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Protective effect of atorvastatin on bone tissue in orchidectomised male albino Wistar rats

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ABSTRACT

Recent studies have shown that atorvastatin influences bone metabolism. We investigated its bone protective effect in orchidectomised rats after 12 weeks of treatment. Eight-week-old rats were divided into 3 groups: sham-operated group, control group after orchidectomy and experimental group after orchidectomy with atorvastatin administration (12 mg/kg/day). Bone mineral density and bone marker concentrations of aminoterminal propeptide of procollagen type I (PINP), osteoprotegerin (OPG), insulin-like growth factor 1 (IGF-1) in serum, and carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), bone alkaline phosphatase (BALP), bone morphogenetic protein 2 (BMP-2) in bone homogenate were measured. Total serum calcium and tibial calcium content was determined. Femurs were used for three-point bending test of the shaft and compression testing of the femoral neck. Bone markers (CTX-I, BALP, BMP-2) in control rats were higher *vs.* sham-operated rats. Atorvastatin reduced CTX-I, BMP-2 and OPG compared to controls. IGF-1 was decreased in control rats *vs.* sham-operated rats; atorvastatin increased IGF-1 *vs.* control rats. Atorvastatin exerts a positive effect on bone metabolism by increasing bone mineral density of the whole body, which had decreased under the effects of orchidectomy. Three-point bending test revealed an increase in maximal load values of the left femurs after atorvastatin administration compared to controls. The diameter of the left femur and length of both femurs were increased after atorvastatin administration compared to controls. Our findings suggest that atorvastatin has a beneficial effect on bone metabolism in orchidectomised rats by decreasing bone turnover, with resulting improvement in bone mineral density and bone biomechanical properties.

1. Introduction

Cardiovascular disease and osteoporosis are among diseases of civilization which have serious health consequences, particularly in the aging population. One of the major risk factors for cardiovascular disease is high blood cholesterol. Hypercholesterolemia can be controlled by several classes of drugs, including statins, but the effect of these drugs on bone metabolism has not yet been fully clarified.

Statins, hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are among the most widely prescribed drugs in the world for the prevention of cardiovascular events and death. Besides lowering serum lipids, statins have recently been shown to exert pleiotropic effects such as improvement of endothelial dysfunction, reduced inflammatory response, stabilization of atherosclerotic plaques, and reduced thrombogenic response, which may not be directly related to cholesterol synthesis (Liao, 2002). Recently-published studies have shown that the mevalonate pathway plays an important role in bone metabolism (Coxon and Rogers, 2003; Hughes et al., 2007; Staal et al., 2003). Statins inhibit bone resorption by directly inhibiting the mevalonate pathway in osteoclasts. Inhibition of the mevalonate pathway by statins leads to decreasing availability of several important intermediate non-steroidal isoprenoid precursors, especially geranylgeranyl pyrophosphate and farnesylpyrophosphate. These isoprenoid precursors are necessary for the posttranslational lipid modification (prenylation) of certain small glutamyl transpeptidase binding proteins – Rho, Rac and Rab – which are essential for the normal function of osteoclasts. Thus, by inhibiting the mevalonate pathway, statins can trigger apoptosis of osteoclasts (Coxon and Rogers, 2003). Mundy et al. (1999) first reported that statins stimulated bone formation *in vitro* and in rodents. This effect was associated with increased expression of the bone morphogenetic protein-2 (BMP-2) gene in bone cells, leading to increased osteoblast differentiation and enhanced bone formation (Mundy et al., 1999). We have confirmed this fact in our previous study: BMP-2 expression in proximal tibia was increased after 8 weeks administration of atorvastatin (3 mg/kg/day) in healthy male albino Wistar rats (Gradosova et al., 2011). *In vitro* studies have demonstrated that statins are able to

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stimulate both bone formation and resorption. In contrast to *in vitro* studies, results of various *in vivo* studies indicate that the antiresorptive effect is more predominant (Jadhav and Jain, 2006). Statins have also been found to increase bone mineral density in humans. Further, study data have suggested that statin use might be associated with a lower risk of fractures, but these data have been inconsistent (Toh and Hernández-Díaz, 2007).

In the present study, markers of both bone formation and resorption were measured to investigate the roles of atorvastatin in bone metabolism, bone mineral density and bone biomechanical properties in orchidectomised rats. The rat after orchidectomy is currently an accepted experimental animal model of male hypogonadism and the subsequently developing osteopenia and osteoporosis (Erben, 2001; Verhas et al., 1986; Wink and Felts, 1980).

2. Materials and methods

2.1. Animals

All animals received humane care in accordance with the guidelines set by the institutional Animal Use and Care Committee of Charles University, Prague, Faculty of Medicine in Hradec Kralove, Czech Republic. The protocol of the experiment was approved by the same committee. The experiments used eight-week-old male albino Wistar rats (Biotest Ltd., Konarovice, Czech Republic). The animals were hosted in groups of 4 in plastic cages. Throughout the experimental period the animals were maintained in controlled conventional conditions (12 hours light and 12 hours dark, temperature 22 ± 2 °C, air humidity 30–70%). Tap water, standard laboratory diet (SLD, VELAS, plc, Lysa nad Labem, Czech Republic) and SLD enriched with atorvastatin (Albert Weber — SEMED, Prague, Czech Republic) were given *ad libitum*. The weight of the rats was monitored once a week.

