Statin Therapy and the Expression of Genes that Regulate Calcium Homeostasis and Membrane Repair in Skeletal Muscle

Annette Draeger,* Verónica Sanchez-Freire,* Katia Monastyrskaya,* Hans Hoppeler,* Matthias Mueller,* Fabio Breil,* Markus G. Mohaupt,† and Eduard B. Babiychuk*

From the Institute of Anatomy,* and the Department of Nephrology/Hypertension,† Inselspital, University of Bern, Bern, Switzerland

In skeletal muscle of patients with clinically diagnosed statin-associated myopathy, discrete signs of structural damage predominantly localize to the T-tubular region and are suggestive of a calcium leak. The impact of statins on skeletal muscle of non-myopathic patients is not known. We analyzed the expression of selected genes implicated in the molecular regulation of calcium and membrane repair, in lipid homeostasis, myocyte remodeling and mitochondrial function. Microscopic and gene expression analyses were performed using validated TaqMan custom arrays on skeletal muscle biopsies of 72 age-matched subjects who were receiving statin therapy (n = 38), who had discontinued therapy due to statin-associated myopathy (n = 14), and who had never undergone statin treatment (n = 20). In skeletal muscle, obtained from statin-treated, non-myopathic patients, statins caused extensive changes in the expression of genes of the calcium regulatory and the membrane repair machinery, whereas the expression of genes responsible for mitochondrial function or myocyte remodeling was unaffected. Discontinuation of treatment due to myopathic symptoms led to a normalization of gene expression levels, the genes encoding the ryanodine receptor 3, calpain 3, and dystrophin being the most notable exceptions. Hence, even in clinically asymptomatic (non-myopathic) patients, statin therapy leads to an upregulation in the expression of genes that are concerned with skeletal muscle regulation and membrane repair.

Statins are generally regarded as the treatment of choice for hypercholesterolaemia. Consequently, it is not surprising that the annual prescription of these drugs has exceeded the 100-million-mark during the past two decades.1,2 Notwithstanding their excellent safety profile, adverse effects on skeletal muscle are by no means infrequent. Since statin-associated myopathy is not consistently coupled with an elevation in the serum levels of creatine kinase, the muscular disturbances often remain undiagnosed.3–5 Although minor in occurrence, their manifestation has a negative bearing on physical activity and patient compliance with this life-saving therapy.6 Recent data demonstrate a genetic susceptibility for statin associated myopathy,7 which can be linked to a single nucleotide polymorphism of the SCLO1B1 gene. Carriers of the SCLO1B1*5 allele are at a twofold relative risk of developing statin-associated myopathy.8 The effects of statins on gene expression in statin-naïve skeletal muscle have previously been investigated in cell culture9–11 and the effects of statin treatment in combination with eccentric exercise studied on skeletal muscle of eight healthy volunteers.12

Observational studies have revealed ~10% of patients to develop statin-associated myopathy.4 But it is not yet known what distinguishes the muscle of these individuals from that of the ~90% who remain asymptomatic. In a previous study of ours, we demonstrated vacuolization of the T-tubular system to be a recurrent feature and to be more prevalent among patients who had been

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Address reprint requests to Prof. Annette Draeger, Institute of Anatomy, University of Bern, Baltzerstr. 2, 3012 Bern, Switzerland. E-mail: draeger@ana.unibe.ch.
clinically diagnosed as having statin-associated myopathy. Patients were identified as having statin-associated myopathy by clinical criteria, consistent with the recommendations of the Muscle Safety Expert Panel. These findings prompted us to investigate the impact of statins on the expression of selected genes involved in the molecular regulation of calcium and membrane repair.

Other gene families, which are potentially affected by cholesterol-lowering treatment are those involved in the regulation of lipid homeostasis and/or mitochondrial function. In addition, the expression of genes involved in myocyte remodeling was assessed to determine the extent of cellular reorganization.

