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New experimental possibilities for statin-associated myopathy diagnosing

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ABSTRACT

Aim. To identify the relationships between structural proteins of myocytes, as well as indicators of antioxidant defense and metabolites of glycolysis against the background of using statins in animals in the experiment for clarifying the diagnosis of statin-associated myopathy.

Materials and methods. The study was conducted on outbred male rats, which were divided into 3 groups. The control group consisted of intact animals, and there were two experimental groups: group 1 – animals with induced hypercholesterolemia, group 2 – animals with induced hypercholesterolemia treated with simvastatin. In the muscles of animals from the studied groups, the content of structural proteins of the sarcomere, titin and nebulin, was analyzed, and the concentration of glycolysis metabolites (pyruvic acid and lactate) and the activity of antioxidant defense enzymes (superoxide dismutase (SOD) and catalase) were determined.

Results. The use of simvastatin in the animals led to a decrease in the content of NT- and N2A-titin isoforms and an increase in the content of the T2-proteolytic fragment. Complete absence of nebulin was also noted, which reflects the presence of dystrophic changes in the muscle tissue. Long-term administration of simvastatin caused metabolic changes in the rats, characterized by impaired supply of cells with molecular oxygen. However, as opposed to the animals with hypercholesterolemia that were not given statins, a decrease in hypoxia-induced shifts was observed. Abnormalities in the performance of the antioxidant defense (AOD) system and multidirectional changes in the activity of the antioxidant pair “SOD – catalase” were noted. The correlation analysis revealed a positive relationship between the content of the NT-titin isoform and the SOD activity and negative correlations between the content of the N2A-titin isoform and the level of lactate, as well as between the T2-proteolytic fragment of titin and the level of lactate.

Conclusion. The study revealed a complex set of biochemical changes in the muscles that underlie myotoxicity of statins during their long-term use. Additional biochemical parameters were found, such as SOD activity and lactate concentration, changes in which, along with the determination of titin and nebulin concentrations, indicating tissue hypoxia, will make it possible to more accurately diagnose statin-associated myopathy.

Key words: statins, statin-associated myopathy, simvastatin, hypercholesterolemia, skeletal muscle, titin, nebulin.

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Новые возможности диагностики статиновой миопатии в эксперименте

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РЕЗЮМЕ

Цель. Выяснить взаимосвязи между структурными белками миоцитов, а также показателями антиоксидантной защиты и метаболитами гликолиза на фоне применения статинов у животных в эксперименте для уточнения постановки диагноза «статиновая миопатия».

Материалы и методы. Исследование проводилось на беспородных самцах крыс, которых разделили на три группы. Контрольная группа – интактные животные и две экспериментальные группы: группа 1 – животные с индуцированной гиперхолестеринемией, группа 2 – животные с индуцированной гиперхолестеринемией, получавшие симвастатин. В мышцах животных исследуемых групп был проведен анализ содержания структурных белков саркомера – титина и небулина, а также определена концентрация метаболитов гликолиза (пировиноградной кислоты и лактата) и активность ферментов антиоксидантной защиты (супероксиддисмутазы (СОД) и каталазы).

Результаты и обсуждение. Применение симвастатина у животных приводило к уменьшению содержания NT- и N2A-изоформ титина, увеличению содержания протеолитического фрагмента Т2, также отмечалось полное отсутствие небулина, что отражает наличие дистрофических изменений в мышечной ткани. Длительное введение симвастатина вызывало у крыс метаболические изменения, характеризующиеся нарушением обеспечения клеток молекулярным кислородом. Однако, по сравнению с животными с гиперхолестеринемией, которым статины не вводили, наблюдалось уменьшение гипоксических сдвигов. Отмечены нарушения в работе системы антиоксидантной защиты, разнонаправленные изменение активности антиоксидантной пары «СОД – каталаза». Проведенный корреляционный анализ выявил положительную зависимость между содержанием NT – изоформы титина и активностью СОД, отрицательные корреляционные зависимости между содержанием N2A-изоформы титина и уровнем лактата, Т2-протеолитического фрагмента титина и уровнем лактата.

Заключение. Выявлен сложный комплекс биохимических изменений в мышцах, лежащих в основе миотоксичности статинов при их длительном применении. Обнаружены дополнительные биохимические показатели – активность СОД и концентрация лактата, изменения которых, наряду с определением титина и небулина, свидетельствующие о гипоксическом повреждении ткани, позволят более точно диагностировать статиновую миопатию.

Ключевые слова: статины, статиновая миопатия, симвастатин, гиперхолестеринемия, скелетные мышцы, титин, небулин.

