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Statins induce biochemical changes in the Achilles tendon after chronic treatment

Letícia Prado de Oliveira*, Cristiano Pedrozo Vieira, Flávia Da Ré Guerra, Marcos dos Santos de Almeida, Edson Rosa Pimentel

Department of Functional and Structural Biology, Institute of Biology, University of Campinas – UNICAMP, Campinas, SP, Brazil

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ABSTRACT

Statins have been widely prescribed as lipid-lowering drugs and are associated with tendon rupture. Therefore, this study aimed to evaluate the possible biochemical changes in the Achilles tendon of rats after chronic treatment with statins. Dosages of statins were calculated using allometric scaling with reference to the 80 mg/day and 20 mg/day, doses recommended for humans. The rats were divided into the following groups: treated with simvastatin (S-20 and S-80), treated with atorvastatin (A-20 and A-80), and the control group that received no treatment (C). Measurements of low-density lipoprotein (LDL) in the plasma were performed. The levels of non-collagenous proteins, glycosaminoglycans (GAGs) and hydroxyproline were quantified. Western blotting for collagen I was performed, and the presence of metalloproteinases (MMPs)-2 and -9 was investigated through zymography. The concentration of non-collagenous proteins in S-20 was less than the C group. There was a significant increase in pro-MMP-2 activity in A-80 group and in active MMP-2 in S-20 group compared to the C group. A significant increase in latent MMP-9 activity was observed in both the A-80 and S-20 groups when compared to C group. In the A-20 group, there was a lower amount of collagen I in relation to C group. In addition, a higher concentration of hydroxyproline was found in the S-20 group than the C group. The analysis of GAGs showed a significant increase in the A-20 group when compared to C group. The treatment induced remarkable alterations in the Achilles tendon and the response of the tissue seems to depend of the used statin dosage. The presence of MMP-2 and MMP-9 is evidence of the degradation and remodeling processes in the extracellular matrix of the tendons. Our results show that statins induce imbalance of extracellular matrix components and possibly induce microdamage in tendons.

1. Introduction

Statins are a group of drugs prescribed for the treatment of hyperlipidemia, and they act by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme involved in cholesterol production. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, and therefore serves as a therapeutic target for the action of statins (Campo and Carvalho, 2007). Consequently, HMG-CoA reductase inhibitors induce a reduction of LDL in the plasma, a reduction of intracellular cholesterol and an increase of the LDL receptors (Maron et al., 2000).

Statins are widely prescribed medications with various clinical benefits, including the prevention of myocardial infarction and the formation of atherosclerotic plaques (O'Sullivan, 2007). Additionally, many studies have shown that the clinical benefits associated with statins are either independent of or indirectly dependent on a reduction in LDL-cholesterol (Campo and Carvalho, 2007). These pleiotropic effects, have been well-studied in an effort to identify additional potential uses for these drugs in the treatment of other pathologies, such as (Liao and Laufs, 2005) hypertension, (Yang et al., 2005), Alzheimer's disease (Campo and Carvalho, 2007), sepsis (Giusti-Paiva et al., 2004) and osteoporosis (Jadhav and Jain, 2006).

Despite being quite effective medicines, statins have some adverse effects, including constipation, headaches, sleep disturbances, and other more serious effects, such as hepatotoxicity and musculoskeletal complications (Hoffman et al., 2012; O'Sullivan, 2007). More recently, cases of tendinitis and tendon ruptures have been associated with the use of statins (Marie and Noblet, 2009). These complications have been observed in several tendons, including the distal biceps (Savvidou and Moreno, 2012), the patellar (Beri et al., 2009), the quadriceps (Nesselroade and Nickels, 2010; Rubin et al., 2011) and the Achilles tendons (Beri et al., 2009; Carmont

* Corresponding author at: Department of Structural and Functional Biology, Institute of Biology, University of Campinas – UNICAMP, CP 6109, 13083-970 Campinas, SP, Brazil. Tel.: +55 19 3521 6117/6115.
E-mail addresses: lelekapr@hotmail.com, leprado.farm@gmail.com (L.P. de Oliveira).

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et al., 2009; Chazerain et al., 2001), the latter of which are commonly injured (Marie et al., 2008).