Materials and Methods

Patients

This study was conducted with the approval of the Ethics Committee of the Canton of Bern, Switzerland. All subjects gave informed consent. Samples of the vastus lateralis muscle were collected from a total of 72 individuals. They belonged to three different groups: Group 1: 20 subjects who had never undergone statin therapy, (mean age 64 ± 13 years; 20% female). 8 of these subjects were partially analyzed in our previous study and 12 subjects took part in a longitudinal study on the effects of eccentric training in the elderly. Their baseline biopsy was used in the present study. Group 2: 14 patients (mean age 60 ± 11 years, 50% female) who had a history of clinically diagnosed statin-associated myopathy, and who had voluntarily discontinued their statin treatment for a minimum of 3 weeks (median 12 weeks, range 3 to 300 weeks) before the time of their biopsy. Eleven biopsies in this group were partially analyzed in our previous study and 3 biopsies were new. Group 3: 38 statin-treated patients (mean age 61 ± 11 years, 37% female), 14 of whom had a clinically diagnosed history of statin-associated myopathy (2 new) and 19 patients who received statin therapy without muscle complaints (6 new).

Subjects were identified as having statin-associated myopathy by clinical criteria, consistent with the recommendations of the Muscle Safety Expert Panel. Patients suffering from statin-associated myopathy were referred to the Department of Nephrology and Hypertension for the evaluation of alternative treatment options. Asymptomatic statin-treated subjects were recruited via newspaper advertisements. The vastus lateralis muscle was biopsied at mid-thigh level. In brief, the lateral portion of one thigh was anesthetized (1% xylocaine) and a small incision made through the skin and underlying fascia and ~50 to 100 mg of tissue biopsied using a 14 gauge needle. The muscle sample was immediately frozen in liquid nitrogen and/or chemically fixed for microscopy.

Since cross-sectional biopsies were used throughout the study, none of the biopsies compares the pre- with the post-treatment condition of any individual patient.

Processing of Tissue

An average of 3 tissue blocks (~3 x 6 mm) per patient were fixed and processed for semithin sectioning as well as ultrastructural analysis in the electron microscope. Whenever sufficient material was available, an additional 1 to 2 blocks were processed for ultracytometric and immunohistochemistry. The polyclonal antibodies against annexin A6 and the dihydropyridine receptor were obtained from Invitrogen (Basel, Switzerland), a polyclonal antibody against dysferlin was from Imgenex (Luceme, Switzerland). Secondary antibodies were purchased from Jackson Laboratories (Milan, La Roche, Switzerland) (Cy3-coupled) and Molecular Probes (Invitrogen; Alexa-coupled). Nuclear labeling was performed using the Hoechst 33342 (Sigma, Buchs, Switzerland) stain.

Analysis of Tissue

The specimens were evaluated by an observer who was skilled in light and electron microscopy but blinded as to the patient groups. On transverse semithin sections, between 200 and 400 individual fibers were analyzed per biopsy. The number of muscle fibers displaying structural abnormalities was expressed as a percentage of the total number of fibers per section in the light microscope. The threshold for significant muscle injury was arbitrarily determined to be ≥2% damaged fibers/biopsy.

RNA Isolation and Gene Expression Studies

Total RNA was isolated from the muscle biopsies using RNeasy Fibrous Tissue Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer’s instructions treating the samples with DNase I (Qiagen) to avoid any genomic DNA contamination. Total RNA concentration was measured using spectrophotometry at 260 nm (DU 530, Beckman Coulter, Nyon, Switzerland), the purity was determined with the 260/280 ratio range and controlled by agarose gel electrophoresis. cDNA was synthesized using random hexamer primers and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland).

Expression studies were performed using validated TaqMan custom Arrays in a 384-well micro fluidic card format allowing simultaneous analysis of 47 human genes and the reference gene 18S rRNA in two replicates. Eight cDNA samples using 100 ng of starting total RNA were analyzed using each card. Forty-one genes were selected for analysis (Table 1).

Reactions were run on the Applied Biosystems 7900HT Fast Real-Time PCR System, at the default setting program (50°C 2 minutes, 94.5°C 10 minutes; 97°C, 30s; 59.7°C, 60s; 40 cycles). Data acquisition was performed according to the manufacturer’s suggestions. Expression values for target genes were normalized to the concentration of 18S rRNA. Gene expression values were calcu-
lated based on the comparative threshold cycle (Ct) method. In short, the Ct data for the selected genes and 18S rRNA in each sample were used to create Ct values ($Ct_{gene}$/$Ct_{18S rRNA}$). Thereafter, $\Delta\Delta Ct$ values were calculated by subtracting the $Ct$ of the calibrator from the $Ct$ value of each target.

