Short Thesis for the degree of doctor of philosophy (PhD)

Effect of fluvastatin on rat skeletal muscle function – in a hypercholesterolamic animal modell

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The Ph.D. defense takes place at 10:00 AM, 7th of June, 2022, online. For participation, please register via e-mail at the following address: somogyi.gergo@med.unideb.hu. Registration process closes at 13:00 PM, 6th of June, 2022. Late registration is not accepted for technical reasons.

Introduction

Hypercholesterolaemia's consequence: atheroschlerosis is the leading cause of death worldwide. The number of patients taking statins for hypercholesetrolaemia is increasing. Nevertheless, a significant amount of this patient is not able to take the medication mainly because muscle adverse effects, thus they are not able to profit the statin's preventing cardiovascular mortality. The statin-induced myalgia or muscle weakness is well-studied, while the exact patomechanism of statin iduced myopathia is still not clearly understood.

1. Skeletal muscle

The skeletal muscle is a tissue specialised for contarction, its main function is changing body position or locomotion. The muscle constriction transforms chemical energy (ATP, glycogen, glucose) into mechanical energy. The process of myogenesis is caracterised by myoblast fusion, which results a multinucleated muscle fibre (myotubule). The striated morphology is resulted from the organisation of myofibrills into sarcomers. The action potential originates from the neurons, and spreads through the sarcolemma and T-tubules into the inner part of the myotubule. The terminal cysts of the sarcoplasmatic reticulum (SR) are localised in close physical proximity to both sides of the T-tubule sytstem. The Dihydropyridin Receptors (DHPR) and Ryanodyne recepotors (RyR) are localised here, in the triad. Every second RyR is in close proximity to a DHPR-tetrad. The calcium-release unit (CRU) is working on both sides of the triad separately, it transforms the electrical stimulus into mechanical response using intracellular calcium (ic. Ca^{2+}) stored in the SR. The calcium used for the muscle contraction is provided by the intracellular storage of the myotubules entirely. Sustained repolaristaion of the sarcolemma blocks the cascade of calcium-release, and the free intracellular calcium is reuptaken to the SR by SERCA pump.

The Ryanodine Receptor is a 2MDa homotetramaer protein, it is a low sensitivity, high conductivity cation channel. In striated muscle primarily functions as a ligand-gated Ca^{2+} channel, responsible for rapid calcium release from the SR. RyR is regulated by ic. calcium-concantration: nanomolic Ca^{2+} concentration activates, micromolic concentration blocks the channel function of RyR. The high Ca^{2+} concentration inside the SR also increases the opening probability of RyR.

2. Elementary Calcium Release Events

Elementary Calcium Release Event (ECRE) is a localised rise in the intracellular Ca^{2+} concentartion can not be separated into smaller units: ECRE is a result of the opneing of one ryanodine receptor cluster. The basic form of ECRE is the calcium spark. The calcium spark is the initial step of the excitation-contraction coupling. The local rise in the Ca^{2+} concentration represneted by sparks fusionates and generalisates and results the global ic. Ca^{2+} transient, which is a common intracellular response for extracellular stiumli. The morphology of the sparks are defined by the related RyR's gating mechanism, thereby provides information about the function (gating and Ca^{2+} flux) of the CRU's.

3. Cholesterol

Cholesterol is an important component of mammalian cell membranes, it modulates the membrane fluidity and permeability. Abnormal blood cholesterol level results in systemic diseases as atherosclerosis. The circulating blood low and high density lipoprotein (LDL and HDL respectively) cholesterols have a basic role in the pathogenesis of atherosclerosis. Cholesterol is synthetised from acetil-coenzim A molecules in the endoplasmic reticulum (ER) of the cells. The rate limiting step is the hydroxymethylglutharyl coenrzyme A (HMG-CoA) - mevalonate transformation catalised by the enzyme HMG-CoA-reductase. The product cholesterol has the most important, blocking effect in the physiological regulation of the HMG-CoA reductase enzyme. Statins are pharmacological competitive inhicitors of the HMG-CoA reductase enzyme. In the skeletal muscle, 80% of the the total membrane cholesterol content is localised the T tuble system, so it has higher cholesterol concentration compared to the surface membarane parts of the plasma membrane. The cholesterol content of the T tubule membranes is modulating the function of the proteins localised here responsible for the electromechanical coupling, eg. the DHPR's.

4. Statins

Scandinavian Simvastatin Survival Study was published in 1994, the research involved 4444 hypercholesterolaemic patinets with heart disease. After a follow up of five years, the blood cholestserol level of the simvastatin users dropped by 35%, meanwhile the risk of dying in myocardial infarction decreased by 42% in the statin group! The statins are a drug family of HMG-CoA reductase inhibitors. Inhibiting the process of atheroclerosis is not the sole result of decreasing blood cholesterol levels: statins have pleiotrpcic effect. Decreasing cardiovascular mortality, statins stabilises atherosclerotic plaques and improves the endothel fuction of arteries. This may be the result of inhibiting isoprenoid production and decrease prenylated proteins. Statis are known as immunomodulants, this effect is also related to small GTP-eses through MHC II. The fluvastatin has anti cancer effect also, it blocks cell proliferation and induces apoptosis in hepatocellular carcinoma.

