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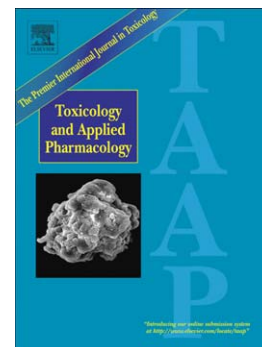
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STATIN-INDUCED MYOTOXICITY IS EXACERBATED BY AGING: A BIOPHYSICAL AND MOLECULAR BIOLOGY STUDY IN RATS TREATED WITH ATORVASTATIN

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Abbreviations

gCl: chloride conductance; PKC: Protein Kinase C; CN: calcineurin; restCa: resting intracellular calcium; MEF2: Myocyte enhancer factor-2; EDL: extensor digitorum longus; CK: creatin kinase; AMPK: AMP-activated kinase

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Abstract

Statin-induced skeletal muscle damage in rats is associated to the reduction of the resting sarcolemmal chloride conductance (gCl) and ClC-1 chloride channel expression. These drugs also affect the ClC-1 regulation by increasing protein kinase C (PKC) activity, which phosphorylate and closes the channel. Also the intracellular resting calcium (restCa) level is increased. Similar alterations are observed in skeletal muscles of aged rats, suggesting an higher risk of statin myotoxicity. To verify this hypothesis, we performed a 4-5-weeks atorvastatin treatment of 24-months-old rats to evaluate the ClC-1 channel function by the two-intracellular microelectrodes technique as well as transcript and protein expression of different genes sensitive to statins by quantitative real-time-PCR and western blot analysis. The restCa was measured using FURA-2 imaging, and histological analysis of muscle sections was performed. The results show a marked reduction of resting gCl, in agreement with the reduced ClC-1 mRNA and protein expression in atorvastatin-treated aged rats, with respect to treated adult animals. The observed changes in myocyte-enhancer factor-2 (MEF2) expression may be involved in ClC-1 expression changes. The activity of PKC was also increased and further modulate the gCl in treated aged rats. In parallel, a marked reduction of the expression of glycolytic and mitochondrial enzymes demonstrates an impairment of muscle metabolism. No worsening of restCa or histological features was found in statin-treated aged animals. These findings suggest that a strong reduction of gCl and alteration of muscle metabolism coupled to muscle atrophy may contribute to the increased risk of statin-induced myopathy in the elderly.

Keywords

ion channels; protein kinase C; senescence; skeletal muscle atrophy; energy metabolism dysfunction.

1. Introduction

Statins (3-Hydroxy-Methyl-Glutaryl Coenzyme A reductase inhibitors) are widely prescribed drugs to reduce plasma cholesterol levels. Their benefit in the hypercholesterolemia as well as in the prevention and treatment of coronary heart disease has been well established (Vaughan and Gotto, 2004). Furthermore, statins exhibit anti-inflammatory and antineoplastic pleiotropic effects that may extend their clinical value (Weitz-Schmidt, 2002 and Demierre et al., 2005). However, statin therapy can be severely limited by adverse effects involving many tissues, especially skeletal muscle. The clinical evidence of statin-associated muscle disorders range from benign myalgia to severe myopathy, up to the life-threatening rhabdomyolysis. Advanced age patients, suffering from senile muscle atrophy and loss of performance (Piccirillo et al., 2014), experience an increased risk of statin-induced muscle disorder. In 2001, the marketed cerivastatin was withdrawn because of the increased rate of mortality in aged subjects during the therapy (Baker and Tarnopolsky, 2001). The causes of skeletal muscle damage are still not fully understood, although various hypotheses have been formulated, including involving the inhibition of cholesterol biosynthesis, such as depletion of ubiquinone or small GTP-binding proteins involved in myocyte maintenance (Vaughan and Gotto, 2004). Our previous studies have identified one possible contributor to statin-induced muscle damage (Pierno et al., 2006, Pierno et al., 2009 and Camerino et al., 2011). We found that statins can affect skeletal muscle function by reducing the resting chloride channel conductance (gCl) in adult rats, through a mechanism independent of their ability to lower endogenous cholesterol. The gCl, carried by the ClC-1 chloride channel, plays a critical role in the stabilization of resting membrane potential and helps to repolarize the sarcolemma after action potentials, allowing a correct contractile activity and maintenance of muscle cell integrity (Imbrici et al., 2015). The reduction of gCl during statin administration is caused both by a reduction of ClC-1 channel expression and by the increased activity of Protein Kinase C (PKC), which reduce ClC-1 function (Pierno et al., 2009 and Camerino et al., 2014). Lipophilic statins can increase calcium ions release

from mitochondria and sarcoplasmic reticulum in rats and humans (Liantonio et al., 2007 and Galtier et al., 2012), and favor PKC-dependent phosphorylation of ClC-1 channels. We also observed that modulators of gene expression, such as Myocyte-enhancer factor-2 (MEF2) and histone deacetylase HDAC, may be associated to ClC-1 and PKC regulation (Pierno et al., 2013). Indeed, it is thought that PKC θ co-operates with calcineurin (CN) in slow-twitch muscles to ensure MEF2 transcriptional activation, which in turn sustains slow muscle gene expression and maintains a low gCl (Camerino et al., 2014 and D'Andrea et al., 2006). Other studies have described that statin treatment may cause muscle atrophy during adulthood through atrogin-1 dependent protein degradation (Cao et al., 2009 and Mallinson et al., 2012). A proteomic study also identified different expression patterns likely involved in the alteration of skeletal muscle function, such as energy production systems, both oxidative and glycolytic enzymes and creatine kinase, which were down-regulated following statin administration. Also alteration of proteins involved in cellular defenses against oxidative stress, such as heat shock proteins, was found (Camerino et al., 2011). At the light of these data, it can be hypothesized that statin-related side effects may be worsened by senescence. Indeed, it is well established that aging process severely affects skeletal muscle function, with a loss of muscle mass which lead to functional disability and loss of independence (Visser et al., 2005). Our previous studies have demonstrated that aging process in rat skeletal muscle is associated to a reduction of resting gCl and ClC-1 mRNA expression. A modification of PKC activity also occurs along with alteration of calcium homeostasis (Pierno et al., 1999 and Fraysse et al., 2006). An increase of MuRF-1 and atrogin-1 (Gumucio and Mendias, 2013) as well as a modification of the glycolytic and oxidative pathways (Akasaki et al., 2014) has been also described during sarcopenia.

Based on the previous findings showing the effect of statins on adult skeletal muscle (Pierno et al., 2009), the aim of this study was to investigate whether the mechanisms involved in skeletal muscle alteration induced by senescence may concur to the increased risk of statin-induced myopathy in the

elderly. We performed a long-term treatment with atorvastatin in adult and aged rats and analyzed a number of statin-sensitive molecular, cellular and functional parameters in the fast-twitch Extensor Digitorum Longus (EDL) and the slow-twitch soleus (Sol) muscles. These include the resting gCl , the expression of $ClC-1$ and modulatory genes, the resting intracellular calcium (restCa) level and expression of related genes and proteins, and the expression of genes involved in atrophy and energy metabolism. We also evaluated the plasma level of atorvastatin in adult and aged treated rats.

2. Methods

2.1. Animal care and atorvastatin treatment

All experiments were performed in accordance with the Italian Guidelines for the Use and Care of Laboratory Animals (D. Lgs 2014 n26), which conforms with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and Guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, and was approved by the Veterinary authority of the Italian Ministero della Salute (D.M. n.126/2009). All animal studies comply with the ARRIVE guidelines. Adult (5-months old) and aged (24-months old) male Wistar rats (Charles River Laboratories, Calco, Italy) were assigned to four experimental groups of five to eight animals each as detailed in the experimental procedure. These animals were housed individually in metabolic cages under veterinary supervision, in an environmentally controlled room under 12 h/12 h light/dark cycle with controlled room temperature and were allowed access to a diet of standard laboratory chow (30 g/day) (Charles River, 4RF21) and water. The experimental groups are as following: (a) a group of 7 adult rats treated with 10 mg/kg/day atorvastatin, (b) a group of 8 aged rats receiving 10 mg/kg/day atorvastatin, (c) a group of 7 adult rats treated with the drug vehicle carboxy-methyl-cellulose (CMC), (d) a group of 8 aged rats treated with CMC. Atorvastatin

was orally administrated to rats using an esophageal cannula for 4–5 weeks. During the treatment, the rats were weighted weekly and vital parameters (general health conditions, body weight, water and food consumption) were observed. Skeletal muscle performance was evaluated daily by testing each rat for the righting reflex (the ability of the rat to turn itself on its four legs from the supine position) (Desaphy et al., 2014). At the end of the treatment period rats were deeply anaesthetized with urethane (1.2 g/kg body weight, i.p.) to allow the dissection of Extensor Digitorum Longus (EDL), Soleus (Sol) and Tibialis Anterior (TA) muscles. These muscles were used immediately for electrophysiological and functional studies or frozen in liquid nitrogen and stored at -80°C for further gene and protein expression analysis. Soon after, the animals were killed with urethane overdose. All efforts were made to minimize animal suffering.

