction developed more heart failure, ventricular tachycardia, or death during hospitalization than patients who were not in statin treatment [13]. Moreover, cerivastatin or atorvastatin withdrawal elicited oxidative stress and impaired endothelium-dependent relaxation in mice [14] and rapid loss of statin-mediated protection in mouse models of cerebral ischemia and thrombus formation [15]. Furthermore, atorvastatin withdrawal facilitated the occurrence of pentylentetrazol-induced seizures, as evidenced by a decrease in the latency to clonic and generalized tonic-clonic seizures [10]. However, evidence for statin withdrawal-induced harmful effects to the brain parenchyma is still lacking.

Considering the widespread use of statins and compelling evidence that statin withdrawal causes important deleterious effects for which there has been no clear prophylactic strategy [16], it becomes important to study the molecular mechanisms underlying such effects. Given the antioxidant activity displayed by statin treatment and in light of the concept that drug discontinuation effects are caused by the biologic adaptation to the drug persisting after the drug is cleared from the body [20], we hypothesized that atorvastatin withdrawal elicits oxidative and nitrosative stress in the rat cerebral cortex. Therefore, the present study aimed to investigate the effects of statin withdrawal on markers of oxidative and nitrosative stress and on the activity of antioxidant and pro-oxidant enzymes in the rat cerebral cortex, in order to shed some light on the molecular mechanisms underlying the deleterious effects elicited by statin withdrawal.

2. Material and methods

2.1. Animals and reagents

Adult male Wistar rats (250–300 g) were used. Animals were maintained under controlled light and environment (12:12 h light-dark cycle, $24 \pm 1^\circ\text{C}$, 55% relative humidity) with free access to water and food (SupraTM, Santa Maria, RS, Brazil). All experimental protocols were designed aiming to keep the number of animals used to a minimum, as well as their suffering. These were conducted in accordance with national and international legislation (guidelines of Brazilian Council of Animal Experimentation – CONCEA – and of U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals – PHS Policy), and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria (process #53/2010).

Atorvastatin were extracted from commercially available capsules (Lipitor®). Its identity and purity were checked by nuclear resonance methods and were >98%. Atorvastatin was chosen because several studies have shown that its withdrawal worsens outcomes in a number of experimental conditions [10,14,15], and because it is the most widely prescribed statin, being used as the reference group in statin safety studies [21,22]. Primary antibodies for mitochondrial SOD and 3-nitrotyrosine (3-NT) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA, catalog numbers sc-30080 and sc-55256, respectively). All the other reagents were purchased from Thermo Scientific Pierce Protein Research Products (Rockford, IL, USA) or Sigma–Aldrich (St. Louis, MO, USA).

Animals were treated with atorvastatin (10 mg/kg) or a corresponding volume of vehicle solution (sterile saline solution – 0.9% NaCl) by daily intragastric gavage for 7 days, and in one group treatment was withheld for 24 h before sample collection. Atorvastatin doses and schedules for administration were chosen based in previous studies [10,15], and treatment schedules were carried out in such a manner that samples from every experimental group were collected in a given session.

2.2. Tissue processing for neurochemical analyses

At the appropriate time according to the schedule described above, animals were killed by decapitation and had their brain exposed by the removal of the parietal bone. Cerebral cortices were rapidly dissected on an inverted ice-cold Petri dish and homogenized in the proper solution for each subsequent neurochemical analyses, as described below.

2.3. Determination of nitrite plus nitrate content (NO_x)

The cerebral cortex was homogenized 1:5 (m/v) with ZnSO₄ (200 mM) and acetonitrile (96%), centrifuged at $16,000 \times g$ for 30 min at 4°C , and the supernatant was collected for NO_x assay according to the spectrophotometric method based on the Griess reaction described by Miranda et al. [23]. The resulting pellet was suspended in NaOH (6 M) for protein determination.

2.4. Slot blot assays

Levels of protein carbonyls, 3-NT and 4-hydroxy-2-nonenal protein-adducts (HNE) were determined by slot blot as described in detail by Joshi et al. [24], except that the cerebral cortex was homogenized 1:10 (m/v) in 50 mM phosphate-buffered saline (PBS; pH 7.4) supplemented with a cocktail of protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 3 μM Aprotinin, 20 μM Leupeptin, 10 μM Bestatin, 1 μM E-64, 4 μM Pepstatin A, 5 mM EDTA) and was centrifuged at $13,200 \times g$ at 4°C for 20 min and supernatants were collected. The specificity for primary antibodies against protein carbonyls, 3-NT or HNE was checked by repeating each experiment with no primary antibody included in the incubation steps. Under these conditions, no staining was seen on the blots, suggesting that there was no non-specific binding of the primary antibodies.