2.2. Experiment

Rats weighing (219 ± 8 g) at the beginning of the experiment were divided into three groups of 8 animals:

1. sham-operated control fed with SLD
2. orchidectomised control fed with SLD
3. orchidectomised rat fed with SLD enriched with atorvastatin (6 mg/25 g of the diet; Atorvastatin-ratiopharm 10 mg, ratiopharm GmbH, Ulm, Germany). The amount of atorvastatin in the diet was calculated according to daily food intake of the rats to deliver about 12 mg/kg/day in male albino Wistar rats after orchidectomy.

At the beginning of the experiment 16 rats underwent bilateral orchidectomy under ether anesthesia. The rats were used as orchidectomised control group and treatment group. Sham-operated group underwent only scrotal incision. On the second day after operation the experimental rats began to receive SLD enriched with atorvastatin and the sham-operated rats and control rats only SLD, both diets *ad libitum*. After 12 weeks, the animals were sacrificed by blood withdrawal from the abdominal aorta under ether anesthesia, and the obtained serum was aliquoted and stored at −80 °C for ensuing biochemical analyses. After sacrifice of the rats, both tibiae and femurs were dissected free of soft tissue, wrapped in gauze moistened with saline, and frozen to −80 °C till required for analysis.

2.3. Analysis of serum and bone homogenates

In the blood serum, the concentration of total calcium was determined on the Modular Roche analyser (Roche Diagnostics GmbH, Mannheim, Germany), with a commercial kit (Roche) using *o*-cresolphthalein complexone method, and the levels of aminoterminal propeptide of procollagen type I (PINP), osteoprotegerin (OPG) and insulin-like growth factor 1 (IGF-1) by the ELISA (Enzyme-Linked Immunosorbent Assay) method.

In the bone homogenate, the levels of the markers carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), bone alkaline phosphatase (BALP) and bone morphogenetic protein 2 (BMP-2) were determined, also using the ELISA method. The homogenate was prepared from the tibia. After animal sacrifice, both tibiae were carefully excised; after removal of all the surrounding skin, muscle and other soft tissue, they were stored at −80 °C until required. The proximal part of the bone (0.1 g) was disrupted and homogenized in 1.5 ml of phosphate buffer (PBS, PAA Laboratories GmbH, Pasching, Austria) with MagNA Lyser instrument (Roche Applied Science, Germany) at 6500 rpm for 20 s and cooled on the MagNA Lyser Cooling Block. This procedure was repeated three times.

The tissue homogenate was centrifuged at 10,000 *g* at 4 °C for 10 min. The supernatant was obtained and stored at −80 °C. The determination of the levels of bone markers used kits from the firm Uscn Life Science Inc., Wuhan, China (PINP, Procollagen I N-Terminal Propeptide, pg/ml; OPG, Osteoprotegerin, ng/ml; IGF-1 Insulin Like Growth Factor 1; ng/ml, CTX-I, Cross Linked C-Telopeptide Of Type I Collagen; ng/ml, BALP, Alkaline Phosphatase, Liver/Bone/Kidney; U/l, BMP-2, Bone Morphogenetic Protein 2; ng/ml).

In the rat tibia the amount of calcium was determined by flame photometry with an EFOX 5053 flame photometer (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany).

2.4. Dual energy X-ray absorptiometry analysis

The rat bone mineral density (g/cm^2) was measured by means of dual energy X-ray absorptiometry (DXA) on a Hologic Delphi A device (Hologic, MA, USA) at the Osteocentre of the Faculty Hospital Hradec Kralove, Czech Republic. Before measurements, a tissue calibration scan was performed with the Hologic phantom for the small animal. Bone mineral density for the whole body, in the area of the lumbar vertebrae and in the area of the femur was evaluated by computer using the appropriate software program for small animals (DXA; QDR-4500A Elite; Hologic, Waltham, MA, USA). All animals were scanned by the same operator.

2.5. Biomechanical testing procedure

Mechanical testing of the rat femoral shaft and femoral neck was done with a special electromechanical custom-made testing machine (Martin Kosek & Pavel Trnecka, Hradec Kralove, Czech Republic) according to methods described in our previous report (Gradosova et al., 2011). For the three-point bending test, the femur was placed on a holding device with the two support points 18 mm apart. A small stabilizing preload to 10 N was used to fix the bone between the contacts. A constant deformation rate of 6 mm/min (Turner and Burr, 1993) was applied in the anteroposterior direction until maximal load failure and breaking strength (maximum load, N; Newton) was recorded. When the bone was broken, the thickness of the cortical part of the bone was measured by means of a sliding micrometer (OXFORD 0-25MM 30DEG POINTED MICROMETER, Victoria Works, Leicester, Great Britain).

The proximal part of the femur was used for compression test of the femoral neck. The diaphysis of the bone was embedded into a container using a methacrylate resin, and a vertical load was applied to the top of the femoral head. A small stabilizing preload to 10 N was applied and then a constant advancement of 6 mm/min until failure of the femoral neck. The breaking strength (maximum load, N) was recorded by the measuring unit (Digitalanzeiger 9180, Burster praezisiosmesstechnik gmbh & co kg, Gernsbach, Germany). All bones were analyzed by the same operator.