2.2. Plasma creatine kinase (CK) level measurement

Blood was collected by cardiac puncture, soon after death, in ethylenediaminetetraacetic acid (EDTA) rinsed tubes and then centrifuged at 600 g for 10 min at 15°C. The plasma was separated and stored at -20°C until assay. Creatine kinase (CK) determination was performed by standard spectrophotometric analysis by using diagnostic kit (Sigma Aldrich, Milan, Italy) (De Luca et al., 2005).

2.3. Measure of resting chloride and potassium conductance in EDL and Soleus muscles and of the Mechanical Threshold for Contraction in EDL muscle by the two-intracellular microelectrode technique.

As previously detailed (De Luca et al., 2005 and Pierno et al., 2007), the EDL and Sol muscles were placed in a 25 mL muscle bath, maintained at 30°C and perfused with normal or chloride-free physiological solution (gassed with 95% O₂ and 5% CO₂; pH= 7.2–7.3). By means of standard two-

intracellular-microelectrode technique, RMP, cable parameters, component conductances and excitability characteristics of treated and untreated muscle fibers were measured in current-clamp mode. Cable parameters, in both normal and chloride-free solutions, were calculated from the electrotonic potential elicited by square-wave hyperpolarizing current pulse (100 ms duration) injected by the current electrode. So, we experimentally determined R_m and its reciprocal in physiological solution as the total conductance (g_m) and in chloride-free solution to measure the potassium conductance (g_K) (Pierno et al., 2006). The mean g_{Cl} was estimated as the mean g_m minus the mean g_K . Chelerythrine (Tocris Bioscience) was applied *in vitro* on EDL muscles dissected from control and treated rats and g_{Cl} was measured 30 min after drug addition. Briefly, the mechanical threshold (MT) for contraction was measured using a two microelectrode “point” voltage clamp method (Pierno et al., 2006). Details are given in the Supplementary Methods.

2.4. Fluorescence Measurements of Resting Intracellular Ca^{2+} concentration in EDL muscle

Fluorescence measurements were performed on small bundles of five to ten fibers lengthwise dissected from mice EDL muscles, as described elsewhere (Frayssé et al., 2003). Briefly, the muscle fibers were incubated with the fluorescent calcium probe fura-2 for 45–60 min at 22°C in physiological solution containing 5 μ M of the acetoxymethyl ester (AM) form of the dye mixed to 10% (v/v) Pluronic F-127 (Molecular Probes, Leiden, The Netherlands). A QuantiCell 900 integrated imaging system (VisiTech International Ltd) was used to acquire pairs of background-subtracted images of the fura-2 fluorescence emission (510 nm) excited at 340 nm and 380 nm. Then it is possible to transform mathematically, fluorescence ratio in $[Ca^{2+}]_i$ values (Frayssé et al., 2003).

2.5. mRNA expression analysis in EDL and Sol muscles by quantitative real-time PCR

From EDL and Sol muscle sample, total RNA was isolated by Trizol reagent (Life-Technologies C.N. 10296010) and quantified by using a spectrophotometer (ND-1000 NanoDrop, Thermo Scientific). To perform reverse transcription, for each sample, 400 ng of total RNA was added to 1 μ l dNTP mix 10 mM each (Roche N.C. 11277049001), 1 μ l Random Hexamers 50 μ M (Life-Technologies C.N. n808-0127) and incubated at 65°C for 5 min. Afterward, 4 μ l 5X First Standard Buffer (life-technologies C.N. Y02321), 2 μ l 0,1 M DTT (life-technologies C.N. Y00147) and 1 μ l Recombinant RNasin Ribonuclease Inhibitor 40 U/ μ l (Promega, C.N. N2511) were added and incubated at 42°C for 2 min. To each solution 1 μ l Super Script II Reverse Transcriptase 200 U/ μ l (Life-Technologies C.N. 18064-014) was added and incubated at 25°C for 10 min, at 42°C for 50 min and at 70°C for 15 min. Real-time PCR was performed in triplicate using the Applied Biosystems Real-time PCR 7500 Fast system, MicroAmp Fast Optical 96-Well Reaction Plate 0.1 mL (Life-Technologies C.N. 4346906) and MicroAmp Optical Adhesive Film (life-technologies C.N. 4311971). The setup of reactions consisted 8 ng of cDNA, 0,5 μ l of TaqMan Gene Expression Assays, (Life-Technologies), 5 μ l of TaqMan Universal PCR master mix No AmpErase UNG (2x) (Life-Technologies C.N. 4324018) and Nuclease-Free Water not DEPC-Treated (Life-Technologies C.N. AM9930) for a final volume of 10 μ l. The RT-TaqMan-PCR conditions were as follows: step 1: 95°C for 20 s; step 2: 95°C for 3 s; and step 3: 60°C for 30 s; steps 2 and 3 were repeated 40 times. The results were compared with relative standard curve obtained by 5 points of 1:4 serial dilutions. TaqMan Hydrolysis primer and probe gene expression assays were ordered by Life-Technologies with the assay IDs reported in the Supplementary Methods. The mRNA expression of the genes was normalized to the best housekeeping gene beta-actin selected among hypoxanthine phosphoribosyltransferase 1 (Hprt1), beta-2 microglobulin (B2m) and beta-actin (Actb) by GeNorm software (Camerino et al., 2015 and Bustin et al., 2009).

2.6. Protein expression analysis by Western Blot

PKC α e PKC θ proteins were isolated according to Camerino et al. (2014). RyR1 protein was isolated according to Bastide et al. (2000). CIC1 protein was isolated according to Papponen et al. (2005) with some modifications. For CIC1 preparation, EDL muscles were homogenized in ice cold buffer containing 20 mM Hepes (pH 7.4), 2 mM EDTA, 0.2 mM EGTA, 0.3 M sucrose and 0.2 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 7000 x g for 5 min at 4°C. The supernatant obtained was centrifuged at 50,000 x g for 1 h at 4°C and the pellet was solubilized in 50-100 μ l of the same buffer. The protein concentration was quantified using Bradford protein assay kit (Bio-Rad). 40 μ g of protein was separated on a 10% SDS-PAGE and transferred onto nitrocellulose membranes for 1 h at 200 mA (SemiDry transferblot; Bio-Rad). Membranes were blocked for 2 h with Tris-HCl 0,2 M, NaCl 1,5 M, pH 7.4 buffer (TBS) containing 5% non-fat dry milk and 0.5% Tween-20, incubated overnight at 4°C with primary antibody. The following dilution of primary antibodies were used: mouse anti-RyR1 (Thermo Scientific Pierce, Clone: 34C) 1:2500; rabbit anti-PKC α (Cell Signaling Antibodies, c.n. 2015) 1:1000; rabbit anti-PKC θ (Cell Signaling Antibodies c.n. 12206) 1:500; rabbit anti-CIC1 (MyBiosource, c.n. MBS714620) 1:200 with TBS containing 5% non-fat dry milk. After three washes with TBS containing 0.5% tween-20 (TTBS) membranes were incubated for 1 h with secondary antibody labeled with peroxidase (1:5000 anti mouse IgG for Ryr1 protein, 1:5000 anti rabbit IgG for PKC α , PKC θ and CIC1 protein, Sigma-Aldrich). Membrane was then washed with TTBS, developed with a chemiluminescent substrate (Clarity Western ECL Substrate, Bio-Rad) and visualized on a Chemidoc imaging system (Bio-Rad). Densitometric analysis was performed using Image Lab software (Bio-Rad). The software allows the chemiluminescence detection of each experimental protein band to obtain the absolute signal intensity. The density volume was automatically adjusted by subtracting the local background. For each sample, the relative intensity was calculated by normalizing the intensity of beta-Actin (diluted 1:200, rabbit Anti Actin, c.n. A2066, Sigma Aldrich) protein band as reference

standard. We used beta-actin as control protein since its expression appeared quite homogeneous among the various samples.