### Statistics

Numerical data are expressed as mean values together with the SEM For mRNA expression analysis, the Normality was studied with the Kolmogorov-Smirnov and Shapiro-Wilk tests ($\alpha$ set to 0.05), with the Lilliefors Significance Correction. Since the genes do not display a normal distribution, the Mann Whitney U-test was performed. The statistical analyses were performed using SPSS version 15 for Windows (Systat Software Inc, San Jose, CA). The level of significance was set at $P < 0.05$. Statistical values were displayed using the GraphPad Prism software version 5.02.

A covariate regression model for the effect of gender and the statin- and myopathy-related condition, respectively, was applied.

### Results

The aim of the present study was to assess the impact of statin therapy on skeletal muscle obtained from non-

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Table 1. Genes Selected for Molecular Analysis

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<th>Genes</th>
<th>Unique identifiers</th>
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<th>Figure 4</th>
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myopathic statin-treated patients. Consequently, the skeletal muscle samples of all individuals (n = 72) were screened in the light microscope for signs of structural injury, arbitrarily defined as significant if more than ≥2% fibers per biopsy were damaged. The findings of this analysis correlated well with the clinical data (Figure 1A). If the size of the biopsy was sufficiently large, then a portion of the material was cryosectioned for a high-resolution immunohistochemical analysis of markers of the T-tubular system21,22 (Figure 1, B–E). Skeletal muscle from 14 statin-treated patients suffering from myopathy (Figure 1A, dark green), as well as from 10 subjects who had discontinued statin therapy (Figure 1A, dark green), displayed vacuolizations within the T-tubular system (Figure 1, B–E). As described previously,13 these injuries were modest, typically confined to the intracellular space and did not extend to the sarcolemma. Age-matched controls who had never been treated with statins did not suffer from myopathy and showed no muscular damage.

The 41 genes that were selected for molecular analysis (Table 1), encode proteins that are implicated in the regulation of [Ca^{2+}], in T-tubular and sarcolemmal structure and membrane repair, in the regulation of lipid metabolism, in myocyte remodeling and in mitochondrial activity.

Initially, we compared skeletal muscle samples of asymptomatic patients without structural damage (n = 19, Figure 2A, red) with samples from subjects who had never undergone statin therapy (n = 20, Figure 2A pink). Despite the absence of T-tubular vacuolation, many of the genes that are implicated in calcium homeostasis were significantly up-regulated in statin-treated individuals. They include genes for the regulation of Ca^{2+} influx: inositol-3 receptors (ITPR1&2&3), ryanodine receptor 3 (RYR3), the dihydropyridine receptor (CACNA1S), for Ca^{2+} uptake: sarco-endoplasmic ATPases (ATP2A2&3) or for Ca^{2+} efflux: the Na/Ca exchanger ATPase (ATP2B1) (Figure 2B). Not affected were the ryanodine receptor 1 (RYR1), the Na/K-exchanger (ATP1A1) and the sarco-endoplasmic ATPase 1 (ATP2A1). Consistent with these findings, the gene-expression levels of the principal Ca^{2+}-dependent proteases of skeletal muscle, calpains 1 & 3, (CAPN1, 3), were likewise raised, whereas those of calpain 2 (CAPN2) remained unchanged (Figure 2B).

Selective marker proteins, such as dystrophin and caveolin 3 are exclusively expressed at the lateral sarcolemma and distinguish the load-bearing part of the plasma membrane from the current-conducting T-tubular membrane system, to which the annexin protein family is
bound in a Ca^{2+}-dependent manner. The membranes of the T-tubular system are particularly susceptible to cholesterol depletion. Genes encoding members of the annexin protein family (ANXA1-7) and their binding partners (S100A6&1&8&11) as well as dyserfin (DYSF) were up-regulated, whereas the expression levels of cavinin 3 (CAV3) remained unchanged (Figure 2C). Although transmission electron microscopy previously showed the sarcolemma to be intact, the gene encoding dystrophin (DMD) was significantly up-regulated (Figure 2C).

