Atorvastatin treatment reduces exercise capacities in rats: involvement of mitochondrial impairments and oxidative stress

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Bouitbir J, Charles AL, Rasseneur L, Dufour S, Piquard F, Geny B, Zoll J. Atorvastatin treatment reduces exercise capacities in rats: involvement of mitochondrial impairments and oxidative stress. J Appl Physiol 111: 1477–1483, 2011. First published August 18, 2011; doi:10.1152/japplphysiol.00107.2011.—Physical exercise exacerbates the cytotoxic effects of statins in skeletal muscle. Mitochondrial impairments may play an important role in the development of muscular symptoms following statin treatment. Our objective was to characterize mitochondrial function and reactive oxygen species (ROS) production in skeletal muscle after exhaustive exercise in atorvastatin-treated rats. The animals were divided into four groups: resting control (CONT; n = 8) and exercise rats (CONT+EXE; n = 8) as well as resting (ATO; n = 10) and exercise (ATO+EXE; n = 8) rats that were treated with atorvastatin (10 mg·kg−1·day−1 for 2 wk). Exhaustive exercise showed that the distance that was covered by treated animals was reduced (P < 0.05). Using dihydroethidium staining, we showed that the ROS level was increased by 60% in the plantaris muscle of ATO compared with CONT rats and was highly increased in ATO+EXE (226%) compared with that in CONT+EXE rats. The maximal mitochondrial respiration (Vmax) was decreased in ATO rats compared with that in CONT rats (P < 0.01). In CONT+EXE rats, Vmax significantly increased compared with those in CONT rats (P < 0.05). Vmax was significantly lower in ATO+EXE rats (∼39%) compared with that in CONT+EXE rats (P < 0.001). The distance that was covered by rats significantly correlated with Vmax (r = 0.62, P < 0.01). The glycogen content was decreased in ATO, CONT+EXE, and ATO+EXE rats compared with that in CONT rats (P < 0.05). GLUT-4 mRNA expression was higher after exhaustive exercise in CONT+EXE rats compared with the other groups (P < 0.05). Our results show that exhaustive exercise exacerbated metabolic perturbations and ROS production in skeletal muscle, which may reduce the exercise capacity and promote the muscular symptoms in sedentary atorvastatin-treated animals.

reactive oxygen species; skeletal muscle; glycogen

STATINS (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are the most effective lipid-lowering drugs available and are the most prescribed medications in developed countries. They impair cholesterol synthesis by inhibiting the synthesis of mevalonate, which is the rate-limiting step in the cholesterol biosynthetic pathway (36). Statins reduce the cardiovascular-related morbidity and mortality in patients with or without coronary artery disease (12). It is generally well accepted that statins have pleiotropic beneficial effects at the cardiovascular level (2, 38), in particular through enhanced bioavailability of vascular nitric oxide synthase (NOS). Overall, statins are a relatively safe family of drugs. However, they may provoke a variety of dose-dependent adverse reactions, ranging from muscle pain to rhabdomyolysis (17). The development of statin-induced rhabdomyolysis is rare, occurring in ~0.1% of patients. However, the occurrence of myopathy has been estimated to range from 1 to 10% (5). Although these reactions represent a relatively low risk of complications, many patients use statin medication, which may increase the proportion of myopathic complications (19). The risk/benefit analysis of statin therapy is further complicated by the finding that exercise increases the risk for statin-induced myopathy. The prevalence of muscular symptoms is as high as 25% among statin users who exercise (5, 31, 42) and may exceed 75% in statin-treated athletes (31).

Little is known regarding the mechanisms by which statins induce skeletal muscle injury (6). Statins have a well-known deleterious effect due to the decrease of metabolic pathways of coenzyme Q synthesis (7). In vitro investigations indicated that simvastatin inhibits the complex I of the mitochondrial electron transport chain (32). Several studies have shown that mitochondrial impairments may be largely implicated in the deleterious effects of statins (13, 33). The role of mitochondria extends far beyond energy production because they constitute important generators of reactive oxygen species (ROS), which can act as second messengers or a source of cellular damage, depending on the amount of ROS that is produced (3, 15, 24, 30). Pathways that regulate metabolism and apoptosis converge in mitochondria and are recognized as critical components in disease (20).