The main side effect of statin therapy (1-10%) are muscle problems, as serious as ceasing statin therapy in many cases. Statin-induced myopathy is a class effect and dose dependendent. It is caracterised by different symptoms (myalgia, myopathy, myositis or myonecrosis) in the proximal, large muscle groups with elevated blood craetine.kinase (CK) level. The most severe and most fearful side-effect of statin therapy is life threatening rhabdomyolysis.

The exact patomechanism of statin-induced myopathy is not clearly understood yet. The cholesterol lovering effect of the statins may result in impaired membrane integrity. Lowering the coenzyme-Q10 (Q10) level inhibits the oxidative phosphorilation in the mitochondria, thus the energy production of the cells may decrease. Lowering the number of prenylated proteins may lead to apoptosis induction. Calcium signalling pathyway is involved in all these processes

5. Coenzyme Q10:

Q10 is a fat-soluable vitamin found in almost all eucariotic cells, mostly in the mitochondria. As part of the electron transport chain, it is an important factor of cellular respiration and ATP synthesis. Blood Q10 inhibits the production of oxidated LDL, thus protects from atherosclerosis. The isoperne parts are syntethised in the same pathway as the cholesterol, hence statins are lowering the blood Q10 levels also (up to 40%). So decreased Q10 thuoght to have a role in statin-associated myopathy.

Aims

Our aim was to examine the patomechanism of statin-associated myopathy, and create a model for it. Since stains are taken by hypercholesterolaemic patients, so we aimed to provide an animal model reliably reproducing this human state. Than we planned to investigate the effect of statins on skeletal muscle in both control and hpercholesterolaemic conditions. We investigated the presence of myopathy with muscle function tests and caracterised it by muscle force measurements. Calcium plays a basic role in intracellular signaling, besides it also necessary for the electromechanical coupling of skeletal muscles. We planned to detect the changes in the intracellular calcium concentartion ($[Ca^{2+}]_i$) inside the muscle fibre using ratiometric flurescent dyes. Investigating the stains effect on electromechanical coupling, we detected elentary calcium release events, and analysed their morphometric parameters (in time, space and intensity). We also considered to examine if the coenzyme Q10 plays a role in the patomechnism of statin-induced myopathy. Therefore we reproduced both our muscle function tests and ECRE examinations in the presence of Q10 supplementation.

Our hypothesis was that statin therapy alters the intracellular calcium-signaling pathway, and the Q10 supplementation protects the statin-induced muscle damage.

Methods

1. Animal model

We used fluvastatin for our experiments, it has a medium intensity and not requires further metabolisation of the active ingredient.

Adult female Fisher rats were fed with different diets based on standard rodent chow. The animals had access to food and water ad libitum. The treatments were at least 21 days long. Control (C) animals had standard rodent chow. The fluvastatin (S) and the Q10 diet was applied also in hypercholesterolaemic (HC) circumstances. The hypercholesterolamic diet was based upon high cholesterol intake with additional cholic acid, which potentiates the absorption of cholesterol, and with a thyrostatic drug thiouracil. Thiouracyl and the resulted hypothyroidism is known to be associated with myopathy itself, so we used a special control group for the HC diets: we compared it to only thiouracyl (TU) containing diet. For statin diet, the fluvastatin dose was 6.4 mg/kg/die, this represents five times the human maximal dose. The Q10 was applied in a dose 10 mg/kg/die, which also exceeds the human maximal dose.

2. Skeletal muscle primary cell culture

The primary skeletal muscle cell culture was prepared from Wistar rats 3-7 days old. The m. quadriceps femoris, m. soleus, and m. gastrocnaemius were removed from the hind legs. After mechanical dissection, we performed enzymatic digestion than seeded tha cells in proliferating media, HAM F15. After 24 hours incubation, the primary cell cultura was divided into two treatment groups randomly: the control cells was put into DMEM differentiating media including 5% horse sera, meanwhile 10 nM fluvastatin was added to the same media for the statin-treated group of cells. The differentiation was followed up to 48 hours.

3. Skeletal muscle cell proliferation and differentiation

The primary skeletal muscle cell culture was detected at 24, 48 and 72 hours after seeding, the cultures was photographed by a digital camera associated with an inverted light microscope. The myogenic nuclei was manually counted in the pictures and we detected the multinucleated cells. So we calculated the changing of the absolut number of the myogenic nuclei depending on the culture time, besides the average number of nuclei in a muscle cell and the fusion index (the number of myogenic nuclei in mononucleated cells compared to the number of myogenic nuclei in multinucleated cells) was analysed. We also calculated the ratio of multinucleated muscle cells compared to mononucleated muscle cells.