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INTRODUCTION

To date, statins are actively prescribed to patients with cardiovascular diseases as effective lipid-lowering agents [1]. However, the use of this group of drugs can cause statin-induced muscle damage – statin-associated myopathy, which can manifest itself in different ways: from mild myalgia to life-threatening rhabdomyolysis [2]. The development of muscle damage symptoms is the main reason for patients' refusing from statin therapy, and, according to some authors, their number reaches 75% within 2 years from the start of therapy [3].

The diagnosis of statin-associated myopathy is complicated by the fact that in some cases it is asymptomatic, or the symptoms are not pronounced and are mostly subjective. There is currently no universal diagnostic test that would make it possible to make such a diagnosis. One of the methods for diagnosing myopathies is to determine the activity of creatine phosphokinase (CPK) in the blood serum. A drastic increase in the CPK activity in blood is an indicator of damage to myocytes. However, often in statin-associated myopathy, an increase in its activity may either not be observed, or is insignificant [4]. Therefore, identification of additional biochemical parameters that would make it possible to increase the efficiency of diagnosing statin-associated myopathy is relevant.

Current literature provides experimental research data proving that the development of muscular dystrophy is accompanied by a decrease in the content of giant sarcomeric proteins titin and nebulin in myocytes due to their increased proteolysis. At the same time, the elasticity and contractility of muscle tissue decrease [5].

In our earlier studies, metabolic changes in erythrocytes and skeletal muscles of rats were presented against the background of prolonged use of simvastatin, which proved the prooxidant effect of statins.

The aim of the study was to identify the relationship between the structural proteins of myocytes, as well as indicators of antioxidant defense and metabolites of glycolysis against the background of statin use in animals in the experiment for clarifying the diagnosis of statin-associated myopathy.

MATERIALS AND METHODS

The study was carried out on outbred male rats aged 12–14 months and weighing 300–350g. The animals were kept in accordance with the Order of the Ministry of Healthcare of the Russian Federation No. 708N of 23.08.2010 "On approval of the rules of laboratory practice" and sanitary rules of the joint venture 2.2.1.3218-14 "Sanitary and epidemiological requirements to the equipment and maintenance of experimental biological clinics (vivariums) of 29.08.2014". During the experiment, the animals were divided into control and experimental groups.

The control group (35 rats) adhered to a standard vivarium diet for 3 months and received 0.5 ml of distilled water through the esophageal tube once a day.

The experimental groups consisted of 70 rats. Essential hypercholesterolemia was induced in these animals by feeding them with a diet rich in animal fats (ghee) and fast-digesting carbohydrates (cane sugar, semolina) for 3 months. It was diagnosed by the level of total cholesterol (CS) on the "Bayer" analyzer (Germany) after the specified period. Then these animals were divided into 2 equal groups. The animals of group 1 received food without added drugs for 2 months, and once a day they received 0.5 ml of distilled water through the esophageal tube. The animals of group 2, in contrast to group 1, received simvastatin (Zocor, 20 mg) once a day for 2 months, (0.0012 g / 100 g of body weight) in the form of an aqueous suspension through the esophageal tube.

The animals were eliminated from the experiment by decapitation. All manipulations were in compliance with generally accepted ethical standards (Protocol No. 21/15 of the Local Independent Ethics Committee of Rostov State Medical University of 10.12.2015).

Fragments of animal skeletal muscles were used for the study. A muscle tissue homogenate was prepared in the ratio of 1 g of tissue : 9 ml of chilled saline, centrifugation was carried out at 3,000 rpm, and the supernatant was used to determine the concentration of glycolysis metabolites and the activity of antioxidant enzymes.

The pyruvic acid (PVA) concentration was determined by the formation of a colored compound upon interaction with 2.4-dinitrophenylhydrazine [6]. The lactate concentration was determined by the color reaction of paraoxidiphenyl with acetaldehyde formed from lactate in the presence of sulfuric and phosphoric acids and copper ions [7]. The activity of superoxide dismutase (SOD) was determined by a method based on the ability of the enzyme to inhibit autooxidation of adrenaline in an alkaline medium at pH = 10.2 [8]. The catalase activity was determined by the decrease in the substrate – hydrogen peroxide per unit time – by the reaction with ammonium molybdate [9].

The study of the content of giant sarcomeric proteins (titin and nebulin) was carried out by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) with the addition of agarose according to R. Tatsumi, A. Hattori (1995) [10] modified by I.M.Vikhlyantsev, Z,A. Podlubnaya (2017) [11] at the Institute for Theoretical and Experimental Biophysics of the Russian Academy of Sciences (Pushchino).

Statistical processing of the obtained results was performed using the STATISTICA 10.0 and Excel Microsoft software. The significance of differences in the considered parameters of the compared groups was assessed using the Student's t-test after checking the distribution for normality using the Shapiro – Wilk test. When conducting the correlation analysis, the

parametric Pearson's coefficient was used, since the samples were in compliance with normal distribution. Differences were considered statistically significant at $p \leq 0.05$. Data are presented as the mean and the standard error of the mean ($M \pm m$).