2.5. NADPH oxidase activity

NADPH oxidase activity was measured according to Thannickal and Fanburg [25], with slight modifications. Cerebral cortex was removed and homogenized 1:10 (m/v) in 50 mM PBS (pH 7.4), then centrifuged at $1000 \times g$ at 4°C for 10 min and the resulting supernatant was used. NADPH oxidase activity was determined by monitoring NADPH consumption for 90 min at 37°C in the presence or absence of the inhibitor diphenyleneiodonium (10 μM).

2.6. Xanthine oxidase activity

The cerebral cortex was homogenized 1:10 (m/v) in 50 mM PBS (pH 7.4) and xanthine oxidase activity was measured in whole homogenates according to the spectrophotometric method described by Zanotto-Filho et al. [26].

2.7. Superoxide dismutase (SOD) activity

For determination of mitochondrial SOD activity, assays were performed in mitochondrion-enriched fractions, which were obtained as previously described by Bhattacharya et al. [27], with some modifications. Briefly, the cerebral cortex was rapidly dissected and homogenized in ice-cold isolation buffer A (100 mM sucrose, 10 mM EDTA, 100 mM Tris–HCl, 46 mM KCl, pH 7.4). The resulting suspension was then centrifuged for 3 min at $2000 \times g$ at 4°C . After centrifugation, the supernatant (S1) was once more centrifuged for 10 min at $12,000 \times g$ at 4°C . The resulting supernatant (S2) was used for determination of cytosolic SOD activity. The pellet (P2) was then resuspended in isolation buffer B (100 mM sucrose, 10 mM EDTA, 100 mM Tris–HCl, 46 mM KCl, and 0.5% bovine serum

albumin free of fatty acids, pH 7.4) and recentrifuged for 10 min at $12,000 \times g$ at 4°C . The final pellet (P3) was then gently washed and resuspended in isolation buffer C (270 mM mannitol, 70 mM sucrose, 0.02 mM EDTA, 20 mM Tris–HCl, 1 mM K_2HPO_4 , pH 7.4) and stored at -20°C . SOD activity was determined in cytosolic (S2) and mitochondrial (P3) fractions as described by Misra and Fridovich [28]. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 37°C .

2.8. Catalase (CAT) activity

CAT activity was determined spectrophotometrically as described by Furian et al. [29]. CAT specific activity was expressed as first-order rate constant, k . Appropriated controls for non-enzymatic decomposition of hydrogen peroxide were included in the assays.

2.9. Glutathione-S-transferase (GST) activity

Cerebral cortex was homogenized in 50 mM phosphate-buffered saline and GST activity was assayed spectrophotometrically according to Habig et al. [30].

2.10. Western blot assays

The cerebral cortex was rapidly dissected and gently homogenized in ice-cold T-PER tissue protein extraction reagent (Thermo Scientific), supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific). Homogenates were centrifuged at $13,200 \times g$ at 4°C for 20 min and supernatants were collected. An aliquot (20 μg protein) of the supernatant was mixed with SDS loading buffer and boiled for 5 min. Proteins were then subjected to a 15% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Membranes were blocked with 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline, containing 0.04% (v/v) Tween 20 (TBS-T) for 1 h and incubated overnight at 4°C with a 1:5000 dilution of anti-mitochondrial SOD antibodies in TBS-T containing 2.5% BSA. Membranes were washed three times in TBS-T and incubated for 1 h with biotinylated anti-rabbit secondary antibody (1:2500, Sigma Aldrich) in TBS-T containing 2.5% BSA. Membranes were washed three more times and incubated for 1 h with a streptavidin peroxidase polymer (1:2500, Sigma Aldrich) in TBS-T containing 2.5% BSA. Blots were developed using 3,3',5,5'-tetramethylbenzidine, dried, scanned, and quantified with ImageJ software.