Simvastatin and atorvastatin are some of the most widely used statins in the treatment of hypercholesterolemia, and they are highly efficacious and tolerable (Maron et al., 2000). However, they can also result in tendinopathies (Marie et al., 2008). Tendinopathies seem to be a rare adverse effect of statins, but it is also thought that many of these types of adverse effects are left unreported to pharmacovigilance centers (Chazerain et al., 2001).

Recent studies have shown that statins are responsible for inhibiting the secretion of metalloproteinases (MMPs) in lung fibroblasts (Kamio et al., 2010) and endothelial cells (Izidoro-Toledo et al., 2011). Furthermore, they reduce the expression of collagen I in smooth muscle (Schaafsma et al., 2011). Of note, tendons are formed primarily by collagen I, and MMPs play an important role in maintaining and remodeling the extracellular matrix in tendons (Kannus, 2000).

In addition to lowering cholesterol and inhibiting MMPs (Izidoro-Toledo et al., 2011), statin use has also been shown to promote apoptosis in fibroblasts (Yokota et al., 2008), further suggesting an association between statin use and tendon rupture. Together, these factors can weaken the integrity of the tendon tissue and thus predispose the tendon to rupture.

Statins are widely prescribed drugs, and investigating the biochemical changes in tendons caused by statins may help to understand the causes of tendinitis and tendon rupture associated with the use of those drugs. Because there are no studies that address the effects of statins on the components of the extracellular matrix in tendons, this study was designed to investigate the effects of chronic oral statin treatment on the Achilles tendon in rats.

2. Methods

2.1. Experimental groups

Animal care was in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and was consistent with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA), was approved by the Ethics Committee on Animal Experiments of State University of Campinas, SP, Brazil and filed under no. 2473-1.

We used 50 male Wistar rats, young adults, weighing on average 300 g. The rats were housed two per cage in a 12 h light:dark cycle at 23 °C, with free access to standard rat chow and water.

The animals were divided into five experimental groups: rats treated with a daily dose equivalent to 20 mg of atorvastatin (pharmanostra, Rio de Janeiro, Brazil) (A-20) or simvastatin (Galena, Campinas, Brazil) (S-20), rats treated with a daily dose equivalent to 80 mg of atorvastatin (A-80) or simvastatin (S-80) and the control group that received no treatment (C). To dilute the statins we used an aqueous solution of 0.5% carboxymethylcellulose. The treatment lasted for 2 months and a gavage was used to administer the solutions (Camerino et al., 2011).

We accounted for the metabolic rate of the animals and used allometric scaling to calculate the necessary drug dosage (Pachaly and Brito, 2001). Thus, it was necessary to use an animal model in which the pharmacokinetic and pharmacodynamics parameters of the drug of interest were known. In this study, we used as animal model the human and animal target Wistar rats. After two months of treatment, the animals were euthanized with isoflurane (Isofurine – Cristália, Itapira, Brazil) and the tendons were collected for biochemical analysis.

2.2. LDL-cholesterol determination

For the determination of LDL-cholesterol in the plasma of the rats an enzymatic assay was used. Through this assay it is possible to measure LDL-cholesterol directly (Kit LDL-C Plus-Roche).

2.3. Extraction procedures

The calcaneal tendon ($n = 5$) was cut in small pieces and treated with 50 volumes of 4 M guanidine hydrochloride (GuHCl) containing 20 mM EDTA, 1 mM PMSF in 50 mM sodium acetate buffer, pH 5.8, for 24 h at 4 °C with mild stirring (Heinergard and Sommarin, 1987). Afterwards, the material was centrifuged (13,000 × *g*, 25 min, 4 °C) and the supernatant was used for non-collagenous protein dosage and Western blotting for collagen I.

2.4. Quantification of proteins

Samples of the extracts of each experimental group were used. Non-collagenous proteins (NCPs) were quantified according to the Bradford method (1976), using bovine serum albumin as standard. The absorbance was measured at 595 nm.