4. Lipid and creatine kinase analysis

The adult Fisher rats was sacrificed during pentobarbital anesthesia. The aorta abdominalis was cannulated and 5 ml blood sample was collected from each animal. The blood sample was analysed in the Laboratory Medicine Institution of University of Debrecen, Clinical Center. The total-, LDL-, HDL cholesterol, the trigliceryde and the creatine kinase levels was detected from the sera with standardised, automated processes.

5. Pathologican, histological analysis

During the section of the adult Fisher rats, we removed the heart, liver, kidneys and the m. extensor digitorum longus (EDL) and m. soleus (Sol) from the hild legs. The organ weights were measured. For histolgical preparation, we used 8% formaldehyde buffer for fixing the sample, than it was bedded with automated process using paraffine wax. Slices 4 μm wide was cut with microtme, they were fixed to slides and stained with hematoxylin and eosin. The muscle slides were investigated for signs of myopathy and the diameters of muscle fibers were recorded. The kidneys were invesitgated for signs of kidey failure, and the liver for fatty liver disease. The Pathology Department of Kenézy Hospital helped our work with the necessary technical equipment and professionality.

6. Muscle force examination

The skeletal muscle force was measured on both fast (EDL) and slow twitch (Sol) muscles. The muscles was put into Krebs soultion perfused experimental chamber. One end of each muscle was fixed to a metallic rod, and the other was attached to a capacitive mechanoelectric force transducer. The muscle was electrically stimulated with two platina electrodes. The passive stretch of muscles was set by the transducer in order to reach the maximal contraction force response for the stimulation. Twiches were elicited by short, 2 ms supramaximal stimuli, 10 twitch was registered with 2 sec delays. For EDL muscles, tetanus was provoked by 200 Hz single pulses 200 ms long, and for Sol muscles 100 Hz frequency for 300 ms long was applied. The duration of twitch or tetanus was determined from the start of the transient till the relaxation to 90% of maximal force. Time To Peak (TTP) was determined from the start of the transient till the ampitudo maximum. Fatigue of the muscles was measured with 150 evoked tetani 2 sec apart, the degree of fatigue was expressed by normalizing the amplitudes of tetani to the first. We investigated the twitch/tetanus ratio since it is known that the slow twitch muscles has higher values.

7. Single fibre preparation

After sacrifice the adult Fisher rats, the m. extensor digitorum communis (EDC) was removed from the front legs in order to detect the resting calcium concentration of the muscle fibers, and the m. flexor digitorum brevis (FDB) for detecting elementary calcium release events. Muscles underwent enzimatic digestion with type 1 collagenase. After washing the digestiong soution in calcium-free soution, the msucles was gently triturated in order to gain single fibers. To recover from this mechanic impact, the fibres were let to rest for 20 minutes in modified Krebs solution than were moved to the experimental chamber using a glass capillary.

8. Detecting the intracellular calcium concentartion ($[Ca^{2+}]_i$) at rest

The intracellular calcium concentartion at rest was detected both in adult Fisher rat's fibers and primary cultured skeletal muscle cells. The fluorescent stain was applied the same manner: Sample was put into 2 ml of normal HEPES-Tyorode (nTyr) solution, and 5 μM Fura-2 AM calcium-sensitive ratiometric fluorescent dye was loaded for 90 minutes. After washing, the sample was put into a perfusion chamber, where the measurement was took place. The excitation was applied at 340 and 380 nm in repeated time sequences and the emitted light was detected at 510 nm using inverted fluorescent microscopy with 40x magnifying oil immersion lens. Using Grynkiewicz et al.'s equation we were able to calculate the free intracellular calcium concentration from the ratio of the dye's calcium-binded and non-binded fluorescent emission.

9. Detecting elementary calcium release events

The cell membranes of the isolated fibers of FDB muscles of the adult Fisher rats were peremabilised using 0.01% saponin for 2-3 minutes and the fibers were loaded with 50 µM Fluo-3 fluorescent dye. After transfering the fibres into a glass-bottomed experimental chamber, into relaxing inner soultion containing calcium-sulfate, we performed measurements using confocal laser scanning microscope, 63x magnifying water-immersion lens, NA 1.2. The excitation was made by an argon ion laser at 488 nm, the emitted light was detected at 520 nm in line scan mode, so the spatial localisation of the changes in fluorescence intensity depending of time was plotted. The line to scan was 512 pixel long paralel to the longitudinal axis of the muscle fiber, with a pixel 0.142 µm x 0.142 µm. The speed of scanning was 2 µs/pixel with 1.54 ms delay between each scans. The recorded image size was 1024x512 pixel, they were analysed by a software developped by our research group in order to auto-detect and analyse the caracteristics of the ECRE's. Describing the calcium-sparks, we applied the following characteristics: amplitude (normalised peak of fluorescent intensity ($\Delta F/F0$), and the calculated change in $[Ca^{2+}]$), duration, time to peak (TTP, represents the length of calcium release from the SR), Full Time at Half Maximum (FTHM, maximal signal intensity, time to get the 50% of amplitude), and Full Width at Half Maximum (FWHM, saptial width at FTHM or 50% of amplitude).