RESULTS AND DISCUSSION

Keeping animals of the experimental groups on a diet enriched with animal fats and carbohydrates led to a statistically significant increase in cholesterol levels compared to the control group. Administration of simvastatin to the animals of group 2 for two months contributed to a decrease in the level of cholesterol in the blood serum to 1.637 mmol / l, which did not differ significantly from the values in the control group.

To assess the structural and functional state of the skeletal muscles of the animals that were injected with simvastatin for a long time, changes in the qualitative and quantitative composition of titin and nebulin in *m. biceps* of animals of the studied groups were studied (Table 1). The levels of titin and nebulin were evaluated in relation to the content of myosin heavy chains.

Table 1

Changes in the content of titin and nebulin in the muscle tissue of animals in the studied groups, $M \pm m$

Groups Parameters	Control group, $n = 35$	Experimental group
		group 2, $n = 35$
T2-fragment	0.113 ± 0.002	0.137 ± 0.0024 $p < 0.001$
N2A-isoform	0.136 ± 0.002	0.094 ± 0.0025 $p < 0.001$
NT-isoform	0.026 ± 0.0015	0.016 ± 0.0017 $p < 0.001$
Nebulin	0.031 ± 0.0023	Absent

Note. p – significance level of differences relative to the parameters in the control group.

The levels of titin and nebulin in *m. biceps* of the animals in group 1 did not differ significantly from those in the control group. At the same time, studies showed that long-term use of simvastatin in the animals with induced hypercholesterolemia caused a decrease in the content of the NT-titin isoform by 38.46% ($p < 0.001$) and N2A-titin isoform by 30.88% ($p < 0.001$). In addition, against the background of simvastatin use in the animals, an increase in the level of the T2-proteolytic fragment by 1.2 times was noted in their muscle tissue, as well as complete absence of nebulin relative to the animals that did not receive the drug.

The obtained results are in line with the studies of modern scientists who prove that myopathies of various origins are accompanied by a decrease in the content of structural proteins of the sarcomeric cytoskeleton, such as titin (primarily its NT-isoforms) and nebulin, and, as a consequence, lead to deterioration of contractile properties of the muscles [5]. Based on this, it can be assumed that a decrease in the level of titin and nebulin with prolonged use of simvastatin is an indicator that reflects the presence of degenerative processes in the muscle.

To elucidate the nature of myotoxicity of statins, an analysis of the parameters of energy metabolism and the activity of antioxidant defense enzymes in the muscle tissue of the animals in the studied groups was carried out. Glycolysis metabolites – pyruvic acid and lactate – are indicators of the efficiency of molecular oxygen supply to the cells. As a result of the study, in the muscles of the animals in group 1, an increase in the concentration of lactate by 73.23% ($p < 0.001$) and a significant increase in the concentration of pyruvic acid by 247.11% ($p < 0.001$) were noted relative to the control group (Table 2).

It can be assumed that the increase in the concentration of glycolysis metabolites may be associated with an excess of fast-digesting and rapidly oxidized carbohydrates in the diet of the rats in this group. At the same time, a very high level of lactate indicates development of tissue hypoxia, which triggers a cascade of metabolic disorders in the muscle tissue of the animals. According to D.A. Kruse (1997), a drastic increase in the concentration of lactate can serve as a marker of the pathological process severity [12].

Table 2

Levels of glycolysis metabolites in the muscle tissue of animals in the studied groups, $M \pm m$

Groups Parameters	Control group, $n = 35$	Experimental group	
		group 1, $n = 35$	group 2, $n = 35$
Lactate (μmol / ml protein)	3.96 ± 0.447	6.86 ± 0.657 $p < 0.001$	4.64 ± 0.491 $p > 0.05$ $p_1 < 0.01$
Pyruvic acid (μmol / ml protein)	2.25 ± 0.024	7.81 ± 0.570 $p < 0.001$	3.28 ± 0.269 $p < 0.001$ $p_1 < 0.001$

Note. p – significance level of differences relative to the parameters of the control group; p_1 – significance level of differences relative to the parameters of group 1.

In the group of animals with induced hypercholesterolemia against the background of simvastatin use (group 2), we observed a decrease in the concentration of lactate by 32.36% ($p_1 < 0.01$) and a fall in the level of pyruvic acid by 58.0% ($p_1 < 0.001$), respectively, as opposed to the animals

in group 1. Relative to the control group, the concentration of pyruvic acid increased by 45.77% ($p < 0.001$), and there was also a slight increase in the concentration of lactate by 17.17% ($p > 0.05$).