2.7. Histological analysis of TA muscle

The fast-twitch TA muscle was dissected from three randomly selected controls and three randomly selected treated rats from each group. Muscles were rinsed in normal physiological solution and immediately frozen in N₂ cooled isopentane. Transversal 6-10 μ m thick section were cut from the mid-belly of the muscle and stained with haematoxylin-eosin. Histopathological analysis was performed with ImageJ and included the evaluation of general homogeneity of tissue architecture in relation to the presence of area of damage (inflammation, necrosis and/or non-muscle area), of centronucleated fibers and of vacuolization (De Luca et al., 2005 and Cozzoli et al., 2013).

2.8. Plasma sample preparation and ElectroSpray Ionization Mass Spectrometry (ESI-MS)

Plasma samples were extracted employing a liquid-liquid extraction technique according to the procedure previously reported with minor modification (Liantonio et al., 2012). Atorvastatin calcium (Sigma Aldrich) was used as standard and the internal standard was clofibrac acid (Sigma-Aldrich). Mass spectrometric detection and quantification were carried out using an electrospray interface and a Q-TOF mass spectrometer (Agilent 6530 Accurate Mass Q-TOF LC/MS, Palo Alto, CA). Ionization was achieved in the negative ion mode. Full-scan mass spectra was recorded in the mass/charge (m/z) range of 50-800, focusing on $[M-H]^-$ peak 213.0340 (IS) and $[M-H]^-$ peak 557.2489 (Atorvastatin). To determine drug plasma concentration, calibration curves were constructed by plotting the ratios of analyte/IS peak vs. nominal concentration of the analyte and analyzed by linear regression analysis. The calibration curve were linear over the concentration

range (5-300 ng/mL), with correlation coefficients (R^2) ≥ 0.9925 . Details are given in the Supplementary Methods.

2.9. Statistical analysis

All data are expressed as mean \pm SEM. The estimates for SEM and number of fibers (n) of gCl were obtained from the variance of gm and gK, assuming no covariance, and from the number of fibers sampled for gm and gK, respectively (Pierno et al., 2009). The analysis of variance (ANOVA) followed by Bonferroni's t test or Fisher's t test was used to evaluate statistical differences between the treated and control groups. A comparison of means between treated and the related control group was evaluated by the unpaired Student's t test.

3. Results

3.1. Atorvastatin chronic treatment and animal health

All the rats (7 treated and 7 untreated adult rats; 8 treated and 8 untreated aged rats) were examined daily over the entire period of the treatment. No signs of adverse effects were observed. In all the animals, the righting reflex was normal during the entire treatment period. The Extensor Digitorum Longus (EDL) and Soleus (Sol) muscle weight was measured in 3 untreated adult and 6 treated adult, in 8 untreated aged and 8 treated aged rats. Although a slight reduction of muscle weight was found in aged as compared to adult animals, no modification was observed between treated vs. untreated animals (Supplementary Fig. 1A). Body weight was slightly reduced either in adult or aged treated rats, particularly at the end of the treatment (Supplementary Fig. 1B).

3.2. Plasma level of Creatine Kinase in atorvastatin treated adult and aged rats.

The creatine kinase (CK) level was measured in the four groups of animals (Table 1). We found an increase of CK, although not significant, in atorvastatin-treated adult rats, with respect to untreated adult rats. Aging process significantly increased CK in control conditions as compared to the adults. CK was further increased in atorvastatin-treated aged animals.

3.3. Modification of electrophysiological properties in EDL and Soleus muscles during aging and effects of atorvastatin treatment.

3.3.1. Component ionic conductances

As previously reported (Pierno et al., 2009), a modification of the component chloride conductance (gCl) was found in the EDL muscle fibers due to atorvastatin treatment. Indeed, the gCl was significantly reduced by 25.6 ± 2.2 % in atorvastatin treated (6 rats, 45 fibers) as compared to untreated adult rats (4 rats, 26 fibers) (Fig. 1A). A significant reduction of resting gCl by 29.4 ± 2.9 % was also found in the EDL muscle of 29-months old aged rats (8 rats, 57 fibers) with respect to the adult ones, as previously reported (Pierno et al., 1999). Atorvastatin treatment further decreased gCl by 26.3 ± 3.6 % in aged animals (6 rats, 42 fibers) with respect to untreated aged animals, demonstrating additive effects (Fig. 1A). In total, the gCl reduction in atorvastatin-treated aged rats with respect to the untreated adult ones was 48.0 ± 2.6 %. In the EDL muscle, the component potassium conductance (gK) accounts for about 10 % of the total sarcolemma conductance at rest. No modification of this parameter was found after statin treatment in adult rats. The gK was 300 ± 44 $\mu\text{S}/\text{cm}^2$ (26 fibers, 4 rats) and 305 ± 18 $\mu\text{S}/\text{cm}^2$ (34 fibers, 4 rats) in control adult and atorvastatin-treated adult rats, respectively. The gK was only slightly increased in aged animals with respect to control adult, being 365 ± 27 $\mu\text{S}/\text{cm}^2$ (50 fibers, 6 rats), but was not modified by

atorvastatin treatment ($350 \pm 22 \mu\text{S}/\text{cm}^2$, 41 fibers, 4 rats). To study the regulation of chloride channels sustaining the gCl, we have tested the effects of *in vitro* application of chelerythrine to excised EDL muscles. The PKC inhibitor induced only a modest increase of gCl in control adult rat EDL fibers, whereas the effect was significant in muscle fibers of adult rats treated with atorvastatin (Fig. 1A). In aged muscles, chelerythrine significantly increased gCl in control but less after statin treatment (Fig. 1A). Nevertheless, by increasing 2-fold chelerythrine concentration, gCl increases up to the untreated aged value. Soleus (Sol) is a postural muscle in which the resting gCl is basically lower with respect to the fast EDL muscle in the adult animals. Here we found a 16% reduction of resting gCl in Sol muscle of adult rats treated with atorvastatin, although less pronounced compared to EDL muscle. Also aging process reduced the Sol muscle gCl by 22% with respect to adults and atorvastatin treatment further reduced Sol muscle gCl by 12% in aged rats (Supplementary Table 1).

3.3.2. Excitability parameters

The most markedly affected parameter was the threshold current (I_{th}) needed to obtain the first action potential. A reduction of I_{th} was observed in EDL of atorvastatin treated adult rats. It was also reduced in aged rats with respect to adults and was further reduced in aged treated rats (Supplementary Fig. 2A). The reduction of I_{th} is consistent with the reduction of gCl and increased excitability. The latency of action potential (Lat) was slightly increased. No significant modification of excitability parameters was found in the slow-twitch Sol muscle, in agreement with the minor sensitivity of this muscle to aging and statin treatment (Pierno et al., 2009 and Fraysse et al., 2006) (Supplementary Fig. 2B).

3.4. Effects of atorvastatin treatment on the Mechanical threshold (MT) for contraction and on resting intracellular calcium level in skeletal muscle fibers of adult and aged rats.

Muscle fibers (EDL) from atorvastatin-treated rats needed less depolarization to contract at each pulse duration and the threshold potential–duration relationship was consequently shifted toward more negative potentials as compared to adult controls (Fig. 1B). The Rheobase values calculated from the fit of experimental points was -63.3 ± 0.2 mV (13 fibers/3 rats) and -72.1 ± 0.6 mV (13 fibers/3 rats, $P < 0.001$) in adult untreated and adult treated rats, respectively. Aging significantly modified the Rheobase with respect to that found in the adults, being -70.5 ± 0.3 mV (16 fibers/5 rats). Nevertheless, it was not further changed by atorvastatin treatment in aged animals, being -71.70 ± 0.4 mV (25 fibers/5 rats). Using the FURA-2 cytofluorimetric technique, we evaluated the effects of statin treatment on the calcium homeostasis of the EDL muscle fibers of aged rats (Fig. 1C). In accord with previous studies (Liantonio et al., 2007 and Fraysse et al., 2006), aged fast-twitch muscle fibers showed a significant increase of resting cytosolic calcium (restCa) with respect to the adults, and atorvastatin treatment increased restCa in the adult rats. However, restCa was not further modified in atorvastatin-treated aged rats as compared to the untreated aged value (Fig. 1C).