10. Statistical analysis

Data from same treatment groups were averaged and standard error of the means (SEM) was calculated. The results are presented as mean ± SEM. Signifacnce of differences was detected by one-way ANOVA variance analysis and Student-Newman-Keuls post hoc method was applied. The resluts gain from the cell cultures were not normally distributed, so we applied Mann-Whitney rank sum test. Confidence interval was set at 95% level, so we considered differences significant at $P<0.05$. For ECRE's n represents the number of sparks, otherwise n represents the number of fibers or muscbles in each treatment groups. For diets inducing hypercholesterolaemia, TU group was considered as reference.

Results

1. Statin treatment of primary skeletal muscle cell culture

After 24 hours culture time, the cells reached almost full confluence in the proliferating media. In control conditions, the cells get spinder formation after another 24 hours in the differentiating media, the cell count decreased for myoblast fusion. Another 24 hours lead to full confulentce on the slides and each filed of view well over 10 nuclei big byotubules were to be seen. With fluvastatin treatment applied after the proliferating phase, the spinster formation of cells were sparse after 24 hours differentiating, and another 24 hours resulted in few spinster shaped cells and most of the cells were round as non-differentiated. Fusioun of myoblasts were rare, and well over 10 nuclei big myotubules were not to be seen during the whole culture time. Myotubules in statin-treated culuters were hardly any 10 nuclei big. Controll myotubules were more frequent and contained more nuclei as well.

Detailed morphometric anlysis: Reference number was the nuclei count detetcted after 24 hours proliferating phase in each treatment groups (self-control), so we determined relative nuclei count. In control dishes, the first 24 hours differentiating culture time resulted a 58% relative nuclei count rise, and after another 24 hours 54% additional increase was observed. But in the S group the first 24 hours resulted only a 3% elevation and another 24 hours revealed a 18% (non-significant) decrease in the relative myogenic nuclei count. Compared to control cultures, the myogenic nuclei count was significantly lower in the S group on both culture days. Fusion index was used to determine the differentiation level of muscle cells. Fusion index was constatly rise both in control and statin groups, meanwhile the S group had significantly lower values on both culture days. Development was characterised also by determine the average nuclei count of the muscle cells. After 48 hours culture time, the control cells contained 2 nuclei on average, and at 72 hours 3 nuclei. Fluvatstain resulted significantly lower values: 48 hours on average monoucleated cells were present and at 72 hours 2 nuclei. The average control muscle cell after 72 hours of cultre had 2-5 nuclei. More than 5 nuclei per cell was found in 20% of the cells and in 10% there were more than 10 nuclei in a cell or myotubule. Meanwhile in the fluvastatin group, afetr 72 hours of cell culture, there was more than 50% mononucleated muscle cells, and 40% of the cells had 2-5 nuclei. Bigger cells with 5-10 nuclei were found only in 6%. Statin treatment resulted less muscle cells altogether. Besides blocking cell proliferation, muscle cell differentiation was also lagged behind the control cultures.

2. Anatomy results

The average weight of the animals slightly differed in the different treatment groups. We observed less relative muscle weight (compared to body weight) in TU, HC and HC+S treatment groups for EDL, Sol and heart muscle also compared to control. If we compared the HC and HC+S groups to TU, there was no significant difference in relative muscle weights. As a result of statin treatment, there was a reduction in relative muscle weights in heart, EDL and more prominently in Sol muscles, compared to control.

The pathological examination reveled no macroscopic organ damage. Microscopic examination showed moderate fatty degeneration of the liver in high cholesterol (HC) diet. Kidneys and heart were intact in all groups. The skeletal muscles did not showed signs of myopathy with light microscopy, the striation was intact and there was no sign of inflammation in the treatment groups. However the muscles in the statin treated groups had a large varitey in the cross-section values of the muscle fibers, this was not able to detect in either other groups. In these statin groups (S and HC+S) the average fiber cross-section diameter was significantly less, the muscle fibers were thinner. C: 1.835 ± 79 μm^2 (n=161), S: 1.448±44 μm^2 (n=288); HC: 2.065±82 μm^2 (n=116); HC+S: 1.664±44 μm^2 (n=226). The HC and the TU groups fiber diameters had not differ significantly from control values. TU: 1.801±65 μm^2 (n=128).