Since in 19 patients the cholesterol synthesis was pharmacologically lowered, genes encoding cholesterol-sensing proteins [sterol regulatory-element-binding-protein cleav-

age-activating protein (SCAP) and hydroxy-methyl-glutaryl-CoA reductase (HMGCR), were, not surprisingly, markedly up-regulated; so too, was the gene for the LDL receptor (LDLR), which mediates the cellular uptake of cholesterol from the serum (Figure 2D). Genes encoding either a transcription factor associated with cholesterol synthesis: sterol regulatory element binding transcription factor 1 (SREBF1), or a protein involved in the esterification of cholesterol (SOAT1) were not up-regulated, neither was the peroxi-

some-proliferator-activated receptor α (PPARA), which en-

codes enzymes that are involved in the intracellular oxida-

tion of fatty acids, including those of the cytochrome p450 pathways (Figure 2D).

Genes encoding proteins that are involved in the re-

modeling of either injured skeletal muscle myocytes, such as an embryonic myosin isoform [myosin heavy-chain 3, (MHH3)] which is synthesized de novo, or extra-

cellular matrix proteins [fibroblast-growth factor receptor 4 (FGFR4)] and collagen type 1 (COL1A1) were not up-regulated; neither was the gene encoding the molec-

ular mediator of muscular atrophy, atrogin 1 (FBXO32).

Likewise unaffected were the gene expression levels of 3 proteins that influence mitochondrial activity: cytochrome c oxidase (MT-CO1), NADH-ubiquinone oxidoreductase (MT-ND1) and ATP synthase 6 (MT-ATP6). The gene encoding α-skeletal muscle actin (ACTA1) was slightly upregulated (P = 0.022; Figure 2E).

In a covariance analysis (Supplemental Table S1 at http://ajp.amjpathol.org) gene expression changes did not appear to be linked to gender. However, some differ-

ences in values for the female gender (Group: No Myop-

athy Never Statin versus No Myopathy Statins) were noted and attributed to the low number of female subjects in that particular group.

The upregulation of genes encoding proteins that are involved in Ca^{2+}-homeostasis, in combination with a di-

lated T-tubular system, might lead to an activation of membrane repair processes, as is implied by the up-

regulation of "membrane repair" genes dyserfin and dys-

trphin. Therefore, ultrathin cryosections of biopsies from untreated subjects (Figure 3, A–F) and from statin-
treated patients (Figure 3, G–L) were analyzed immuno-

tochemically. Immunostaining for dyserfin showed the sarcolemma to be uninterrupted in untreated and statin-
treated patients alike (Figure 3, B, C, H, I). However, in the statin-treated patients, dyserfin was also detected at intracellular sites that did not coincide with the annexin A6-demarcated T-tubular system (Figure 3, H and I). We observed that a redistribution of dyserfin was more ob-

vious in patients whose T-tubular system displayed signs of vacuolization.

In patients who had chosen to discontinue their course of treatment with statins (Figure 4A, n = 14, turquoise), an overall normalization of the gene expression profiles was observed. Notable exceptions relating to Ca^{2+} homeostasis (Figure 4B) were the genes encoding the ryanodine 3 rece-

ptor (RYR3), the SERCA 3 pump (ATP2A3) and calpain 3 (CAPN3), all of which were up-regulated. With the exception of dystrophin (DMD), the expression levels of genes encoding proteins of the sarcolemma and the T-tubular system