2.11. Immunoprecipitation and mitochondrial SOD nitration analysis

The cerebral cortex was rapidly dissected and gently homogenized as described above for western blot assays, and 500 μg of protein were subjected to immunoprecipitation and subsequent determination of nitrated mitochondrial SOD content by slot blot according to Mamo et al. [31], with some modifications. Briefly, homogenates were precleared with protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 h and incubated overnight with a 1:250 dilution of anti-mitochondrial SOD antibodies or control IgG. Next, samples were incubated for 2 h with protein A/G-agarose beads and subjected to centrifugation at $1000 \times g$ for 1 min. Pellets were collected, washed three times with protein extraction reagent, and resuspended in SDS loading buffer. After boiling for 5 min, samples were centrifuged at $1000 \times g$ for 1 min and the supernatant was used for detection of 3-NT and mitochondrial SOD by slot blot, as described above.

2.12. Protein determination

Protein content was measured colorimetrically by the method of Bradford [32] or by the bicinchoninic acid method using a commercially available kit (Thermo Scientific Pierce BCA Protein Assay Kit). Bovine serum albumin (1 mg/mL) was used as standard.

2.13. Statistical analyses

Data were analyzed by parametric one-way ANOVA and expressed as mean + standard error of the mean. *Post hoc* comparisons were performed using the Bonferroni test. A probability of $P < 0.05$ was considered significant.

3. Results

Since atorvastatin withdrawal has been shown to decrease nitric oxide levels in endothelial cells [18], our first experiment was aimed to investigate the effect of atorvastatin treatment or withdrawal NOx levels in the rat cerebral cortex. Nitrite and nitrate are stable metabolites of nitric oxide, and the sum of nitrite plus nitrate content (NOx) may provide information about the nitric oxide levels. Interestingly, statistical analysis (One-way ANOVA followed by Bonferroni test) revealed a significant decrease in NOx levels following atorvastatin treatment ($32.18 \pm 8.07\%$) or withdrawal ($34.65 \pm 6.17\%$) [$F(2,31) = 5.248$; $P < 0.05$] (Fig. 1).

Given the above described change in NOx levels and since direct and indirect antioxidant activity has been reported for atorvastatin [3–5], we decided to investigate the effect of atorvastatin treatment or withdrawal on the levels of selected markers for oxidative or nitrosative damage. Whereas a significant increase ($33.4 \pm 11.25\%$) in 3-NT immunoreactivity was detected following atorvastatin withdrawal [$F(2,21) = 5.421$; $P < 0.05$] (Fig. 2A), no significant differences in the levels of protein carbonyls [$F(2,29) = 0.1357$; $P > 0.05$] (Fig. 2B) or HNE [$F(2,28) = 0.8280$; $P > 0.05$] (Fig. 2C) were found.

Considering that protein nitration (as shown by 3-NT levels) is typically caused by peroxynitrite, and the main source of this radical is the reaction between superoxide anion and nitric oxide, we decided to investigate the effect of atorvastatin treatment or withdrawal on the NADPH oxidase and xanthine oxidase activities, two important cellular sources of superoxide anion. Interestingly, we found a significant increase ($39.99 \pm 9.77\%$) in NADPH oxidase activity [$F(2,28) = 3.554$; $P < 0.05$] in the rat cerebral cortex following atorvastatin withdrawal (Fig. 3A). On the other hand, no significant differences regarding xanthine oxidase activity were found [$F(2,18) = 0.2629$; $P > 0.05$] (Fig. 3B).

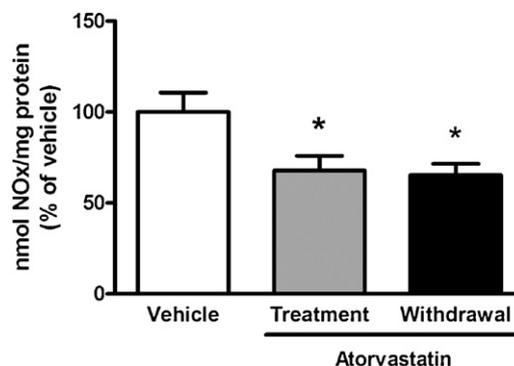


Fig. 1. Effect of oral atorvastatin treatment or withdrawal on NOx content in the rat cerebral cortex. Data are mean + standard error of the mean for $n = 10$ –12 per group. The asterisk indicates a significant difference from the vehicle group ($P < 0.05$ – one-way ANOVA followed by the Bonferroni test).