Statins and exercise interfere with muscular metabolism and ROS production (13, 19, 34). Indeed, intense and prolonged exercise induces oxidative stress, notably at the mitochondrial level, and causes oxidative damage to proteins and lipids in the contracting skeletal muscle (25). However, exercise may act as an important signal to stimulate the adaptation of muscle antioxidant systems via the activation of redox-sensitive signaling pathways (17). Because statin treatment and exercise training are used to reduce cardiovascular risks, their association should be recommended. Therefore, it is important to evaluate the effects of physical exercise with statin treatment in animals to determine whether this association induces deleterious effects in skeletal muscle (29).

The aim of this study was to characterize whether exercise triggered or exacerbated the muscular deleterious effects of statins via a mechanism that implicated glucose metabolism, mitochondrial impairments and/or ROS production.
In the present study, we demonstrated that atorvastatin treatment in rats decreased exercise capacities and muscular glycogen content, exacerbated muscular oxidative stress, and inhibited activation of mitochondrial respiration after exhaustive exercise.

MATERIALS AND METHODS

Animals. Experiments were performed on adult male Wistar rats (Depré, France) weighing 250–300 g. The rats were housed in a neutral temperature environment (22° ± 2°C) on a 12:12-h photoperiod and provided food and water ad libitum. All experiments were performed in agreement with the Guide for the Care and Use of Laboratory Animals that was published by the US National Institutes of Health (NIH Publication no. 85–23, revised in 1996) and was approved by our local ethics committee (AL/02/11/05/08).

Compound and administration. Atorvastatin (Tahor) was generously provided by Pfizer and was diluted in phosphate-buffered solution (PBS 1×). After 1 wk of acclimation, 34 rats were randomly divided into four groups as follows: 1) animals that were treated with PBS 1× (CONT; n = 8), 2) animals that were treated with atorvastatin (ATO; 10 mg·kg−1·day−1, n = 10), 3) animals that were treated with PBS 1× and subjected to an exhaustive exercise before death (CONT + EXE; n = 8), and 4) rats that were treated with atorvastatin (10 mg·kg−1·day−1) and subjected to an exhaustive exercise before death (ATO + EXE; n = 8). ATO and ATO + EXE animals were treated with atorvastatin for 2 wk by oral gavage via a cannula.

Treadmill exercise. Animals in the CONT + EXE and ATO + EXE groups were acclimated to a treadmill (Treadmill Control, Letica, Spain) 1 wk before initiation of the study. Acclimation consisted of 10 min at 40 cm/s two times a week. The exercise test consisted of running for 40 min at 40 cm/s followed by an increase in speed (5 cm/s) every 2 min until exhaustion, which was defined as the animal touching the electrified grid at the rear of the treadmill two times for 5 s.

Tissue processing. On day 14, animals were anesthetized with an intraperitoneal injection of 0.1 ml pentobarbital sodium per 100 grams of body weight. The superficial part of the plantaris muscle was excised and cleaned of adipose and connective tissues. Muscles were immediately used for the study of respiratory parameters. The blood was excised and cleaned of adipose and connective tissues. Muscles were immediately used for the study of respiratory parameters. The blood was collected by cardiac puncture immediately before treatment in rats decreased exercise capacities and muscular glycogen content, exacerbated muscular oxidative stress, and inhibited activation of mitochondrial respiration after exhaustive exercise.

Immunoblot analysis. Total rabbit anti-actin (Sigma) and total rabbit anti-β-actin (Bio-Optica, Italy) were used as specific detection antibodies. The blots were developed using a horseradish peroxidase-conjugated anti-rabbit antibody (Dako, Glostrup, Denmark) and 10 min at 40 cm/s two times a week. The exercise test consisted of running for 40 min at 40 cm/s followed by an increase in speed (5 cm/s) every 2 min until exhaustion, which was defined as the animal touching the electrified grid at the rear of the treadmill two times for 5 s.