3. Blood lipid and creatine kinase analysis

The control cholesterol, triglycerol and CK values were within the reference range and HDL/LDL ratio showed also the high HDL doinance usual for rats. HC diet caused more than sevefolds elevation in blood cholesterol and the proportion of lipoproteins also changed: there was the atherogenic LDL dominance likewise to humans and the anti-atherogenic HDL was only 40% of total cholesterol. Statin treatment did not changed the proportion of the lipoprotiens, according to literature in rats HMGCoA reductase treatment decrease the triglycerol contentration in blood. We also were able to detect decrease in trigliycerol value, however it has not reached signicant level probably because of low sample size.

There was a significant rise in the CK values of statin treated animals: the S group had 1,5x higher values compared to C. In HC+S, the blood CK was threefold elevated, which is a sign of myopathy. According to Statin Muscle Safety Task Force 2014 Classification, it fits into the category of slight myonecrosis so we considered muscle damage proven. We were not able to detect elevated CK nor TU, nor HC diet groups. We considered CK as skeletal muscle originated for the lack of any sign of macro- or microscopic heart damage (eg. myocardial infarction). If we normalise the CK values to muscle weight, eg. EDL average weight, the elevated CK in statin treated groups rise higher since it was detected with lower muscle weights.

4. Intracellular calcium concentration ($[Ca^{2+}]_i$) at rest

In primary cell cultures, the ($[Ca^{2+}]_i$) at rest was significantly lower in S group compared to C. The myotubules had lower values compared to satellite cells. However, fibers isolated from adult animals showed opposite results: long-term fluvastatin treatment caused significantly elevated resting $[Ca^{2+}]_i$ level compared to control. This elevation was independent of cholesterol levels. The HC diet has risen the $[Ca^{2+}]_i$ of the fibers significantly, and HC+S diet resulted a more prone significant elevation compared to TU or HC groups. TU treatment alone significantly decreased the resting $[Ca^{2+}]_i$ level compared to control.

5. Muscle force measurements

The muscle force of slow twitch Sol and fast twitch EDL was investigated. The muscle contractions in HC and TU groups had similar time characteristics. Meanwhile HC+S group differed. The TTP in statin treatment did not differed significantly for EDL or Sol. The duration of the contractions were significantly shorter for statin-treated Sol both in case of twitch or tetani, and the tendency was the same for EDL, but the difference was not statistically significant. In HC+S animals, the amplitude of muscle force was significantly lower for twitch and tetani, for EDL and Sol both compared to HC (HC and TU amplitudes did not differed significantly). Onte that the HC+S muscles had significantly lower fiber cross-section area compared to HC. Normalising the muscle force to muscle fiber cross-section area, we were not able to detect significant difference in the EDL or Sol muscle force between the HC+S and the HC animals (nor twitch nor tetanus). Therefore we consider muscle mass waist for the reason behind the decrease in absolute muscle force in HC+S animals, and the fibers contracting capacity did not changed. (EDL: HC twitch 17.6±3.1 mN/mm^2 vs. HC+S 17.2±3.3 mN/mm^2, HC tetanus 31.5±5.8 mN/mm^2 vs. HC+S 34.5±7.8 mN/mm^2. Sol: HC twitch 7.7±0.7 mN/mm^2 vs. HC+S 7.0±0.7 mN/mm^2, HC tetanus 22.5±2.4 mN/mm^2 vs. HC+S 20.2±4.5 mN/mm^2). Muscle fatigue did not differed significantly: TU muscle forces (n=5) dropped to 0.63±0.03 of maximal force, vs. HC muscle force (n=6) decreased to 0.72±0.03, vs. HC+S (n=8) to 0.76±0.0. Meanwhile HC+S Sol muscles showed significantly greater fatigue compared to HC or TU. In TU muscles (n=7), the force decreased to 0.79±0.05, in HC (n=6) to 0.80±0.03, vs. HC+S muscle force (n=9) dropped to 0.64±0.06. Summarizing our findings, hypercholesterolaemic state did not altered the muscle function, the kinetics and the force of the contractions did not differed from the reference (TU) group. Statin treatment decreased the length of muscle contractions both for twitch and tetanus: in slow twitch Sol muscles, the difference was statistically significant. Fluvastatin treatment significantly decreased the force of muscle contractions (along with elevated resting $[Ca^{2+}]_i$ concentrations).

Q10 diet resulted no significant difference in muscle force in either types of muscle from control (Q10: n=5, C: n=10). We did not find difference in the twitch/tetanus ratio between Q10 and C. There were no difference in the amplitude of twitch or tetanus in HC and HC+Q10 muscles either. HC+S+Q10 muscle forces did not differed significantly from HC+S. The kinetics of the contractions were similar, duration and TTP did not differed significantly in any treatment pairs compared. Summarizing our findings, we were not able to detect statistically significant effect of Q10 in case of fast twitch EDL or slow twitch Sol muscle's force or kinetics of contractions. Q10 diet failed to alter the fluvastatin's effect on muscle force.