Figure 2. Changes in the gene expression (mRNA) levels of key marker proteins within the skeletal muscle biopsies of non-myopathic patients under-

going statin therapy. A: Color-coded display of the two categories of patients whose biopsies were analyzed for the mRNA levels of genes encoding proteins indicated in B–E. These patients included the 20 controls who had never been treated with statins (pink) and the 19 asymptomatic ones who were receiving statin therapy (red). B–E: The mRNA expression levels of 41 genes were deter-

mined using a custom Taqman microarray system. The symbols (color-coded as in a) depict the Fold Differences (reference group: subjects who had never received statin therapy (pink)) for each gene product, normalized to the level of 18S RNA (mean values ± SEM). B: Most of the genes implicated in Ca^{2+}-

homeostasis are up-regulated (ITPR1&2&3, RYR1&3, ATPA2&3, CACNA1A, CAPN3). Several proteases (calpains 1&3, CAPN1&3) are up-regulated. C: Genes encoding structural proteins of the T-tubular system: members of the annexin family (ANXA1&2&4&6, S100A6, S100A10), for dysferlin (DYSF) and for annexin protein family (ANXA1-7) and their binding part-

ners (HMGCR), as well as the LDL receptor (LDLR) are highly significantly up-regulated. D: Cholesterol-sensors SREBP cleavage-activating protein (SCAP) and the HMG CoA reductase (HMGCR), as well as the LDL receptor (LDLR) are highly significantly up-regulated. E: Genes that are often associated with the remodeling of muscle fibers (FGFR4, COL1A1, and FBXO32), or with mitochondrial activity (MT-CO1 and MT-ND1 and MT-ATP6), or actin (ACTA1) are not significantly upregulated. For identification of gene products and P values see Table 1.
membranes (Figure 4C), as well as cholesterol-sensors (Figure 4D), were similar to those in the biopsies of individuals who had never undergone statin therapy. Interestingly, 2 genes (ND1, ATP6) encoding proteins that are involved in mitochondrial activity, were down-regulated in patients who had discontinued statins (Figure 4E).

Discussion

The underlying cause of statin-associated myotoxicity is unknown, although many different mechanisms have been proposed. These include membrane destabilization, a dearth of GTP-binding proteins, mitochondrial dysfunction, tRNA immaturity, interference with the ubiquitine-proteasome pathway, and the up-regulation of a skeletal muscle atrophy gene. The T-tubular system represents a site of structural weakness in the skeletal muscle of patients who are undergoing statin therapy. Consistent with their role in mediating excitation-contraction coupling, the channels of the T-tubular system surround individual myofibrils and link these with the calcium stores of the sarcoplasmic reticulum. The T-tubular system is more vulnerable to cholesterol depletion than is the sarcolemma or the sarcoplasmic reticulum, and its structural integrity is readily compromised.

We have previously demonstrated that T-tubular injury can occur during statin therapy and that it can accom-
Several genes that are implicated in Ca\(^{2+}\) homeostasis and those that are known to play a role in plasma membrane repair. The comparison of statin naïve subjects with patients who discontinued statin therapy, constitutes an attempt to assess a potential restitution in gene expression after a cessation of therapy.

**Statins and Ca\(^{2+}\) Homeostasis**

Since our preliminary histopathological characterization permitted a discrimination between damaged and undamaged skeletal muscle biopsies, we were able to assess the influence of statin therapy on the expression of genes independently of the clinical findings.

Our data reveal ongoing statin therapy to interfere with the expression of genes that are involved in the maintenance of intracellular Ca\(^{2+}\)-homeostasis in skeletal muscle. This finding is consistent with the report of abnormal in vitro contracture tests in muscle from statin-treated patients\(^{28}\) and with the results of an experimental study using statin-treated rodents, which showed the resting cytosolic levels of calcium to be elevated in myocytes, without an accompanying increase in the sarcolemmal permeability to this cation.\(^ {29}\)

In skeletal muscle, an increase in the level of sarcoplasmic calcium interferes with excitation–contraction coupling,\(^ {30}\) which might account for the muscular weakness that is a characteristic trait of statin-associated myopathy. Furthermore, prolonged exposure to high calcium concentrations could activate the skeletal muscle calpains. An activation of calpain in response to treatment with simvastatin has been noted in cultivated human skeletal muscle.\(^ {10}\) For calpain 3 in particular, a Ca\(^{2+}\)-dependent cleavage of the molecule is known to be indispensable for its proteolytic activity.\(^ {31}\)