3.5. Expression level of mRNA encoding selected genes in EDL and Soleus muscles of atorvastatin treated adult and aged rats

3.5.1. CIC-1 chloride channel, Protein Kinase C, enzymes and transcription factors involved in the regulatory pathway

We explored the modifications of CIC-1 mRNA expression by Real-Time qPCR in muscles of atorvastatin treated rats. We found that CIC-1 transcript amount was slightly reduced in EDL muscles of aged animals with respect to the adults. Statin treatment slightly reduced CIC-1 mRNA level in the adult animals, while the reduction was significant in treated aged animals compared to untreated aged (Fig. 2). No modifications of CIC-1 mRNA were observed in Sol muscle (Supplementary Fig. 3). We also evaluated mRNA expression of PKC α and PKC θ , both involved in

the modulation of CIC-1 channel activity (Camerino et al., 2014). In EDL muscle, PKC α mRNA was significantly increased in aged animals, but no modification was found in statin treated animals either in adult or aged. Conversely, PKC θ mRNA was not modified in aged animals, but was increased in adult and aged treated rats with respect to their own control (Fig. 2). In Sol muscle, an increase of PKC θ mRNA was observed in aged treated muscles, which may contribute to the observed reduction of gCl. Since a relationship was found between PKC and expression of some gene transcription modulators, such as MEF2 and HDAC (Camerino et al., 2014), we evaluated their involvement in statin induced muscle damage. MEF2 has been found to be increased during myotonia (Wu and Olson, 2002) and due to PKC θ activation (Camerino et al., 2014 and D'Andrea et al., 2006). Here we found an increase of MEF2 mRNA in untreated aged as well as in statin treated adult and aged rats. Slight modifications were found in Sol muscle (Supplementary Fig. 3). However HDAC was not modified (Fig. 2).

3.5.2. Calcium handling genes involved in skeletal muscle contractile function

We also focused on the expression of genes involved in calcium release and re-uptake, such as the ryanodine receptor-1 (RyR-1) and the sarco-endoplasmic reticulum Ca²⁺ ATPase (SERCA) type 1 and 2. The mRNA expression of RyR-1 was significantly increased in aged EDL (Fig. 2) and Sol muscles with respect to adults (Supplementary Fig. 3), but was not modified by statin treatment (Liantonio et al., 2007). The mRNA levels of SERCA1 and SERCA2 were unchanged in EDL muscle. An increase of SERCA1, typical of fast-twitch muscles, was found in Sol muscle during aging with respect to adult control. The SERCA-1 mRNA level in statin treated old rats was more similar to that of the adult. The calcium-binding protein parvalbumin is mainly expressed in fast-twitch muscles and contributes to muscle relaxation after contraction (Berchtold et al., 2000). Parvalbumin mRNA was only slightly increased in EDL of statin-treated rats, but was markedly increased in Sol muscle of old rats and of statin-treated old rats (likely to buffer calcium release).

Calcineurin (CN), a calcium dependent phosphatase, significantly increased in the EDL of statin-treated adult rats. A slight increase was observed in statin treated aged rats. In contrast, in Sol muscle, a significant increase was observed only in aged animals.

3.5.3. *Atrogenes and autophagy-related genes*

The mRNA expression of atrogen-1 and MuRF-1 was significantly increased during aging in both EDL and Sol muscles, as expected (Gumucio and Mendias, 2013 and Serrano et al., 2011). A significant increase of atrogen-1 mRNA was observed in EDL muscle of atorvastatin-treated adult rats (Fig. 2). However, atrogen-1 or MuRF-1 were not further increased in atorvastatin-treated aged rats. In contrast, the mRNA expression of autophagy-related genes, BNIP3 and LC3, was similar in EDL and Sol muscles of adult, aged, and treated animals.

3.5.4. *Muscle metabolism-related genes*

We previously found a down-regulation of beta-enolase (ENO3), a glycolytic enzyme, in statin treated rats (Camerino et al., 2011). Here we found a slight decrease of ENO3 mRNA in EDL of adult rats treated with atorvastatin and of untreated aged rats (Fig. 2). A marked decrease was observed in EDL of statin-treated aged animals, suggesting a stronger modification of glycolytic pathway. No modification was found in Sol muscle (Supplementary Fig. 3). A significant increase of pyruvate kinase M2 isoform (PKM2) was observed in EDL muscle of treated adult and aged rats. In addition, PKM2 mRNA was significantly decreased in untreated aged EDL muscle. To explore mitochondrial activity and energy production pathways, we evaluated mt-NADH dehydrogenase subunit 5 (mtND5) gene expression. We found a significant decrease in mRNA expression only in aged animals treated with atorvastatin with respect to untreated aged. Also the AMP-activated kinase (AMPK), known to be involved in energy muscle process, was slightly increased after statin

treatment in both situations. The level of RhoA mRNA was significantly decreased following statin treatment in aged treated rats suggesting major effect of statin during aging.

3.6. Expression of CIC-1 channel protein, Protein Kinase C and Ryanodine receptor in EDL muscle of atorvastatin treated adult and aged rats

Consistent to the reduction of mRNA expression, we demonstrated that the level of the CIC-1 channel protein was reduced in adult rats treated with atorvastatin with respect to untreated adults (Fig. 3). Also a decrease of CIC-1 protein, although not significant, was observed in aged compared to adult animals. Interestingly, a marked decrease was found in atorvastatin-treated aged rats, which sustains the reduction of gCl observed (Fig. 3). We also evaluated the expression of two PKC protein isoforms mainly expressed in skeletal muscle. The two isoforms of PKC were not modified by aging process (Fig. 3). However, the PKC θ protein was significantly increased in adult treated muscles, in line with the mRNA changes, but was slightly increased in aged treated muscles, in contrast to the significant increase in the mRNA level. The PKC α protein was slightly increased in adult and aged treated rats (Fig. 3). Despite the measured changes in RyR mRNA, the amount of RyR-1 protein was only slightly modified after atorvastatin treatment either in adult or aged rats (Supplementary Fig. 4).

3.7. Histological analysis of TA muscle of atorvastatin treated adult and aged rats

We performed a histological analysis of TA muscle sections from untreated and statin treated adults and aged animals at the end of the treatment period. Representative pictures of the histological profile of TA muscle of adult and aged atorvastatin treated rats are shown in Fig. 4. Despite the inter-individual variability within groups (3 rats/group examined), a marked increase of the non-

muscular area over the total area was observed in treated and untreated aged animals with respect to both groups of adult rats. According to previous studies (Serrano et al., 2011 and Miljkovic et al., 2015), the area of damage in aged muscles was identified as the area occupied by infiltrates (due to inflammation) and by non-muscular tissue, likely represented by fibrotic or adipose tissue. A qualitative analysis showed the presence of cell infiltrates particularly in untreated aged muscles. The presence of centronucleated (CNF) fibers, isolated or in clusters near necrotic fibers, was also observed in aged untreated and aged treated rats indicating possible degeneration/regeneration process. No marked change in centronucleated fibers was observed as a consequence of the statin treatment (Fig. 4). However, a specific vacuolation was present in statin-treated adult rats, as already described by others (Westwood et al., 2005), and in aged treated rats as a consequence of statin treatment. In our experimental conditions, the vacuoles show peculiar inclusions, appearing as regular and symmetric mineralization. These vacuoles occurred with low incidence in adult treated rats, and their number slightly increased in aged treated rats.

3.8. Plasma atorvastatin content in treated adult and aged rats

To verify possible modification in pharmacokinetic properties of atorvastatin due to aging, we measured its content in the plasma of the four groups of animals. High levels of atorvastatin were found in plasma of both adult and aged treated animals, being null the content in the untreated animals. No significant difference of atorvastatin mean content was found between adult and aged statin treated animals (Supplementary Fig. 5). It is however of note a higher variability within aged treated animals, which suggests some variation in plasma protein content, in the volume of distribution, and/or enzymatic activity in a number of aged animals.