6. Elementary calcium release events

FDB muscle fibers fisolated from statin treated animals presented significantly (p=0.033) more calcium sparks compared to C. Spark frequency in S group (n=17) was 2.24±0.2 Hz/sarcomer, vs. C: 1.59±0.1 Hz/sarcomer, n=21. Most of the morphometric parameters (amplitude, FWHM, TTP) of sparks reamined unaltered in S group, however, we detected slightly, but significantly (p=0.002) longer sparks after statin treatment: duration was 39,3±0,3 ms, n=3962, vs. C 37,8±0,4 ms, n=1605. Summarising our findings: fluvastatin elevates the spark ferquency meanwhile the morphometric parameters remain mainly unchanged.

The HC diet did not altered the spark frequency (HC: 1.55±0.17 Hz/sarcomer, n=21, vs. TU: 1.59±0.1 Hz/sarcomer, n=21, p=0.871). However all the examined morphometric parameter showed significant alterations. The spark amplitude in HC diet decreased (HC: 0.31±0.002, n=2239, vs. TU: 0.34±0.003, n=1667, p<0.001). The FWHM also decreased in the HC group (HC: 1.25±0.02 μm vs. TU: 1.33±0.02 μm, p=0.002). Rise Time for HC sparks was longer (HC: 17.5±0.3 vs. TU: 15.7±0.3 ms, p<0.001), and the duration was also longer (HC: 41.1±0.4 vs. TU: 37.8±0.4 ms, p<0.001). Summarising our finding, the HC diet resulted smaller calcium saprks in space and intensity, while in time sparks were longer (slower) but ouccured with same frequency as in control conditions.

HC+S fibers revealed higher spark frequency (HC+S: 2.38±0.26 Hz/sarcomer, n=18, vs. HC: 1.55±0.17 Hz/sarcomer, n=21, p=0.006). So we detected the same effect of statin treatment as we observed in normocholesterolaemic conditions. Spark amplitude was higher in HC+S group (HC: 0.31±0.002 vs. HC+S: 0.35±0.002, p<0.001). FWHM was also higher (HC+S: 1.36±0.02 μm vs. HC: 1.25±0.02 μm, p<0.001), TTP decreased (HC: 17.5±0.3 ms vs. HC+S: 16.0±0.2 ms, p<0.001). Duration of sparks also decreased with statin compared to HC diet only (HC: 41.1±0.4 vs. HC+S: 39.5±0.3 ms, p=0.003). Summarising our findings statin treatment in HC condition resulted higher spark amplitude, spatially larger and in time shorter calcium sparks compared to HC diet. Comparing HC+S results to normocholesterolaemic S results, we were not able to detect significant differences in the morphometric parameters of the sparks. So we consider as fluvastatin reversed or annihilated the alterations caused by the HC diet.

Q10 treatment did not resulted significant alteration in the calcium spark frequency (C: 1.15±0.2 Hz/sarcomer n=11, vs. Q10: 1.19±0.21 Hz/sarcomer, n=11, p=0.989). We detected significantly (p=0.001) fewer sparks in S+Q10 animals compared to S, the spark frequency decreased to the control saprk range in S+Q10 fibers (S+Q10: 1.11±0.12 Hz/sarcomer, n=12, vs. S: 2.33±0.21 Hz/sarcomer, n=15). The amplitude of sparks decreased in Q10, to 0.908 (p<0.001) compared to C. Other morphometric parameters of sparks remained unaltered by the Q10 treatment. Summarising our findings, Q10 tretament reversed the statin treatment's effect of rising calcium sparks and decreased the spark amplitude, meanwhile remained all other parameters unaltered. We observed same effect of Q10 diet with statin as in control conditions.

Discussion of the new scientific results, consequences

1. Animal model

Statin associated myopathy is more common than clinical trials report, it affects 9-20% of patients taking statins compared the reported 1-5%. Myopathy leads to ceasing the statin therapy (50% in one year), thus these patients did not benefit the cholesterol-lowering and the cardiovascular morbidity and mortality decreasing effect of the drug. Ultrastructural skeletal muscle damage can be detected even in asymptomatic patients.

Our rat animal model has blood cholesterol high enough to represent human conditions. We found two- to fivefold increase in blood cholesterol level in rats in response to high fat diets, and thyroid function blockage resulted even modest elevations. We combined the two methods and achieved an almost eightfold elevation in the blood cholesterol level of the animals. Thyroid function blockage is known to impair skeletal musle function, so we used a special control group also with animals have only thyroid function blocking diet. Our results assents that skeletal muscle function impairment is the result of the applied statin therapy and is not in association with the hypothyroid state of the animals.