Total cholesterol, HDL cholesterol, and CPK determination in plasma. Blood was collected by cardiac puncture immediately before death in ethylenediaminetetraacetic acid (EDTA)-rinsed tubes and then centrifuged at 3,400 rpm for 15 min at 4°C. The plasma was separated and stored at −20°C until analysis. The analysis of plasma biochemical parameters was performed with randomly selected plasma samples. Total cholesterol and HDL cholesterol were evaluated using standard spectrophotometric analysis with a diagnostic kit (Biomerieux, Marcy l’Etoile, France), and creatine kinase was assessed using a diagnostic kit (Bioassay Systems).

Study of muscle mitochondrial respiration. This technique was used to determine global mitochondrial function, reflecting both and reflecting the density and functional properties of mitochondria in the muscle (23, 46). The mitochondrial respiration was studied in saponin-permeabilized fibers as previously described to assess the architecture of the mitochondria. Baseline readings were recorded in the presence of the complex I substrates glutamate and malate (5 mM; 2 mM, V0). After the determination of V0, the maximal fiber respiration rates were measured at 22.1°C under continuous stirring in the presence of saturating amounts of adenosine diphosphate (ADP) as a phosphate acceptor (2 mM; Vmax). The acceptor control ratio (ACR) was Vmax/V0 and represented the degree of coupling between oxidation and phosphorylation. When Vmax was recorded, the electrons flowed through complexes I, III, and IV. Complex I was inhibited with amitral (2 mM), and complex II was stimulated with succinate (25 mM; Vmax). Mitochondrial respiration in these conditions was evaluated using complexes II, III, and IV. After the experiments, the fibers were frozen and dried for 15 min at 150°C. The respiration rates were expressed as micromoles O2 per minute per gram dry weight.

Dihydropyridine staining for ROS measurements. Serial sections (10 μm thick) of frozen muscles were cut using a cryostat microtome and thaw-mounted onto glass slides. After air-drying, the slides were incubated (30 min at 37°C) with 2.5 μM dihydropyridine (DHE) in PBS 1×. DHE produced red fluorescence when oxidized to ethidium bromide (EtBr) by superoxide anions (21). After staining, the sections were rinsed, air-dried, mounted in Vectashield (Vector Laboratories, Burlingame, CA) and placed under a coverslip. The slides were examined under an epifluorescence microscope (Nikon Eclipse E800) with a ×20 epifluorescence objective. The emission signal was recorded using a Zeiss 573–637 nm filter. We analyzed the micrographs with Adobe Photoshop 6.0. The data are reported as a percentage of the control group.

Glycogen content. We prepared samples as previously described by Murat and Serfatty (22). We homogenized the samples on ice using 10–30 mg/ml tissue in 0.025 M citrate (pH 4.2) containing 2.5 g/l NaF. After centrifugation (15,000 g for 5 min) to remove debris, we measured the glycogen content using the EnzyChrom glycogen assay kit (Bioassay Systems). We analyzed each sample in duplicate. The data are expressed in micrograms per milliliter of muscle.

Quantitative real-time polymerase chain reaction (q-RT-PCR).

Total RNA was obtained from muscles using the Trizol reagent (Invitrogen Life Technologies, Rockville, MD), as previously described (44) and following the manufacturer’s instructions. RNA was stored at −70°C before performing the reverse transcription reaction. The cDNA was synthesized from total RNA using the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers. To perform the real-time PCR reaction, cDNA was combined with 10 μM of each primer (sense and antisense) and SYBR green (Invitrogen Life Technologies) as a fluorescent dye and H2O. The real-time PCR measurements of individual cDNAs was performed in triplicate using SYBR green dye to measure duplex DNA formation with the LightCycler System (Roche Diagnostics, Meylan, France). The sequences of the primers were designed using information that was published in the public database GeneBank of the National Center for Biotechnology Information (NCBI). The following primer sets were used: GLUT-4 forward, 5′-GGCTGTTGTCATACCAGCCTTCAGC-3′ and GLUT-4 reverse, 5′-CCATTGGGCTCAAGCATTCTC-3′ (GeneBank NM_012751). Quantification of the gene expression was performed using a previously described method (16). β-actin was used as an internal control. The β-actin mRNA content was not affected by treatment or acute exercise. The amplification efficiency of each sample was calculated as previously described (27).