4. Discussion

The risk of myopathy induced by statin therapy is higher in aged than in adult subjects (Moßhammer et al., 2014 and Stroes et al., 2015). Thus, statin associated muscle symptoms may limit their use and may contribute to the discontinuation of a needed therapy. Unfortunately, the lack of an adequate number of clinical trials does not allow to identify the correct dose for aged patients, and statins are instead prescribed as in the adults, despite the age-related changes in pharmacokinetics and pharmacodynamics. The reason of the increased risk of myopathy in the elderly is not known but the age-related decline of muscle function and impairment of regenerating capacity with metabolic implications may play an important role (Janssen et al., 2005). In addition, a modification of ion channel activity and expression may be also critical (Pierno et al., 1999). The resting gCl, important for sarcolemma stabilization, is strongly modified either by statin effects or aging process. This parameter is sustained by the CIC-1 chloride channel, which loss-of-function mutations can lead to myotonia congenita, a disease characterized by muscle rigidity due to impaired muscle relaxation after contraction (Imbrici et al., 2015). The reduction of gCl measured in the fast-twitch EDL muscles of statin-treated adult rats is due, on one hand, to a reduction of channel transcription and expression, and on the other hand, to an increased basal PKC-dependent phosphorylation of the expressed channels (Pierno et al., 2009). Similar effects are induced by aging process (Pierno et al., 1999 and De Luca et al., 1994). Strikingly, aging and statin treatment showed additive effects on CIC-1 mRNA and protein expression, which were further decreased in treated aged rats compared to untreated aged rats. This effect is unlikely to be associated to age-related alteration of drug pharmacokinetics, because no significant modification in plasma atorvastatin content was found between aged and adult treated rats. Electrophysiological experiments with chelerythrine suggest that the channel is basically more phosphorylated in treated aged rats, likely due to an increased activity of PKC θ isoform (Camerino et al., 2014). Consequently, a huge 48 % reduction of the gCl was observed in muscle fibers of treated aged rats.

Such a reduction may be compared to the condition of dominantly-inherited myotonia congenita. Yet, we found no evidence of myotonic action potential runs in muscle fibers, likely due to the concomitant functional alteration of other ion channels (Pierno et al., 1998). However, the reduced gCl likely contributes to the reduction of the threshold current to elicit an action potential (I_{th}), which may likely predispose sarcolemma to electrical instability under stress condition, such as prolonged or intense exercise. Interestingly, the PKC θ protein expression was significantly increased by statins in adult and slightly in aged animals. Since the ClC-1 channel is a target of PKC θ (Camerino et al., 2014), its increased expression likely contributes to the effects of statins on the gCl. Beside the direct modulation of ClC-1 channels through phosphorylation, it is possible that the increased expression of PKC θ may be involved in the reduced expression of ClC-1. Indeed, it has been shown that PKC θ co-operates with CN in the regulation of the activity of MEF2, a modulator of phenotypic gene expression in muscles (Marrocco et al., 2014 and Al-Khalili et al., 2004). Our previous studies showed a modification of gCl together with phenotype-specific genes, CN and MEF2, in PKC θ -null mouse muscles, supporting the role of PKC θ in their regulation as well as in the tuning of muscle phenotype (Camerino et al., 2014). Accordingly, we found a significant increase of MEF2 expression, together with a moderate increase of CN, either in adult or aged treated rats with respect to the adult value. Thus, an increase of PKC θ activity may reduce the gCl through phosphorylation of ClC-1 channels, leading to sarcolemma instability, MEF2 activation and further reduction of gCl due to the reduction of ClC-1 expression. Accordingly, an increase of MEF2 transcriptional activity was also found in myotonic muscles (Wu and Olson, 2002). However, the same amount of MEF2 increase was found either in adult or in aged excluding an additional effect during aging. Since statin treatment induced an increase of restCa in fast-twitch muscles of adult rats (Liantonio et al., 2007 and Sirvent et al., 2012), we examined if the restCa increase is amplified in statin treated aged rats. Although a marked increase of restCa occurs in the untreated aged animals, no further modification of restCa was found in statin-treated aged rats.

Accordingly, the Mechanical Threshold for contraction, an index of excitation-contraction coupling that is dependent on restCa, and on the complete contractile apparatus was shifted toward more negative potentials due to aging process but was not further modified by statin treatment. Thus, the restCa increase may not be directly involved in the higher sensitivity of aged muscle to statins. According to previous studies (Camerino et al., 2011), atorvastatin treatment also modifies the glycolytic and oxidative metabolism in adult rats. Here, we found that these metabolic pathways are more affected by statin in aged rats. The down-regulation of mRNA level of beta-enolase, a glycolytic enzyme responsible for the formation of phosphoenol-pyruvate (PEP), is a clear sign of the glycolytic sufferance particularly evident in the aged treated rats, in which muscle tissue is already compromised by aging process. The down-regulation of the glycolytic enzymes is a symptom of energy production failure, and contribute to muscle damage. Accordingly, hereditary muscle glycogenosis in humans are characterized by defective glycolytic enzymes, including beta-enolase, and leads to different degree of myopathy, ranging from cramps to myoglobinuria (Musumeci et al., 2012 and Comi et al., 2001). Interestingly, an increase of Pyruvate kinase M2 (PKM2) isoform was found either in adult or aged rats treated with atorvastatin. The PKM2 is produced in rapidly dividing cells and in cancer cells (Cairns et al., 2011), its increase is responsible for the accumulation of the upstream glycolytic metabolites with a shift of glucose metabolism toward alternative pathways (pentose phosphate pathway) responsible for sustained macromolecule biosynthesis and required for increased proliferation. It should be noted that PKM2 was significantly decreased in aged muscles suggesting that atorvastatin may have an additional regenerative potential likely through a reactivation of a number of satellite cells. Accordingly, atorvastatin has been shown to improve the post-infarct microenvironment by inhibiting the RhoA/ROCK pathway, thus facilitating the survival and therapeutic efficacy of implanted stem cells (Zhang et al., 2014). In fact, we found a down-regulation of RhoA in atorvastatin-treated aged rats, supporting a possible beneficial role of atorvastatin on muscle regeneration that needs further

investigation. A modification of mitochondrial function was observed only in aged animals treated with atorvastatin. Indeed, the significant decrease of mt-ND5 mRNA observed in these animals is an index of an impairment of energy production system and mitochondrial respiratory capacity. The mitochondrial gene mt-ND5 encode for the NADH dehydrogenase subunit 5 and is required to catalyze electron transfer to ubiquinone. Such a perturbation in energy metabolism and ATP synthesis may have a profound impact on the contractile function. Accordingly, in both adult and aged treated rats, we observed a slight increase of AMP-activated protein kinase (AMPK) mRNA level, a crucial sensor of energy status, which is known to be activated during metabolic stress, hypoxia and ATP consumption (Sun et al., 2006). The activation of AMPK may increase myofibrillar protein degradation through the expression of atrogen-1 and MuRF1 (Nakashima et al., 2007). A CK increase in blood also supports statin-induced muscle damage, particularly evident in aged treated animals. As already demonstrated (Serrano et al., 2011 and Ryall et al., 2008), the histochemical analysis showed numerous areas of damage, such as infiltrates or fibrotic tissue deposition, in aged muscles. However, a characteristic vacuolation was observed, although with low incidence, in both statin treated adult and aged animals. Although further studies are needed to better characterize these inclusions, similar findings were observed in *Drosophila* and in human muscle during sarcopenia (Demontis and Perrimon, 2010 and Piccirillo et al., 2014). These inclusions may represent the accumulation of protein aggregates due to ubiquitin proteasome induced muscle degradation, as also reported as an effect of glucocorticoids therapy and suggested to be an effect of statin treatment (Hanai et al., 2007). Statin myotoxicity seems to affect type II, glycolytic myofibers more than type I, oxidative fibers (Westwood et al., 2005 and Schaefer et al., 2004). Our results are in line with these findings, indeed the reduction of gCl found in Sol muscle of aged rats treated with atorvastatin was less marked than in EDL likely because of the lower expression of CIC-1 with respect to the fast one (Pierno et al., 2007 and Desaphy et al., 2005). Also

muscle metabolism seems to be less affected. Sol muscle is an oxidative muscle and the high number of mitochondria may compensate the energy request.