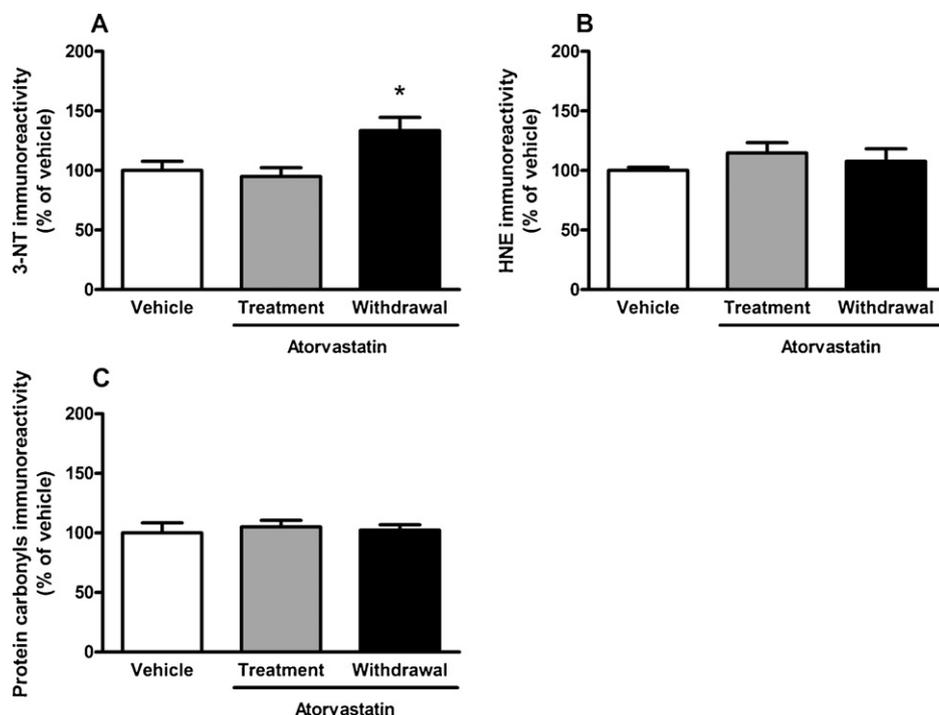


Fig. 2. Effect of oral atorvastatin treatment or withdrawal on immunoreactivity for (A) 3-nitrotyrosine (3-NT), (B) 4-hydroxy-2-nonenal (HNE) and (C) protein carbonyls in the rat cerebral cortex. Data are mean + standard error of the mean for $n = 8-12$ per group. The asterisk indicates a significant difference from the vehicle group ($P < 0.05$ – one-way ANOVA followed by the Bonferroni test).

Superoxide anion can be enzymatically dismutated to a more stable hydrogen peroxide by cytosolic and mitochondrial SOD activities, which are the major ROS detoxifiers of the cell. Therefore, in light of the currently reported increase in NADPH oxidase

activities in the rat cerebral cortex following atorvastatin treatment or withdrawal. Interestingly, atorvastatin withdrawal decreased mitochondrial SOD activity (by $71.42 \pm 18.12\%$) [$F(2,13) = 10.37$; $P < 0.05$] (Fig. 4A), without altering cytosolic SOD activity [$F(2,12) = 0.4280$; $P > 0.05$] (Fig. 4B). Hydrogen peroxide

