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

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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.

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 pentylenetetrazol-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 ± 1 °C, 55% relative humidity) with free access to water and food (Supra^{TM}, 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 (NOx)

The cerebral cortex was homogenized 1:5 (m/v) with $ZnSO_4$ (200 mM) and acetonitrile (96%), centrifuged at 16,000 × *g* for 30 min at 4 °C, and the supernatant was collected for NOx 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 phenylmethanesulfonylfluoride, 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 × *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 × *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 × *g* at 4 °C. After centrifugation, the supernatant (S1) was once more centrifuged for 10 min at 12,000 × *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