5. Conclusions

In conclusion, our study provides novel information regarding the causes of the increased risk of myopathy that elderly patients experience with statin therapy. The ClC-1 channel appears a susceptible parameter, the function of which may be worsened in aged treated animals. Beside, oxidative and glycolytic metabolism alteration as well as sarcopenia makes the muscle fibers of the treated aged animals more sensitive to stress conditions and to myopathy. A scheme of the proposed mechanism is represented in Fig. 5. All these findings suggests caution with statin therapy in aged individuals. The identification of these biological markers of skeletal muscle damage may pave the way for new therapies. For instance inhibitors of PKC, allowing the recovery of gCl, could diminish the statin-induced muscle damage. Also the restoration of the energetic pathways, i.e. through Coenzyme Q10 supplementation or AMPK-targeted pharmacological strategies, may be of importance.

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Conflicts of interest

None.

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Table 1. Creatine kinase (CK) activity measured in adult and aged atorvastatin treated rats

Groups	n rats	CK level (U/l)
Adult	5	522 ± 150
Adult treated	5	2336 ± 576
Aged	8	2616 ± 371*
Aged treated	5	2938 ± 715*

From left to right was reported the group of animals examined, the number of plasma samples from n rats, CK level (U/l). Significant differences were found by using one-way ANOVA test followed by Bonferroni t-test (F=4.6, df=3/19; P<0.02) (*vs. Adult, P<0.05 or less)

Figure Legends

Fig. 1. Effects of atorvastatin treatment on resting chloride conductance (gCl), contractile parameters and calcium homeostasis in EDL muscle of adult and aged rats. (A) Resting gCl measured in extensor digitorum longus (EDL) muscle fibers from 6 adult and 6 aged rats treated with atorvastatin (ATOR), and from 4 adult and 8 aged untreated rats. White and black bars represent the mean \pm SEM of gCl, measured in untreated and treated muscles, respectively, in the absence of chelerythrine (from 26–57 muscle fibers from the number of rats, as indicated). Statistical analysis by ANOVA showed significant differences in gCl ($F = 36$, $dF = 3/166$, $P < 0.001$). Bonferroni's t-test showed significant differences *vs. Adult untreated ($P < 0.001$) and #vs. Aged untreated ($P < 0.001$). The effect of *in vitro* application of chelerythrine on gCl of untreated and atorvastatin-treated adult and aged rats is showed in grey and hashed. Each bar represents the mean \pm SEM of gCl measured in muscle fibres of untreated and treated adult and aged rats in the presence of chelerythrine (1–2 μM) from 8–12 fibers of the indicated number of rats. °Significantly different vs. its own control by Student's t-test (at least $P < 0.05$). (B) The mechanical threshold (MT) for contraction measured in the EDL muscle fibers of atorvastatin-treated adult ($n = 3$) and aged ($n = 5$) rats with respect to untreated (CTRL) adult ($n = 3$) and (ctrl) aged ($n = 5$) rats. The threshold voltage/duration relationship was obtained by plotting the values of threshold potential (in millivolts) needed to obtain myofiber contraction as a function of the voltage pulse duration (in milliseconds). Each point is expressed as the mean value \pm SE of 13–25 fibers. The curves fitting the experimental points have been obtained using the equation described in the “Methods” section. From the fit of experimental points the rheobase voltage was calculated and reported in the “Result” section. (C) Resting cytosolic calcium concentration (restCa) measured in EDL muscle fibers of atorvastatin-treated adult ($n = 3$) and aged ($n = 3$) rats with respect to untreated (CTRL) adult ($n = 3$) and (CTRL) aged ($n = 3$) rats. RestCa was measured in skeletal muscle by using fura-2 fluorescence method. Each bar represents the mean value \pm SEM of 30, 40,

29 and 33 fibers, respectively, from the indicated number of animals. Statistical analysis by ANOVA showed significant differences in restCa ($F = 6.9$, $dF = 3/128$, $P < 0.001$). Bonferroni's t-test showed significant differences *vs. untreated adult (at least $P < 0.01$).

Fig. 2. Real-time PCR analysis of gene expression in EDL muscle of adult and aged rats treated with atorvastatin. Effects of aging and atorvastatin administration on gene expression level in EDL muscles of rats. Histograms show quantification of transcript levels performed with real-time PCR, for gene related with CIC-1 regulation, calcium signalling, atrophy, autophagy and muscle metabolism normalized by the β -actin gene. The bars indicate the fold change in gene expression, in relation to aging, between Aged and Adult rats (*Aged Vs. Adult*), in relation to atorvastatin administration in adult between Adult Ator and Adult untreated rats (*Adult Ator Vs. Adult*) or atorvastatin administration in aged, between Aged Ator and Aged untreated rats (*Aged Ator Vs. Aged*). Also the results obtained in aged ATOR vs adult were compared (*Aged Ator Vs. Adult*). Each bar was measured from the mean \pm S.E.M. of 6 Adult, 5 Adult Ator, 5 Aged and 5 Aged Ator animals. *Statistical analysis was performed for each groups using one-way ANOVA and multiple comparisons by Fisher (at least $P < 0.05$).

Fig. 3. Protein expression in EDL muscle of Adult and Aged rats treated with atorvastatin. (A) Representative Western blot showing the expression level of CIC-1, PKC α and PKC θ protein in muscle tissues. The blots were reacted with specific antibodies. β -actin was used to normalize the blot. (B) The densitometric analysis of each experimental band was performed using ImageLab software. Histograms show quantification of relative protein levels. Relative intensity was calculated by normalization of the absolute intensity of target protein with the absolute intensity of β -actin, as reference standard, and are represented as arbitrary units (AU). Each bar represents the

mean \pm SEM of 6 adult untreated, 5 adult treated, 5 aged untreated, 5 aged treated rat muscles.

Statistical analysis was performed using ANOVA followed by Fisher's t-test (F= 6.01, dF 3/8,

P<0.02 for CIC-1; F = 1.35, dF=3/9, NS for PKC α ; F= 8.26, dF= 3/7, P<0.02 for PKC θ)

*Significantly different with respect to CTRL adult (at least P<0.05).

Fig. 4. Histological analysis in TA muscle of Adult and Aged rats treated with atorvastatin. (A) Samples of haematoxylin-eosin staining showing the morphological profile of Tibialis Anterior (TA) muscle from adult and aged rats treated with atorvastatin (adult ATOR and aged ATOR), and from adult and aged untreated (adult CTRL and aged CTRL) rats. The sections from aged muscles clearly show an increase of the non-muscular area (such as infiltrates, fibrotic or adipose tissue). The sections of atorvastatin treated muscles show the vacuoles with inclusions. The images are at 10 \times magnification (B) Histograms show the total percent area of damage calculated as the sum of the % total nonmuscle area and (C) the percent of centronucleated fibers (CNF) compared to normal fibers (NF) in the different experimental conditions. Each bar is the mean \pm SE for 9–15 fields from 3 muscles per group.

Fig. 5. Scheme depicting the proposed mechanism of statin-induced myopathy. Statin increase PKC activity and reduce the gCl through phosphorylation of CIC-1 channels, leading to sarcolemma instability, MEF2 activation and further reduction of gCl due to the reduction of CIC-1 expression. Old individuals are more sensitive to statin effect because similar modifications (increase of PKC activity, CIC-1 reduced expression) were typically found in aged muscle. A marked alteration of the expression of glycolytic and mitochondrial enzymes together with muscle atrophy and CK increase demonstrates a concomitant impairment of muscle function and increased risk of myopathy during aging.