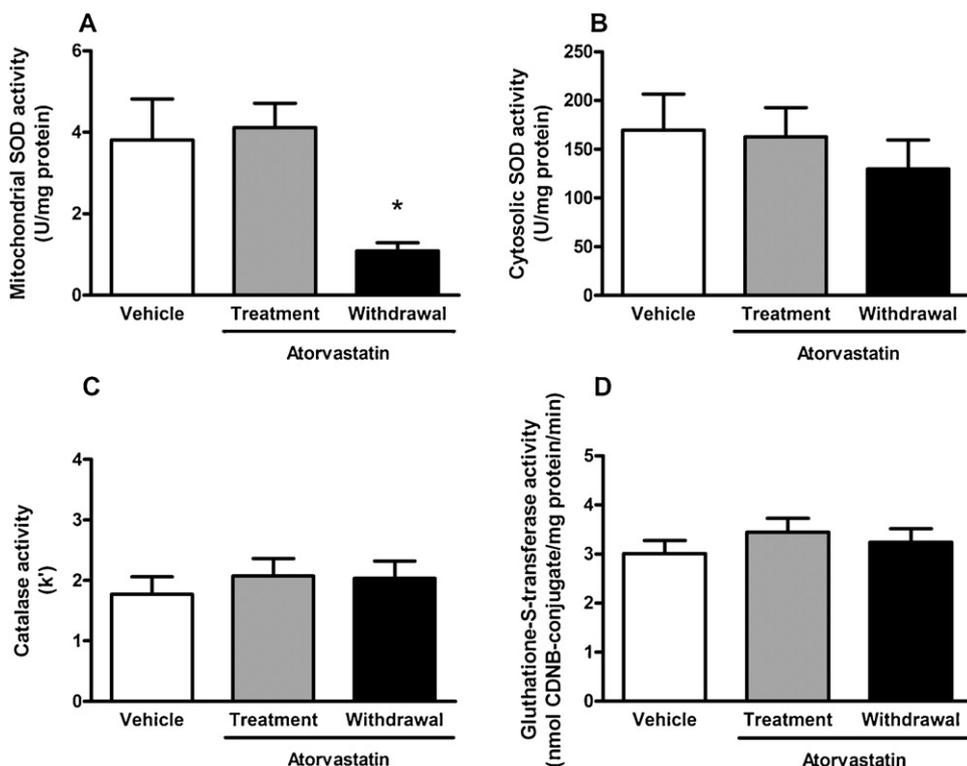


Fig. 3. Effect of oral atorvastatin treatment or withdrawal on activity of (A) NADPH oxidase and (B) xanthine oxidase in the rat cerebral cortex. Data are mean + standard error of the mean for $n = 7-11$ per group. The asterisk indicates a significant difference from the vehicle group ($P < 0.05$ – one-way ANOVA followed by the Bonferroni test).

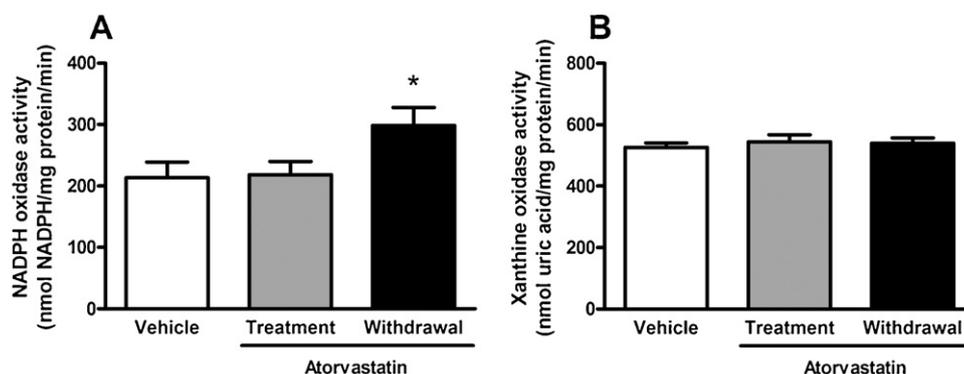


Fig. 4. Effect of oral atorvastatin treatment or withdrawal on activity of (A) mitochondrial SOD and (B) cytosolic SOD, (C) catalase and (D) glutathione-S-transferase in the rat cerebral cortex. Data are mean + standard error of the mean for $n=4-7$ per group. The asterisk indicates a significant difference from the vehicle group ($P<0.05$ – one-way ANOVA followed by the Bonferroni test).

may then be converted into water and oxygen by enzymes CAT and/or through the glutathione system. In this context, we measured CAT and GST activities in the rat cerebral cortex after atorvastatin treatment or withdrawal. However, no significant differences were found regarding CAT [$F(2,17)=0.3044$; $P>0.05$] (Fig. 4C) or GST [$F(2,17)=0.6098$; $P>0.05$] (Fig. 4D) activities.

In order to shed some light on the mechanisms underlying the currently reported decrease in mitochondrial SOD after atorvastatin withdrawal, we measured the expression and level of nitration of mitochondrial SOD protein, since peroxynitrite-mediated nitration of selected tyrosine residues in its polypeptide chain greatly decreases its catalytic activity [33,34]. Interestingly, atorvastatin withdrawal increased mitochondrial SOD immunoreactivity (by $72.2 \pm 26.59\%$) [$F(2,15)=4.145$; $P<0.05$] (Fig. 5A). In addition, atorvastatin withdrawal increased the 3-NT/mitochondrial SOD ratio (by $109.8 \pm 17.2\%$) [$F(2,11)=4.039$; $P<0.05$] (Fig. 5B), indicating increased levels of nitrated mitochondrial SOD.