Statistical analysis. The data are expressed as the means ± SE. Statistical analyses were performed using Student’s t-test, one-way or two-way ANOVA followed by Tukey’s test (GraphPad Prism 5, Graph Pad Software, San Diego, CA). The statistical significance is displayed as *P < 0.05, **P < 0.01, or ***P < 0.001.

RESULTS

Atorvastatin treatment increased plasma creatine kinase levels but decreased total cholesterol. Total cholesterol (TC), HDL cholesterol, and CPK were measured in groups of male rats that were treated with atorvastatin with (ATO + EXE) or without exercise (ATO) and in control groups with (CONT + EXE) or without exercise (CONT). After 2 wk of treatment, TC decreased by 20% in the ATO group compared with the CONT group (P < 0.01; Table 1). Atorvastatin treatment decreased
TC by 15% in the ATO+EXE group compared with that in the CONT+EXE group (P < 0.05). As expected, HDL cholesterol levels were not significantly different between the groups (Table 1).

Plasma creatine kinase (CPK) was used as a marker of muscular impairment. CPK increased by 89% in the ATO group (P < 0.001; Table 1) and 46% in the CONT+EXE group compared with the CONT group (P < 0.05). However, no differences were detected between the ATO and ATO+EXE groups.

Atorvastatin treatment decreased endurance capacities. We performed an exhaustive treadmill exercise in animals that were untreated or treated with atorvastatin to test their endurance capacities. The distance that was covered by animals that were treated with statins was significantly lower compared with that in untreated animals (P < 0.05; Fig. 1A). The blood lactate level was largely increased after this exhaustive exercise in a similar manner in both groups of rats (P < 0.001; Fig. 1B).

Oxidative stress was increased in the glycolytic plantaris muscle after atorvastatin treatment. To examine the effects of this treatment on ROS production in vivo, we performed dihydroethidium staining in the plantaris muscle.

ROS levels were increased by 60% in the ATO group compared with the CONT group (Fig. 2, top). The ROS content was increased by 110% in ATO+EXE compared with ATO animals and by 226% in ATO+EXE compared with CONT+EXE animals. However, the ROS content was not different between the CONT and CONT+EXE groups, showing that ROS content increased in statin-treated skeletal muscle after exhaustive exercise.

Atorvastatin impaired mitochondrial function in the glycolytic plantaris muscle. We examined the effects of atorvastatin and exhaustive exercise on maximal respiration rates of skinned fibers in the plantaris muscle. The Vmax and Vsucc were significantly lower in the ATO group compared with those in the CONT group (P < 0.01 and P < 0.05; Fig. 3, A and B, respectively). Similarly, the Vmax and Vsucc were significantly lower in the ATO+EXE group than those in the CONT+EXE group (−39%, P < 0.001 for Vmax and −25%, P < 0.05 for Vsucc; Fig. 3, A and B, respectively). In the CONT+EXE group, the Vmax and the ACR significantly increased compared with those in the CONT group (P < 0.05). However, these two parameters were not increased in the ATO+EXE group compared with those in the ATO group (Fig. 3, A and C, respectively). Additionally, the distance that was covered by rats significantly correlated with Vmax (r = 0.62, P < 0.001; Fig. 4).

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**Table 1. Plasma analysis**

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<thead>
<tr>
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<th>CONT</th>
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<th>CONT+EXE</th>
<th>ATO+EXE</th>
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<tr>
<td>Total cholesterol, mg/dl</td>
<td>54.1 ± 2.8</td>
<td>43.4 ± 1.2</td>
<td>48.5 ± 2.1</td>
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<tr>
<td>HDL cholesterol, mg/dl</td>
<td>29.2 ± 1.1</td>
<td>34.5 ± 1.6</td>
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<tr>
<td>CPK, U/l</td>
<td>345.1 ± 44.8</td>
<td>654.8 ± 34.5</td>
<td>505.0 ± 43.8</td>
<td>657.2 ± 27.0</td>
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Results were expressed as means ± SE. *P < 0.05; **P < 0.01, and ***P < 0.001; control (CONT) group vs. CONT+exercise (EXE) group; *CONT group vs. ATO+EXE group; CONT+EXE group vs. ATO+EXE group, and #CONT group vs. CONT+EXE group.