2. Myopathy

We observed myopathy caused by fluvastatin treatment based upon the decrease of skeletal muscle fiber diameters and muscle force. Hypercholesterolaemic animals receiving statin had elevated resting $[Ca^{2+}]_i$ underlying the skeletal muscle damage, confirmed by the elevated blood CK concentration, a spencific muscle damage marker. Using different statin-associated myopathy classifications, we can drew the same conclusion: Statin Muscle Safety Task Force declares myopathy with moderate myonecrosis based upon the muscle weakness and the CK, whereas the European Atherosclerosis Association classifies as SAMS + minor CK elevation. We confirmed myopathy both with functional examinations and biomarker analysis.

We observed reduced cell proliferation and elongend differentiation after fluvatstatin treatment in primary skeletal muscle culture. After a certain skeletal muscle damage it is expected regeneration processes to start. Skeletal muscle regeneration in adults starts from precursor cells located under the lamina bsaslia of the fibers. We observed these essential processes to block upon statin treatment: both the initial proliferation and cell differentiation were prevented. So based upon our results of the primary cell culture we concluded that regeneration is impeded for damaged skeletal muscles in statin treatment. In our opinion, this is not maintained by the cholesterol depletion of the sarcolemma, for it is known that acute cholesterol depletion increase chicken skleteal muscle cell proliferation.

Alterations of calcium homeostatis was associated with statin myopathy earlier. Structural weakness of T-tubule system was reported in statin treated patients: after cholesterol depletion T tubules were more prone to damage compared to the sarcolemma or other parts of the SR. Acute fluvastatin treatment is also known to alter calcium spark activity in skeletal muscle. RyR3 receptor was up-regulated in satatin associated myopathy patients. The 3 isoform of RyR is more sensitively activated by calcium, so overexperssion is related to elevated intracellular calcium concentration at rest. Our findings are concordant as we detected definitely elevated resting $[Ca^{2+}]_i$ in skeletal muscle fibers of rats underwent chronic fluvastatin treatment. This result refers myopathy along with the observed CK elevation in blood. Chronic fluvtatstin treatment resulted in marked decrease of the maximal muscle force of twitch or tetanus both in fast and slow twitch muscles. Note that fluvastatin treatment decreased the muscle fiber and altogether the whole muscle diameter, and after normalising to muscle diameter we were not able to detect statistically significant difference in the muscle forces of statin treated and control animals. This reveales that statin treatment results in muscle waste and cross-bindings in musle fibers upon activation remains unchanged. Besides muscle force, the kinetics of muscle contractions also altered with the chronic fluvatstin treatment mostly in slow twitch Sol muscles. The significantly shorter contraction time relates to alterations in the activation process.

3. Elementary Calcium Release Events (ECRE)

Our results showed hypercholesetrolaemic rats had smaller and slower calcium sparks, meanwhile the frequency of saprks remained unchanged. Elevated blood cholesterol is related to elevated cholesterol concentartion in the cell mambranes. A former study done on heart and smooth muscle with arteficially low membrane cholesterol content revelaed an association between the membrane cholesterol concentration and parameters of calcium sparks. In respect of this finding, we conclude that the altered spark parameters detected in our experiments is the consequence of the altered membrane fluidity of the skeletal muscles, which was caused by the cholesterol-rich diet resulted elevated blood cholesterol concentration in the rats. Note that we permeabilised the cell membranes of the isolated skeletal muscles by applying saponine for a short time. Since our result are in consistence with the cited heart and smooth muscle result, we are tend to confirm our hypothesis that saponine treatment has neglectable as any effect on the spark parameters.

The applied fluvastatin treatment elevated the spark frequency, and this effect was consequently observable in the skeletal muscle of normo- and hypercholesterolaemic rats either. Fluvatstain did not alter the characetristics of the sparks besides the slight decrease in spark duration. Involvation of calcium signaling pathways are long surmised in the pathomechanism of statin associated myopathy. Simvastatin was reported to trigger intracellular calium transients in healthy human skeletam muscle cells, and that elevates the calcium leaks from SR of isolated skeletal muscles of healthy and RyR muatnt mice. Acute, in vitro application of simvastatin was reported to disturbe calcium homeostasis and rose the amplitude of sparks in healthy human and rat skeletal musle. However our results with chronic fluvastatin treatment did not showed statistically significant alterations of calcium spark amplitudes. This difference may be caused by the different way of application of the fluvastatin (acute, in vitro vs. 4 weeks long, in vivo). If the length or way of statin treatment alters their effect on spark amplitudes, this does not reveals why spark frequency increased in our statin group. Interspecies difference may be stand behind this finding since a study was published recently using similar rat model where statin treatment elevated spark ferquency and duration, whereas statin user patients with symptomatic myopathy was showed to have dcereased spark frequency. Note that in asymptomatic statin users were not able to detect alteration in spark frequency which finding underlines the role of calcium homeostatis in statin associated myopathy. In our experiment we observed the statin-related elevation of spark frequency in every treatment pairs differing only in the presence or absence of fluvastatin (except the Q10 treated animals, see later). Therfore we conclude that this is the most specific, major effect of fluvastatin in our experimental model. In this model fluvastatin treatment decreased the blood cholesterol concentration of hypercholesterolaemic rats to the reference level. Therefore we consider the difference observed between HC and HC+S sparks is not representing the direct effect of fluvastatin, and the decrease of cholesterol concentration is supposed to be the reason that HC+S sparks were similar to controls (in aplitude, duration etc.).