4. Discussion

Statins are cholesterol-lowering agents acting primarily through competitive inhibition of HMG-CoA reductase, but several experimental and clinical studies have shown that statins display a myriad of beneficial effects which appear to be independent from its cholesterol-lowering action, the so-called pleiotropic effects of statins. As such effects appear common to several vascular and non-vascular diseases, statin treatment has been emerged as a potential add-on therapy for several conditions [35]. For instance,

statins alter general immunological responses by altering post-translational protein prenylation [2,36], modulate inflammation by inhibiting monocyte-endothelium interactions [2,36], and increase production and bioavailability of endothelium-derived nitric oxide by decreasing Rho GTPase responses [2,36]. In addition, antioxidant actions play a pivotal role in the pleiotropic effects of statin treatment. In fact, atorvastatin treatment reduced lipoperoxidation, protein oxidation and nitration, and increased GSH levels in parietal cortex of aged beagles, a well-established preclinical model of Alzheimer's disease [5]. Moreover, atorvastatin treatment significantly increased the antioxidant enzyme biliverdin reductase-A protein levels, phosphorylation and activity, with a concomitant decrease in oxidative stress in the parietal cortex of aged beagles [37]. In the same experimental model of Alzheimer's disease, atorvastatin treatment increased heme oxygenase-1 expression in the parietal cortex of aged beagles, and a significant negative correlation between heme oxygenase-1 protein levels and oxidative stress parameters has been found [38].

On the other hand, studies in vascular endothelial and smooth muscle cells and in laboratory animals as well as clinical studies revealed that discontinuation of statin treatment not only led to a rapid loss of its vascular protective actions, but was also followed by a rebound deterioration of vascular health. Importantly, there is no consensus on how to treat patients undergoing statin withdrawal or a clear preventive strategy [39]. Therefore, understanding of the molecular mechanisms underlying the deleterious effects arising after abrupt cessation of statin therapy are of fundamental importance. The molecular basis for the adverse cardiovascular effects

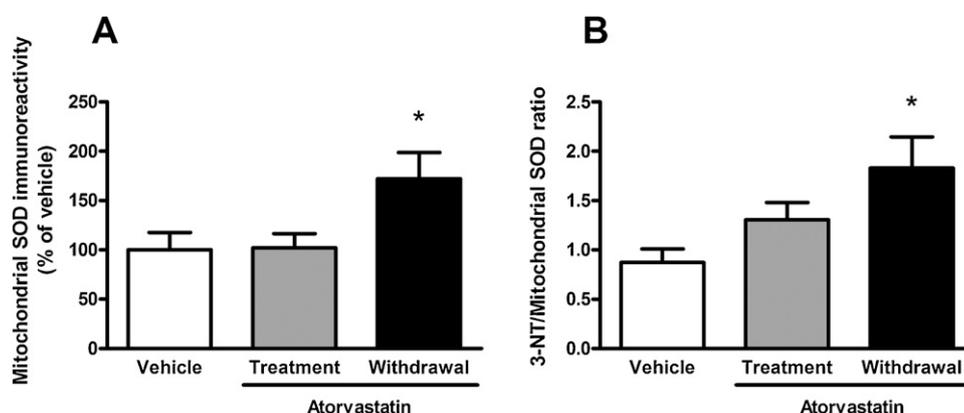


Fig. 5. Effect of oral atorvastatin treatment or withdrawal on (A) immunoreactivity of mitochondrial SOD protein and (B) 3-NT/mitochondrial SOD ratio in the rat cerebral cortex. Data are mean + standard error of the mean for $n=4-6$ per group. The asterisk indicates a significant difference from the vehicle group ($P<0.05$ – one-way ANOVA followed by the Bonferroni test).

of statin withdrawal has been established [39], but it is not clear whether such mechanisms for statin withdrawal-induced harmful effects can be generalized to other tissues than the cardiovascular system. In this context, we aimed to investigate the effects of atorvastatin withdrawal on oxidative and nitrosative stress parameters in the rat cerebral cortex. Accordingly, in the present study we showed that abrupt cessation of oral atorvastatin treatment elicited protein nitration (as measured by increase 3-NT immunoreactivity) in the rat cerebral cortex.