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Fig. 1. Endurance test and plasma analysis. A: endurance, which was evaluated by the average running distance to exhaustion on a treadmill, was performed in rats that were untreated or treated with atorvastatin (CONT+EXE and ATO+EXE, respectively). B: blood lactate concentration was measured before and after acute exercise in rats that were untreated or treated with atorvastatin. The values represent means ± SE; n = 8 animals/group; *P < 0.05 and ***P < 0.001.

Fig. 2. Atorvastatin increased reactive oxygen species (ROS) levels in the plantaris muscle. Quantification of ROS production and representative photomicrographs of sections of the plantaris muscle in different groups that were labeled with the oxidative dye HE (red fluorescence when oxidized to EtBr by O₂⁻, magnification at ×20). The data represent means ± SE; n = 8–10; ***P < 0.001.
Atorvastatin treatment and exhaustive exercise decreased glycogen content. The glycogen content was decreased by 44% in the ATO group compared with that in the CONT group ($P < 0.05$). After exhaustive exercise, the glycogen content was decreased in the CONT+EXE group compared with that in the CONT group. The glycogen content was not decreased after exhaustive exercise in the ATO+EXE group compared with that in the ATO group (Fig. 5).

GLUT-4 mRNA expression was not different between the CONT and ATO animals. However, this GLUT-4 mRNA expression increased in CONT+EXE animals after exhaustive exercise ($P < 0.05$).

**DISCUSSION**

Our study showed that treatment with atorvastatin for 2 wk had the following effects: 1) impaired mitochondrial function, reduced glycogen content of skeletal muscle, and decreased endurance capacities of rats; 2) exacerbated ROS content blunted the mitochondrial activation and GLUT-4 expression in skeletal muscle after exhaustive exercise.

Recently, Krishnan and Thompson (14) concluded in a review that there are insufficient data to determine whether statins affect exercise performance, even if some evidence suggests that these drugs may reduce muscle strength in
older patients and alter energy metabolism during aerobic exercise. In our study, we showed that atorvastatin treatment reduced exercise capacities and impaired complexes I-II-III of the mitochondrial respiratory chain in the skeletal muscle of rats. Interestingly, we identified a correlation between the exercise capacity and muscular oxidative capacity, suggesting that atorvastatin-induced mitochondrial dysfunction reduces exercise capacity. Fatigue resistance in muscles is largely dependent on muscular oxidative energy production (8, 11). In addition, a lower muscular oxidative capacity is associated with a lower endurance capacity (46). Moreover, we showed that statin treatment depleted the glycogen stores in muscular fibers, which may contribute to the diminution of the exercise capacity in rats. Interestingly, it has been proposed that glycogen-depleted muscle fibers are more susceptible to statin-induced damage than glycogen-rich fibers (35). The reduced glycogen content in muscles following atorvastatin treatment may be explained by reduced glycogen synthesis. Indeed, we hypothesized that muscle glucose uptake was reduced and/or glycogen synthase activity was modified after statin treatment. Similarly, there are some defects in muscle glycogen synthase activity and a reduction in glycogen content in diabetes (41). Interestingly, statins disrupt the early events in insulin signaling (18), and atorvastatin worsens glucose tolerance in streptozotocin-induced diabetic rats.

Moreover, atorvastatin may decrease glucose uptake by reducing the amount of GLUT-4 in the plasma membrane, which may contribute to the decrease in the glycogen content (37).

Phosphorylated glycogen synthase is typically maintained in an inactive state. Glycogen synthase kinase 3 (GSK3) has been identified as the kinase that is responsible for phosphorylating glycogen synthase. In addition, insulin is known to activate glycogen synthase by inactivating GSK3 (28). Interestingly, simvastatin increased expression of phospho-GSK3β (4), which may increase phosphorylation of glycogen synthase to reduce the glycogen content. These findings were confirmed in our study using treated skeletal muscle.