**Statins and Membrane Repair**

The up-regulation of dysferlin, as well as of members of the annexin family and their S100 binding partners, indicates that Ca\(^{2+}\)-dependent, dysferlin-mediated membrane repair mechanisms are operative.\(^ {32}\) In addition to its customary sarcolemmal location, an intracellular, presumably vesicular, accumulation of dysferlin has been described in the skeletal muscle of limb-girdle dystrophy patients,\(^ {33}\) which accords with our own data. Interestingly, numerous members of the annexin family of genes were up-regulated in the biopsies of asymptomatic patients who were undergoing statin therapy. However, to date, only annexin A1 has been implicated in membrane repair mechanisms.\(^ {34,35}\) It was surprising that not only well-described repair genes such as dysferlin, and genes encoding several annexins, which bind Ca\(^{2+}\)-dependently to the structurally vulnerable T-tubular system,\(^ {21}\) are up-regulated, but also the gene encoding dystrophin. This points to structural damage within the sarcolemma, which was not apparent in our electron micrographs.
Skeletal Muscle After Discontinuation of Statin Therapy

The discontinuation of statin therapy led to an overall normalization of the gene expression profiles. Notable exceptions were the genes for the ryanodine receptor 3, the sarcoplasmic ATPase 3, calpain 3 and dystrophin. In our previous study, the expression levels of the ryanodine receptor 3 and the sarcoplasmic ATPase 3 genes were elevated in structurally damaged skeletal muscle.13 Since all of the individuals in this group of patients had discontinued the course of treatment owing to muscular problems, we cannot exclude the possibility that several or all of these genes were up-regulated already before statin therapy, rather than being so in consequence of the treatment and remaining thus after its cessation.

An elevation in the gene expression levels of the ryanodine receptor 3 and/or the sarcoplasmic ATPase 3 in skeletal muscle might denote a group of individuals who are potentially susceptible to myotoxicity with a tendency toward already elevated sarcoplasmic Ca2⁺-levels. Conceivably, the raised gene expression levels of calpain 3 coincide with a continued high level of enzyme activity in the wake of a persistent calcium leak.

Statins and Mitochondrial Gene Expression

Several studies have reported mitochondrial impairment and postulated that mitochondrial dysfunction may underlie statin-associated myotoxicity (rev. by26,36). Since in our study, several genes encoding proteins implicated in mitochondrial activity were not affected by statin therapy, we are unable to confirm this observation. However, the number of mitochondrial genes we investigated was small; besides, previous findings relating to molecular regulation of mitochondrial activity in cultured cells37,38 or animal models39 may not be readily translatable to human skeletal muscle in vivo.

In our electron micrographs the skeletal muscle mitochondria did not show signs of structural alteration, however, we found 2 genes implicated in mitochondrial activity to be down-regulated in the muscle of patients who had discontinued treatment, which might indicate a pathological reaction.

A recent report showed the upregulation of a gene implicated in skeletal muscle atrophy (FBXO32) in patients with statin-associated myopathy.25 However, our human patient cohort was perhaps too small to yield statistically reliable data, since we were unable confirm this result.

An in vitro study addressing the effects of statin treatment on the expression of a broad spectrum of genes, identified 4 genes, all related to cholesterol metabolism to be up-regulated, including HMGCoA reductase, which accords with our own results.9 A study by Laaksonen et al40 used whole genome expression profiling of skeletal muscle of 12 statin-treated individuals and 6 placebo controls before and after an 8-week treatment. Their study concluded that high-dose simvastatin correlated with the muscle expression of arachidonate 5-lipoxygenase-activating protein. However, this study did not include material from myopathic patients.

Limitations

An important caveat in the interpretation of our results needs to be mentioned. Due to the limited quantities of available biopsy material, we were unable to ascertain whether the observed changes in gene expression corresponded with changes in protein synthesis. Although the changes in gene expression were highly significant, their magnitude was modest (one- to threefold).

Notwithstanding these reservations, our data point to a fundamental deficiency in the regulation of Ca²⁺-homeostasis within the skeletal muscle of patients who are undergoing statin therapy. This circumstance should be considered in the development of a new generation of statins whose administration would not be associated with myotoxicity.

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