2.5. Hydroxyproline quantification

After washing in PBS (phosphate-buffered saline—5 mM phosphate buffer, 0.15 M NaCl and 50 mM EDTA), fragments from the tendons were immersed into acetone for 48 h and then into chloroform:ethanol (2:1) for 48 h. Fragments were weighed and hydrolyzed in 6 N HCl (1 mL for each 10 mg of tissue) for 16 h at 110 °C. The hydrolysate was neutralized with 6 N NaOH, and 20 μL of each sample was treated with chloramine T solution, as described by Stegemann and Stalder (1967) and Jorge et al. (2008), with some modifications. The absorbance was measured at 550 nm in a spectrophotometer. Hydroxyproline concentrations from 0.2 to 6 μg/mL were used for a standard curve.

2.6. Agarose gel electrophoresis

The fragments of the tendons were dehydrated, and sulfated glycosaminoglycans were released from proteoglycans by digestion with a papain solution (Merck, Darmstadt, Germany) (40 mg/g of dry tissue) containing 100 mM sodium phosphate buffer, pH 6.5, 40 mM EDTA, and 80 mM β-mercaptoethanol (Harab and Morão, 1989). The reaction was stopped by the addition of 4 mM iodoacetic acid for 1 h. The sulfated GAGs were precipitated in ethanol and separated by agarose gel electrophoresis (0.6%) in 0.05 M propylenediamine according to Dietrich and Dietrich (1976).

2.7. Quantification of sulfated GAGs

The samples digested by papain solution were used to quantify the GAGs of the tendons of the different experimental groups. The quantification was determined using the dimethylmethylene blue method (Farndale et al., 1986) using chondroitin sulfate as the standard. The absorbance was measured at 540 nm using an Asys Expert Plus Microplate Reader (Biochrom, Holliston, MA, USA).

2.8. Western blotting for collagen I

For collagen I detection, were precipitated 10 μg of total protein from the guanidine extract, using a solution containing 1 M sodium acetate buffer pH 7.4 (100 mL) and 9 volumes of ethanol (1350 mL) for 24 h at 4 °C. After three washes (150 mL acetate buffer 1 M sodium pH 7.4 and 1350 mL of ethanol), the precipitate obtained was dried at 37 °C and resuspended in reducing sample buffer (0.5 M Tris–HCl pH 6.8, 26% glycerol, 20% SDS, 0.1% Bromophenol Blue). Proteins tendon subjected to electrophoresis on SDS-polyacrylamide (6%) were transferred to nitrocellulose membrane, as described by Towbin et al. (1979).

The membranes were transferred to the device Snap i.d. (Millipore, Billerica, USA). They were blocked with BlØk-CH reagent for 15 s and then the membranes were incubated with primary antibody (C2456 – Sigma–Aldrich for collagen I) at a dilution of 1:500 for 10 min. The membranes were washed three times in TBS. Incubation with secondary antibody (A8786 – anti-mouse Sigma–Aldrich and A2306 – anti-rabbit Sigma–Aldrich) was performed for 10 min and washed again. The revelation was performed with DAB (dimethylaminobenzaldehyde). The band densitometry was made using the Scion Image software Alpha 4.0.3.2 (Scion Corporation).

2.9. Zymography

Metalloproteinase analyses were made according to Marqueti et al. (2006). The samples were incubated in extraction buffer (Tris–HCl 50 mM pH 7.4, NaCl 0.2 M, Triton X-100 0.1%, $CaCl_2$ 10 mM and protease inhibitor 100 μL/10 mL) at 4 °C for 24 h. For MMP-2 and MMP-9 analysis were applied 20 μg of proteins in each lane of sodium dodecyl sulfate (SDS) – 10% polyacrylamide gels prepared with 2 mg/mL gelatin. Then, the gel was incubated in incubation buffer (Tris–HCl 50 mM pH 8.4, 5 mM de $CaCl_2$ e 1 μM de $ZnCl_2$) overnight at 37 °C. Gels were stained with Coomassie Brilliant Blue R-250 and bleached (methanol solution 30% and 10% acetic acid in water). Finally, the gel was placed in shrinking solution (30% methanol and 3% glycerol). The band densitometry was made using the Scion Image software Alpha 4.0.3.2 (Scion Corporation).

2.10. Statistical analysis

All results were expressed as mean ± standard deviation. The results were analyzed by analysis of variance (ANOVA-one-way) followed by Tukey test. The level of significance was $p < 0.05$. The analysis was carried out in GraphPad Prism 3.0 program. It was used $n = 5$ for each technique.