ACCEPTED MANUSCRIPT

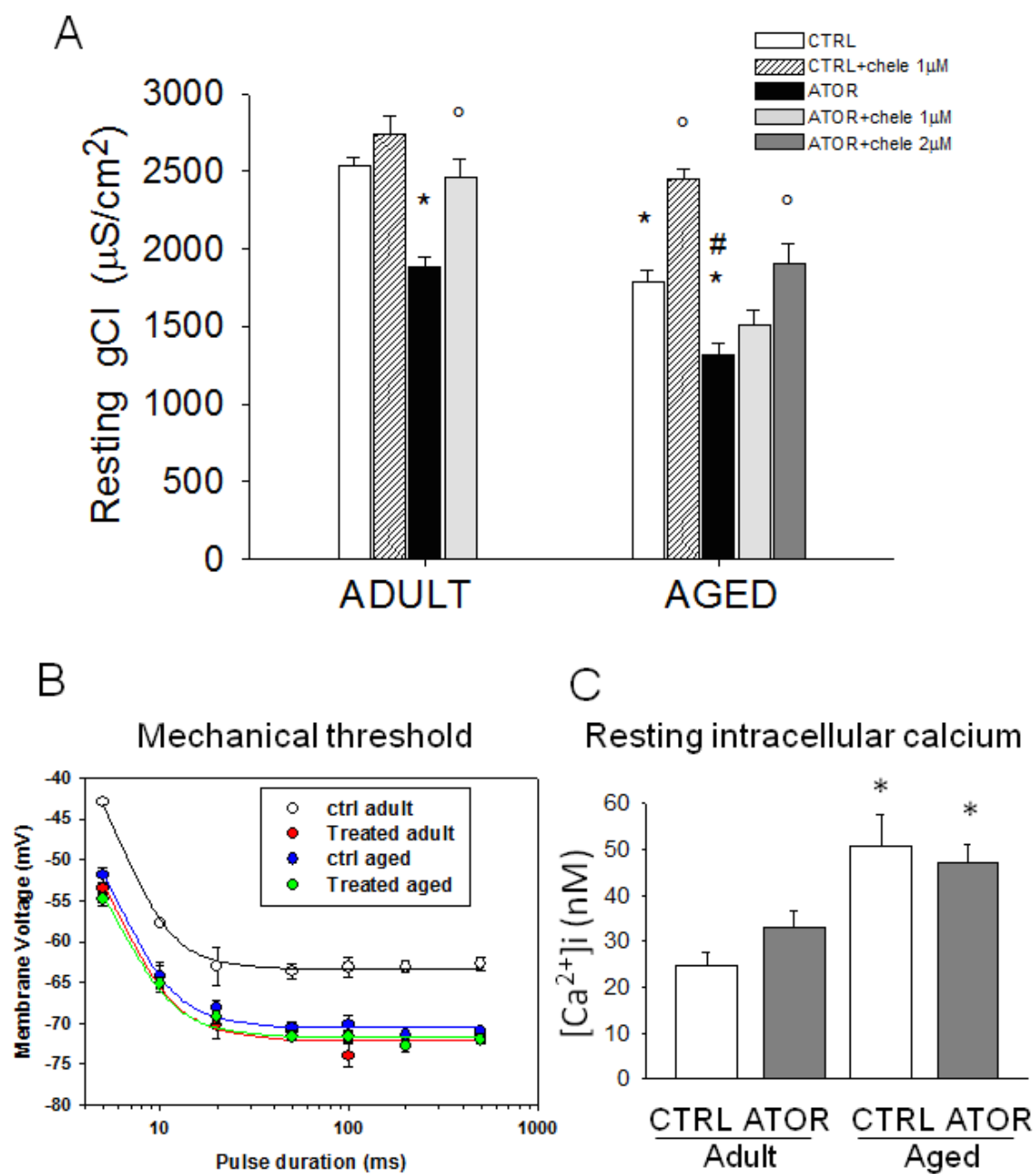


Figure 1

EDL

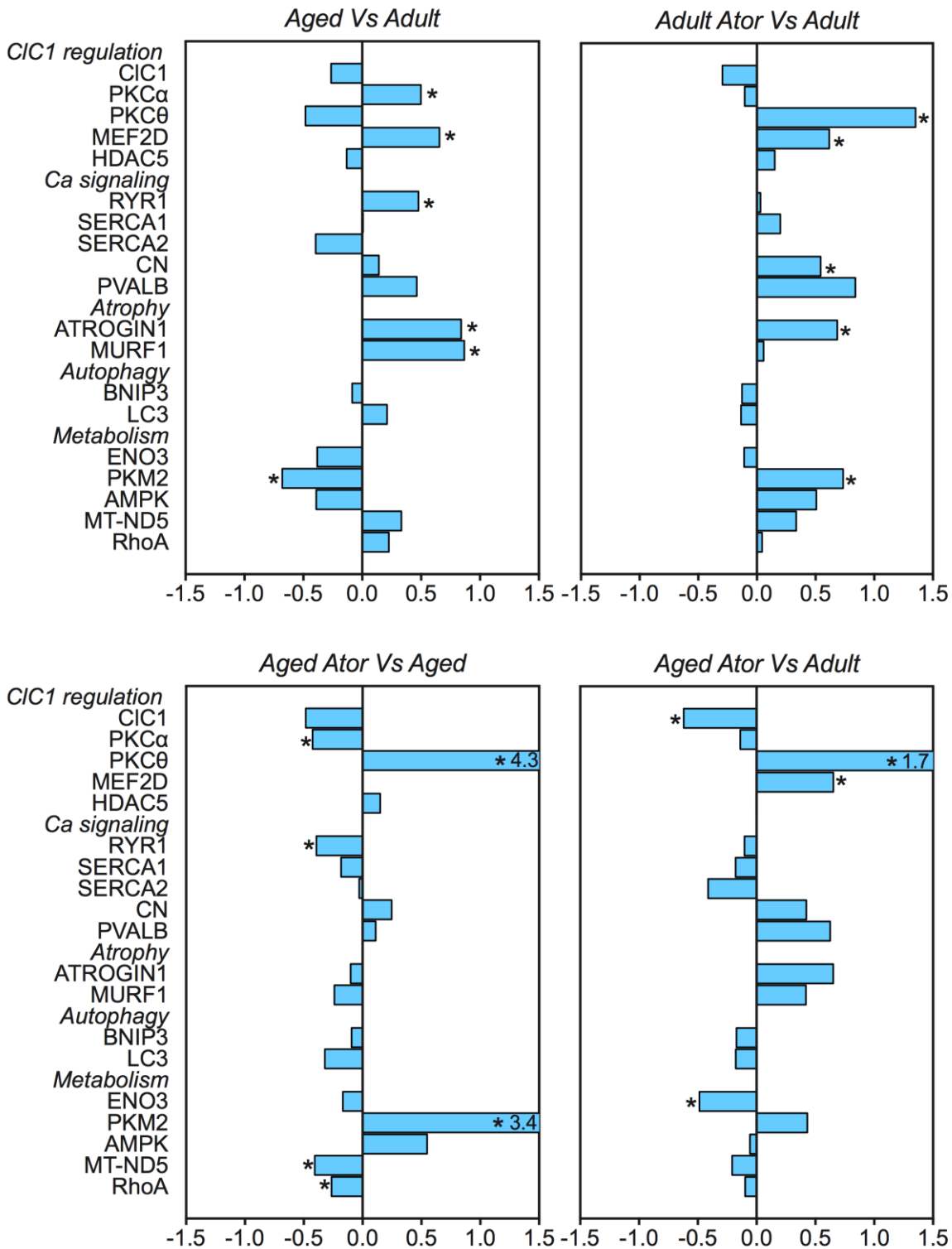
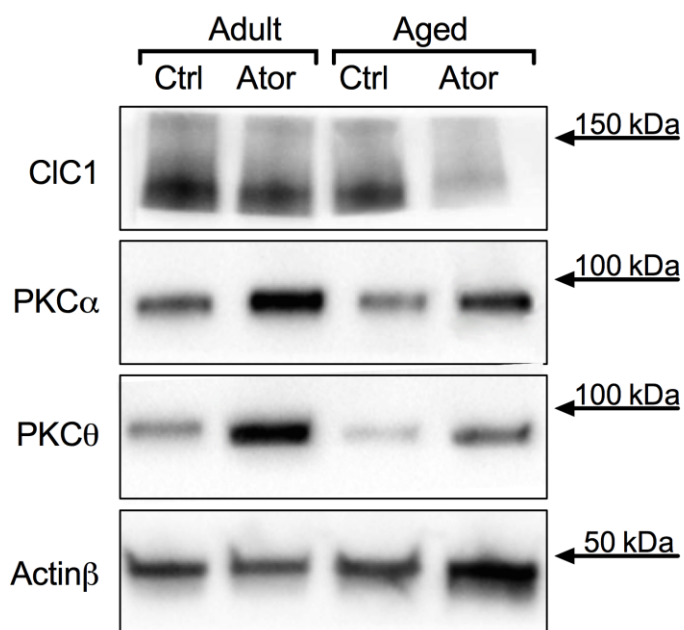