CPK was increased after exhaustive exercise or atorvastatin treatment. However, atorvastatin treatment did not elevate the CPK response to exercise. This result is in accordance with another study that has been performed with lovastatin (39). Eccentric exercise appears to exacerbate the CPK plasmatic level in statin-treated patients (40).

Mitochondrial function in skeletal muscle was impaired after statin treatment, which was in agreement with two studies that showed that several statins impair mitochondria (13). Moreover, in vitro investigations have shown that simvastatin inhibits complex I of the mitochondrial electron transport chain (32). Altogether, mitochondrial impairments may be largely implicated in the deleterious effects of statins (33).

Cellular energetics and homeostasis are challenged during exercise. According to previous data, we showed that exhaustive exercise improved maximal respiration in nontreated animals. This result is in agreement with a study that showed that maximal respiration is increased by 23% from a resting state to exhaustion in humans, which may indicate mitochondrial enzyme activation following an increase in mitochondrial calcium (43). Interestingly, atorvastatin prevented this activation of maximal mitochondrial respiration, which may contribute to reductions in the endurance capacity of these rats. The lactate concentration at the end of exercise was not different between treated and nontreated animals, suggesting that lactate produced in the muscle during exercise was due to glucose uptake from the blood following GLUT-4 translocation to the plasma membrane.

GLUT-4 plays an important role in muscular glucose uptake during exercise (10), and it may be modified by statins (9). After exercise, the activation of GLUT-4 expression was abolished in animals that were treated with atorvastatin, showing a dysregulation in the mechanisms that improve muscular glucose transport. Our results suggest that statins alter muscular metabolism via glucose uptake until reaching mitochondrial homeostasis during exercise.

We found that statins increased ROS production in muscles of treated animals. This finding clearly demonstrated that atorvastatin treatment shifted the cellular redox environment to a more oxidized state. In cell culture, simvastatin breaks down the antioxidant defense system to induce oxidative stress and apoptotic cell death (26). Inhibition of HMG-CoA reductase inhibits the mevalonate pathway. In addition to reducing cholesterol biosynthesis, this inhibition reduces the synthesis of isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate (1). These intermediates are involved in the positive modulation of several nonsteroid isoprenoids (e.g., heme A, coenzyme Q10, and dolichols) that are related to antioxidant status. A reduction in these nonsteroid isoprenoids induces oxidative stress (33). Inhibition of the mevalonate pathway may explain the higher ROS production in the skeletal muscle in atorvastatin-treated animals.

Interestingly when these treated animals performed exhaustive exercise, ROS production was increased. Because statins reduced the antioxidant system such as glutathione, the antioxidant power of myocytes was also reduced. Under these conditions and during exhaustive exercise, redox homeostasis is perturbed and oxidative stress is highly increased (26). The augmentation of hydroxyl radicals, superoxide anions, or hydrogen peroxide may cause damage to lipids, proteins, and DNA, which impairs mitochondrial function in skeletal muscle. This phenomenon may trigger some deleterious effects in skeletal muscle, which is responsible for muscle pain and myalgia development after physical exercise.

Accordingly, it is known that physical exercise, when coupled to statin treatment, is not well tolerated (19, 29). The results of our study in animals may provide some pathophysiological mechanisms explaining this intolerance in humans. These adverse reactions have been shown in specific populations of patients (i.e., sedentary patients who take statins and athletes who trained for several hours per day). Although exhaustive exercise was not well tolerated in sedentary animals, we hypothesized that endurance training that was well calibrated at low or moderate intensity, which may be coupled with statin treatment, was beneficial by inducing muscular adaptations (i.e., improvement of the antioxidant power of fibers; Ref. 45) and reducing the potential muscular deleterious effects of statin treatment without overwhelming antioxidant capacities.
In conclusion, our results show that exhaustive exercise, when coupled with atorvastatin treatment, induced perturbations in cellular homeostasis, leading to a strong augmentation of oxidative stress and inhibition of maximal mitochondrial respiration in skeletal muscle. This study provides some important explanations for the mechanisms behind muscle soreness after atorvastatin treatment.

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GRANTS

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REFERENCES