Nitric oxide is a little reactive radical produced by nitric oxide synthase isozymes (NOS) which display a myriad of physiological and pathological effects [40]. When nitric oxide interacts with oxygen and superoxide anion, nitrosylation and nitration occur, respectively. With respect to nitrosylation, nitric oxide interacts with oxygen, electron acceptors or metals to produce nitrosium ion, which can subsequently interact with protein thiols such as cysteine residues in a reversible manner [41]. Given its reversibility, protein nitrosylation has been considered a physiological post-translational modification which regulates protein function in a similar way to phosphorylation [41]. On the other hand, nitric oxide interacts with superoxide to generate the highly reactive peroxynitrite, leading to tyrosine nitration of proteins [42]. Regarding this point, increasing evidence suggest a strong relationship between protein nitration and the pathophysiology of several diseases, including inflammatory, neurodegenerative, and cardiovascular disorders [42]. Protein nitration tyrosine residues can block protein phosphorylation by interfering with tyrosine residues, thus modifying cell signaling pathways [42].

Regarding the source of superoxide for peroxynitrite formation, we found that statin withdrawal increased NADPH oxidase, an important cellular source of superoxide. Interestingly, while NADPH oxidase inhibition has been suggested as a major pleiotropic mechanism of statin treatment [2,4,36], statin withdrawal has been associated with a rebound increase in NADPH oxidase [14]. The mechanisms underlying NADPH oxidase activation triggered by statin withdrawal are multiple, and include increased plasma membrane targeting of NADPH oxidase catalytic subunits through rac1-GTPase and geranylgeranylation [4]. In this context and in agreement with the current literature we found an increase in NADPH oxidase activity in the cerebral cortex of rats from statin withdrawal group, indicating that NADPH oxidase could be an important source of superoxide in this condition.

Interestingly, we found a decrease in NOx content in the rat cerebral cortex following atorvastatin treatment or withdrawal. Considering atorvastatin treatment, there is evidence that statins increase endothelial NOS activity, through augmented enzyme expression by lengthening the half-life of its mRNA [36], activation of protein kinase B/Akt pathway, which in turn activates endothelial NOS by enzyme phosphorylation [36] and restoration of eNOS activity through reduction of caveolin-1 abundance [36]. Since we found a decrease in NOx content, it is possible that the statin effect on endothelial NOS does not occur in the cerebral cortex. However, since nitric oxide production is subjected to direct feedback inhibition of NOS by nitric oxide [43], decreased nitric oxide levels may be, at least in part, explained by this mechanism. One alternative explanation comes from the fact that statins downregulate activity both neuronal NOS and inducible NOS isoforms [44], which are the predominant NOS isoforms in the brain parenchyma [43]. Moreover, decreased NOx content following atorvastatin withdrawal could be also explained by scavenging of nitric oxide by superoxide originating from NADPH oxidase, resulting in increased peroxynitrite formation and protein nitration. Additionally, it should be noted that peroxynitrite can decompose to yield nitrate and/or nitrite (*i.e.* NOx) by several enzymatic and non-enzymatic mechanisms [45]. For instance, it has been shown that peroxynitrite decomposes at neutral pH *via* protonation to peroxynitrous acid to yield nitrate

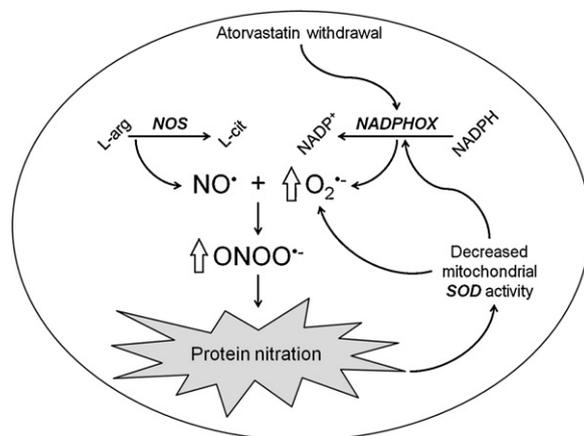


Fig. 6. A proposed scheme for the effects of atorvastatin withdrawal in the rat cerebral cortex. Atorvastatin withdrawal increases NADPH oxidase activity (Fig. 3) and therefore superoxide production. Superoxide anion reacts with nitric oxide, decreasing nitric oxide levels (Fig. 1) and generating the highly reactive peroxynitrite, which ultimately causes protein nitration (Fig. 2). A possible target of peroxynitrite is mitochondrial SOD (Fig. 5), which decreased activity (Fig. 4) contributes to maintenance of increased superoxide levels. In addition, decreased mitochondrial SOD activity may contribute to NADPH oxidase activation [47], resulting in a feedforward cycle. L-Arg, L-arginine; L-cit, L-citrulline; NOS, nitric oxide synthase; NO•, nitric oxide; NADP⁺, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADPHOX, NADPH oxidase; O₂^{•-}, superoxide anion; ONOO•, peroxynitrite; SOD, superoxide dismutase.