Figure 2

A



B

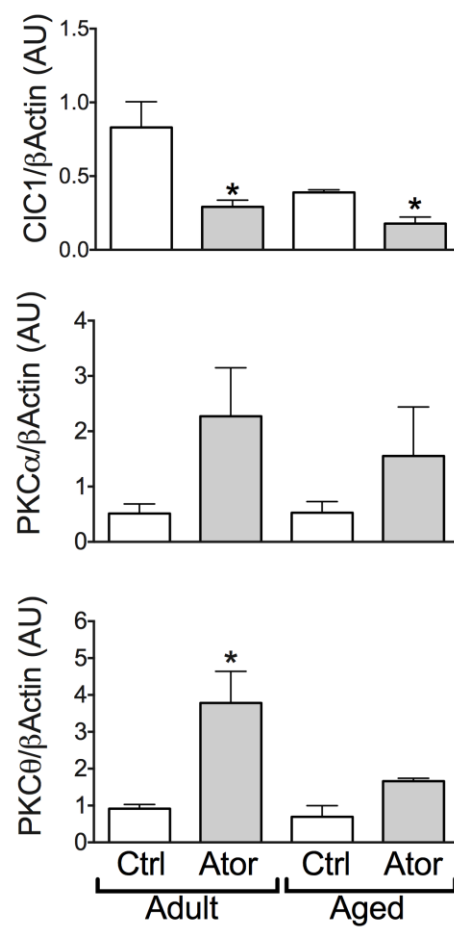


Figure 3

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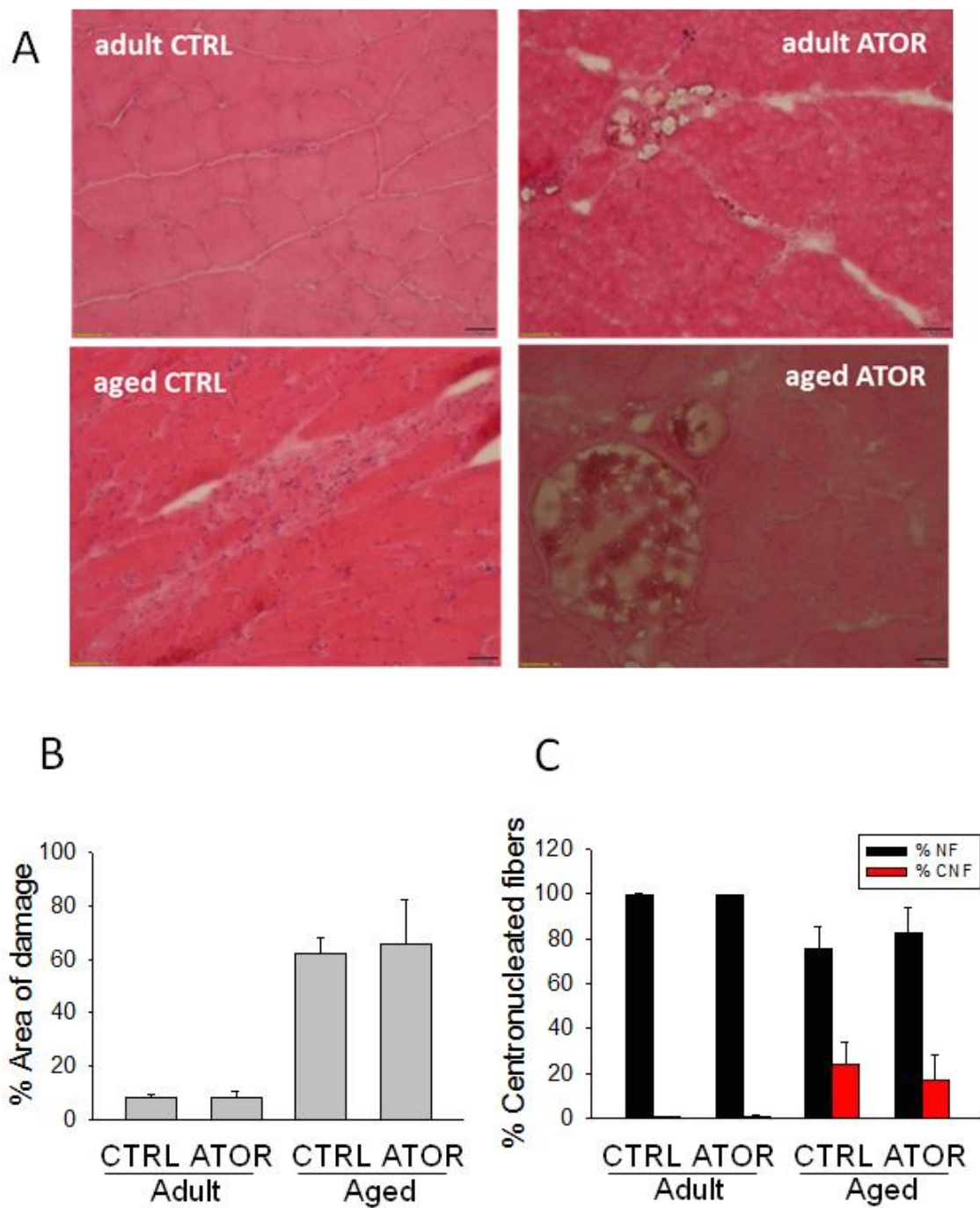


Figure 4

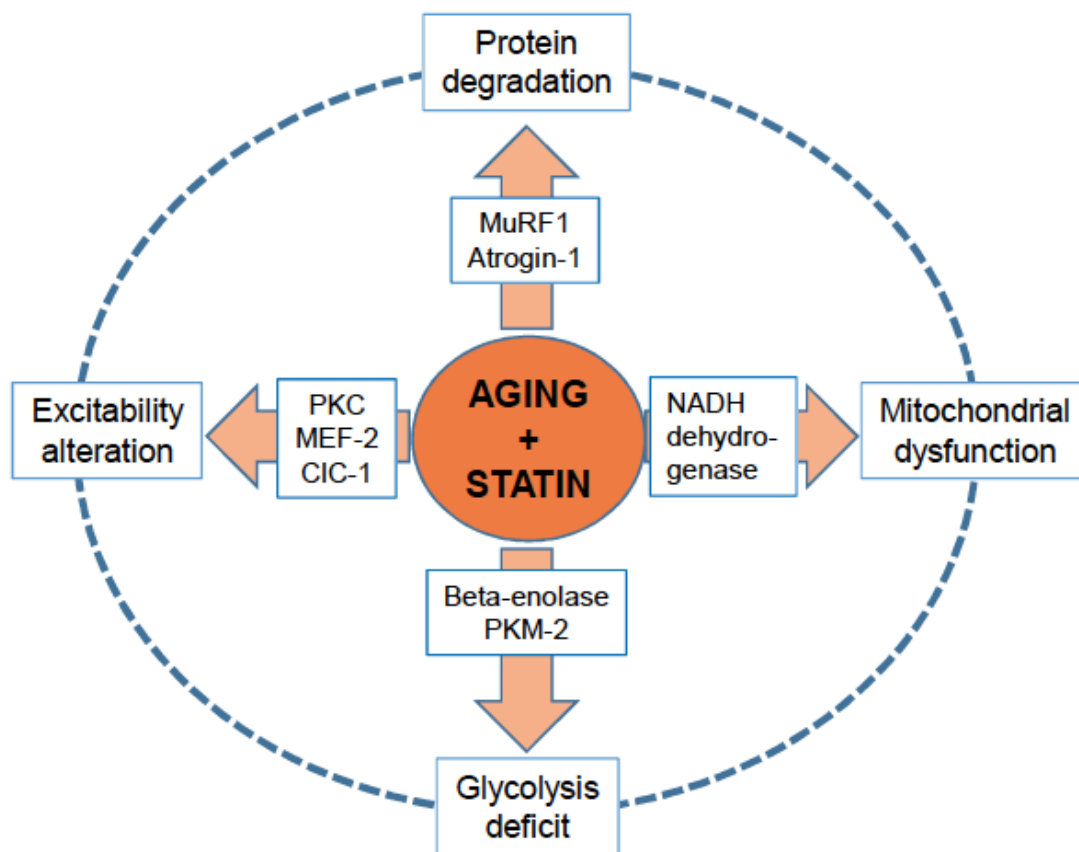


Figure 5

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Highlights

- This work characterizes the causes of atorvastatin related myotoxicity in aged rats
- Skeletal muscle chloride channel ClC-1 is a target of statin-induced side effects
- ClC-1 dysfunction is worsened by aging process
- Age-related changes of energy production contribute to statin-induced myotoxicity
- These findings suggest caution with statin therapy during aging

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