Based on the obtained data, it can be concluded that in the animals of group 2, a tendency to normalization of carbohydrate metabolism was observed against the background of simvastatin use, compared with the animals of group 1. However, compared with the animals of the control group, the concentration of glycolysis metabolites still remained elevated. On the one hand, preservation of an increased level of glycolysis metabolites may reflect the inferiority of adaptive mechanisms and require additional measures aimed at reducing hypoxia-induced shifts. On the other hand, attention is drawn to the pronounced increase in the level of pyruvic acid, as opposed to the lactate concentration. According to the experimental data of Z.I. Mikashinovich (1989), an increase in the level of pyruvic acid under conditions of hypoxia improves microcirculation in tissues and contributes to a decrease in pathobiochemical shifts [13].

SOD and catalase play an important role in the antioxidant defense of almost all cells in the body, including myocytes. As a result of the study, in the group of animals with induced hypercholesterolemia, an increase in the catalase activity by 82.66% ($p < 0.001$) was noted, and the SOD activity did not significantly change relative to the control group (Table 3).

Table 3

Activity of SOD and catalase in the muscle tissue of animals in the studied groups, $M \pm m$

Groups Parameters	Control group, $n = 35$	Experimental group	
		group 1, $n = 35$	group 2, $n = 35$
Superoxide dismutase [conv. unit / mg protein]	0.446 ± 0.049	0.500 ± 0.046 $p > 0.05$	0.219 ± 0.024 $p < 0.001$ $p_1 < 0.001$
Catalase [mKat / mg protein]	1.494 ± 0.211	2.729 ± 0.162 $p < 0.001$	2.786 ± 0.438 $p < 0.001$ $p_1 > 0.05$

Note. p – significance level of differences relative to the parameters of the control group; p_1 – significance level of differences relative to the parameters of group 1.

The revealed increases in catalase activity in the animals with hypercholesterolemia with unchanged SOD activity, according to O.I. Dotsenko et al., are possibly associated "with the activation of peroxisomal reactions or other parallel processes that require increased catalase activity" [14]. Administration of simvastatin to the animals with experimental hypercholesterolemia contributed to a decrease in the SOD activity by 56.2% ($p_1 < 0.001$), while the catalase activity remained practically unchanged relative to the parameters of the animals that did not receive simvastatin. Relative to the parameters of animals in the control group, a significant decrease in the SOD activity by 50.89% ($p < 0.001$) was revealed, and the activity of catalase increased by 86.47% ($p < 0.001$).

According to the literature, such a multidirectional change in the activity of the antioxidant pair "SOD – catalase" is also a sign of hypoxia development in the muscle tissue [14]. The antioxidant pair "SOD – catalase" is characterized by cross regulation, when high activity of one of the enzymes inhibits the activity of the other by the feedback mechanism. A decrease in the SOD activity poses a potential danger of oxidative damage to the most important macromolecules, due to a decrease in disproportionation of the superoxide anion radical and its transformation into a more aggressive hydroxyl radical by the Haber – Weiss reaction.

A correlation analysis was carried out to reveal the relationship between the parameters of carbohydrate energy metabolism, the activity of antioxidant defense enzymes, and giant sarcomeric proteins (titin and nebulin) in the muscle tissue of animals with induced hypercholesterolemia against the background of long-term use of simvastatin. As a result, a positive correlation was found between the content of the NT-titin isoform and the SOD activity, $r = 0.34$, $p = 0.05$ ($p \leq 0.05$). Negative correlations were also noted between the content of the N2A-titin isoform and the level of lactate, $r = –0.35$, $p = 0.04$ ($p \leq 0.05$), and between the content of the T2-proteolytic fragment of titin and the level of lactate, $r = –0.35$, $p = 0.04$ ($p \leq 0.05$).

CONCLUSION

According to the literature, titin, a giant protein of the sarcomeric cytoskeleton, is a template for correct assembly of proteins [15]. The study showed that the use of simvastatin in animals with induced hypercholesterolemia leads to a decrease in the content of giant proteins – titin and nebulin – in myocytes due to their increased proteolysis. As a result of degradation of structural proteins, a decrease in the contractile ability of skeletal muscles occurs, which is observed in pathological conditions, such as muscular dystrophies [16]. On this basis, the levels of titin and nebulin in the muscle tissue can be used as an early marker of statin-associated myopathy.

The analysis of energy metabolism parameters and their relationship with the dynamics of titin isoforms in animals after prolonged administration of simvastatin made it possible to determine a number of indicators that informatively reflect destructive changes in the muscle tissue. The revealed additional biochemical indicators, such as the SOD activity and the lactate level, along with the determination of titin and nebulin concentrations, indicating hypoxic processes in tissues, will make it possible to more accurately diagnose statin-associated myopathy.

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