[45]. In addition, heme proteins, such as hemoglobin and myoglobin, catalyze the isomerization of peroxynitrite to nitrate, and enzymes like peroxiredoxins, cyclooxygenase-1 and glutathione peroxidase may decompose peroxynitrite to yield nitrite [45]. In this context, it is also possible that NOx decrease reflect at least in part impairment in one or more pathways of nitrate and/or nitrite generation from peroxynitrite decomposition.

Importantly, in the present study we showed that atorvastatin withdrawal decreases mitochondrial superoxide dismutase. To the best of our knowledge, this is the first report of decreased mitochondrial SOD follow statin withdrawal. This enzyme is responsible for superoxide anion dismutation in water and hydrogen peroxide, and it is a key scavenger of superoxide in the mitochondrial matrix. Mitochondrial SOD is vital to life in an oxygen-rich atmosphere, as demonstrated by several studies in various *in vivo* models with reduced mitochondrial SOD expression or enzyme activity [46]. In addition, altered expression or enzyme activity of mitochondrial SOD has been linked to many disorders [46], suggesting an important role for mitochondrial SOD in disease development. Indeed, the presently reported decrease in mitochondrial SOD activity adds a new mechanism underlying the deleterious effects of statin withdrawal. In this context, our present results are in agreement with the emerging concept of the existence of a cross talk between mitochondria and cytosolic NADPH oxidases [47] (Fig. 6). In fact, it has been demonstrated that increased levels of mitochondrial superoxide activate cytosolic NADPH oxidases, increasing superoxide production in the cytoplasm [48]. Since NADPH oxidase-derived superoxide stimulate protein kinase C ϵ within the mitochondrial matrix [49] and leads to depolarization of mitochondrial membrane potential followed by mitochondrial ROS formation and respiratory chain dysfunction [49], such interplay constitutes a feedforward cycle in which the NADPH oxidases increase mitochondrial reactive species, which further activate the cytoplasmic NADPH oxidases and increase cellular superoxide production, triggering mitochondrial impairment and diminishing nitric oxide bioavailability [47].

Regarding the mechanism for decreased mitochondrial SOD activity, it has been demonstrated that this enzyme is a target for peroxynitrite-mediated nitration, which ultimately decreases its

activity [33,34]. In fact, mitochondrial SOD appears to be hypersensitive toward nitration, accounting for as much as 20% of total protein nitration under conditions of oxidative stress [50]. In light of our present results, it is plausible to propose that decreased mitochondrial SOD activity elicited by atorvastatin withdrawal may be a result of nitration of selected tyrosine residues in its polypeptide chain, since abrupt discontinuation of atorvastatin treatment increased 3-NT/mitochondrial SOD ratio, indicating that levels of nitrated mitochondrial SOD are increased in the rat cerebral cortex following atorvastatin withdrawal. Interestingly, we found that mitochondrial SOD immunoreactivity was increased after atorvastatin withdrawal, suggesting increased expression of this enzyme in the rat cerebral cortex. Considering the decrease in mitochondrial SOD activity following atorvastatin withdrawal, an increase in protein levels could represent a cellular attempt to compensate decreased enzyme activity, which ultimately revealed ineffective possibly because nitration of key tyrosine residues in mitochondrial SOD following atorvastatin withdrawal.

5. Conclusion

In summary, in the present study we showed that atorvastatin withdrawal elicits oxidative/nitrosative damage in the rat cerebral cortex and that this effect may be mediated by increased NADPH oxidase activity and decreased mitochondrial SOD activity. A proposed scheme of our present results is shown in Fig. 6. Although additional studies are necessary to evaluate the clinical implications of our findings, it is possible that antioxidant strategies targeting NADPH oxidase or mitochondrial SOD activities may constitute potential targets for prophylaxis and/or treatment of the deleterious effects of statin withdrawal to the brain.

Acknowledgments

Work supported by grants from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (ARD 10/0685-8, ARD 11/1630-1 and PRONEM 11/2082-4) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (141164/2010-7) and by fellowships from Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) (to C.V.O., V.R.F. and L.R.R.), CNPq (to L.M.P., L.M.R. and L.F.F.R.) and FAPERGS (to J.G.). The authors thank Dr. Carlos Fernando Mello for kindly providing laboratory facilities.

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