The most important result of the Q10 treatment was that applied it with fluvastatin, the spark frequency was decreased. Therefore Q10 abolished the spark freqency raising effect of statin treatment. Note that the amount of calcium released during an elementary calcium spark remained unchanged. Suppose that alteration of calcium homeostasis, or more precisely the alterations in the frequency of sparks is related to stain induced myopathy, our results strengthen the hypothesis that taking Q10 decrease the potential of the prevalence of skeletal muscle pathologies in statin (fluvastatin) users, athough results of clinical use of Q10 therapy is controverisal in statin induced myopathy. Since it is reported that elevation of the concentration of reactive oxygen spesies (ROS) increase calcium spark frequency in permeabilised skeletal muscles, and also known that Q10 supplementation decrease the ROS concentration in rat skeletal muscle, we consider ROS to have a role in the pathomechanism of statin induced myopathy. Althogh we were not able to detect macroscopic muscle function alteration after Q10 supplementation neither in control, HC or HC+S diet.

Summarising our results, fluvastatin consistently and specifically elevated calcium spark frequency in skeletal muscle cells, regardless of the cholesterol concentration in the animal's blood; and this effect was abolished by the Q10 supplementation in the diet. These results strenghten the role of calcium signaling in the pathomechanism of statin induced myopathy, and beisdes offers an explanation for the positive effect of Q10 supplementation in statin therapy: how Q10 can alleviate the myopathy simpoms of statin user patients.

Summary

Statins are effective drugs in cardiovascular prevention. The exact pathomechanism of statin associated myopathy is still not revealed. We created a hypercholesterolaemic animal model. In our experiments we applied chronic fluvastatin and/or Q10 in the diet of laboratory rats, and investigated the alterations in skeletal muscle force and calcium homeostasis. We detected a sevenfold rise in the blood cholesterol level of the HC rats compared to control. (C: 1.5±0.1 vs. HC: 10.7±2.0 mmol/l; n=15 and 16 respectively). LDL/HDL ratio is also increased (C: 0.29±0.02 vs. HC: 1.56±0.17), it was antagonised by statin treatment, which also elevated the blood CK concentration. The proliferation rate and fusion capacity of myotubules in primary cell cultures decreased with statin applied, and resting intracellular calcium concentration was decreased in the staellita cells and myotubules also. However, the adult skeletal muscle fibers had elevated resting $[Ca^{2+}]_i$ after the statin treatment (C: 116±4 nM vs. S: 151±5 nM; n=33 and 34 respectively). The cross section area of the m. extensor digitorum longus (EDL) fibers decreased in S animals, and muscle force of EDL and m. soleus (Sol) decreased, duration of twitch and tetanus also shortened in Sol. HC condition did not altered, while S treatment increased calcium spark frequency, which was antagonised by Q10 whereas the amount of calcium released by a single spark remained unchanged. We concluded tha fluvastatin treatment specifically and and consequently elevated calcium spark frequency in skeletal muscle fibers indepedent of the blood cholesterol concentration, and this effect was antagonised by the Q10 supplementation. Upon statin treatment, our results shows signs of myopathy characterised by decreased muscle force and elevated blood CK concentration. We also concude that calcium homeostasis plays a role in statin associated myopathy and Q10 has a potential protective effect in statin associated myopathy.

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List of publications related to the dissertation

1. Vincze, J., Jenes, Á., **Füzi, M.**, Almássy, J., Németh, R., Szigeti, G., Dienes, B., Gaál, Z., Szentesi, P., Jóna, I., Kertai, P., Paragh, G., Csernoch, L.: Effects of fluvastatin and coenzyme Q10 on skeletal muscle in normo- and hypercholesterolaemic rats.
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3. Lőrincz, I., Szánthó, E., Simkó, J., Szabó, Z., Barta, K., **Füzi, M.**, Szigeti, G.: A fokozott arrhythmiarizikó új markere: a mikrovolt T-hullám-alternáns patomechanizmusa és vizsgálati módszerei.
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