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Cardiometabolic Risk

Simvastatin Effects on Skeletal Muscle

Relation to Decreased Mitochondrial Function and Glucose Intolerance

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Objectives Glucose tolerance and skeletal muscle coenzyme Q_{10} (Q_{10}) content, mitochondrial density, and mitochondrial oxidative phosphorylation (OXPHOS) capacity were measured in simvastatin-treated patients (n = 10) and in well-matched control subjects (n = 9).

Background A prevalent side effect of statin therapy is muscle pain, and yet the basic mechanism behind it remains unknown. We hypothesize that a statin-induced reduction in muscle Q_{10} may attenuate mitochondrial OXPHOS capacity, which may be an underlying mechanism.

Methods Plasma glucose and insulin concentrations were measured during an oral glucose tolerance test. Mitochondrial OXPHOS capacity was measured in permeabilized muscle fibers by high-resolution respirometry in a cross-sectional design. Mitochondrial content (estimated by citrate synthase [CS] activity, cardiolipin content, and voltage-dependent anion channel [VDAC] content) as well as Q_{10} content was determined.

Results Simvastatin-treated patients had an impaired glucose tolerance and displayed a decreased insulin sensitivity index. Regarding mitochondrial studies, Q_{10} content was reduced ($p = 0.05$), whereas mitochondrial content was similar between the groups. OXPHOS capacity was comparable between groups when complex I– and complex II–linked substrates were used alone, but when complex I + II–linked substrates were used (eliciting convergent electron input into the Q intersection [maximal ex vivo OXPHOS capacity]), a decreased ($p < 0.01$) capacity was observed in the patients compared with the control subjects.

Conclusions These simvastatin-treated patients were glucose intolerant. A decreased Q_{10} content was accompanied by a decreased maximal OXPHOS capacity in the simvastatin-treated patients. It is plausible that this finding partly explains the muscle pain and exercise intolerance that many patients experience with their statin treatment. (J Am Coll Cardiol 2013;61:44–53) © 2013 by the American College of Cardiology Foundation

Statins (3-hydroxy-3-methyl-glutaryl coenzyme A reductase [HMG-Co A reductase] inhibitors) are widely used in the treatment of hypercholesterolemia (1). Hypercholesterolemia is a major risk factor for development of stroke and coronary heart disease (1,2). Statins are reported to have beneficial effects (reduces the production of reactive oxygen species [ROS] and increases antioxidant capacity) on heart muscle, whereas the opposite is observed in skeletal muscle from human subjects (3). Adverse effects of statin treatment on skeletal muscle (from myositis to rhabdomyolysis) have been reported, but the mechanism behind these myopathic changes is not fully understood (4). Muscle pain is a less severe side effect of statin treatment, but it is still quite discomforting for the patients, and the prevalence ranges from 10% in patients who are sedentary to 75% in athletes (5). The incidence of myopathy reported in randomized controlled trials is much lower than 10% (6); a part of the reason for this discrepancy is the definition of myopathy. In many randomized controlled trials, the definition of myopathy is an increased creatine kinase (CK) concentration of more than 10 times the normal range (7). It has been reported that myopathy (biopsy confirmed) can occur without elevation in CK levels (8). In observational studies, it has been reported that around 10% of patients receiving statins develop myopathy (9,10). These muscle symptoms are clinically of major importance for several reasons, not

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least because the symptoms considerably reduce treatment compliance of the patients. Statins act via the mevalonate pathway to reduce the formation of cholesterol, but impingement of this pathway may also affect the formation of the mitochondrial cofactor, coenzyme Q_{10} (Q_{10}) (11). In hypercholesterolemic patients treated with simvastatin, the content of Q_{10} in skeletal muscle was reduced, and this was paralleled by a reduction in mitochondrial content (estimated by citrate synthase [CS] activity and mitochondrial DNA content), but only when a lipophilic statin was used (simvastatin) (12,13). A lower dose of simvastatin (20 mg/day) did not produce measurable changes in skeletal muscle Q_{10} content (14,15). The acute effect of statin treatment on mitochondrial oxidative phosphorylation (OXPHOS) capacity in human skeletal muscle has been investigated, and a decrease in OXPHOS capacity with complex I–linked substrates was reported, whereas respiration with complex II–linked substrates was unaffected (16). Recently, the same group investigated mitochondrial function in patients treated with different statins (lipophilic and hydrophilic) and reported the same results: decreased complex I OXPHOS capacity in the patients and no differences in complex II–linked OXPHOS capacity (17). However, the study groups were not matched for maximal oxygen uptake (physical fitness), which is problematic because exercise training is the most powerful stimulus for mitochondrial biogenesis (18), and differences in mitochondrial content (which was not measured in the study [17]) would have had a major influence on the respiratory results.

There is a paucity of studies of skeletal muscle mitochondrial function in hypercholesterolemic patients in long-term treatment with statins. The present study was therefore undertaken to test whether Q_{10} content, maximal OXPHOS capacity, mitochondrial content, and mitochondrial substrate sensitivity were affected in patients in long-term treatment with a cholesterol-lowering drug (simvastatin).

Recently, it has been reported that statin treatment impairs insulin sensitivity and increases the prevalence of type 2 diabetes (19–22); and it seems that lipophilic statins (i.e., simvastatin, atorvastatin, fluvastatin, lovastatin) have a more pronounced effect on insulin sensitivity compared with hydrophilic statins (i.e., pravastatin, rosuvastatin) (23). A secondary aim of the present study was to test whether these findings of decreased insulin sensitivity would be reflected in a simple oral glucose tolerance test (OGTT) in patients treated with simvastatin.

Methods

Participants. Twenty male subjects were recruited to participate in the study: 10 patients with hypercholesterolemia, in medical treatment with simvastatin (10 to 40 mg/day: 10 mg/day, n = 1; 20 mg/day, n = 4; and 40 mg/day, n = 5) for at least 12 months (average: 5 years) (recruited via the Unit for Inherited Cardiac Diseases, Department of Cardiology, Rigshospitalet), and 10 healthy control subjects. The groups were matched for age, weight, body mass index, fat percentage (total and abdominal), and maximal oxygen uptake ($\dot{V}O_{2MAX}$) (Table 1). The patients with hypercholesterolemia took 1 dose of medication per day in the evening and did not take any other medication, and control subjects did not take any medication. Medication was not withdrawn prior to the test days. Subjects with a family history of type 2 diabetes were excluded from the study. Muscle pain was evaluated with the visual analog scale. After the subjects were recruited and the blood and skeletal muscle were analyzed, 1 of the control subjects turned out to have impaired glucose tolerance; therefore, data from this subject were omitted, leaving 9 in the control group.

Abbreviations and Acronyms

ADP = adenosine diphosphate

C_{50} = substrate sensitivity

CK = creatine kinase

CS = citrate synthase

HAD = beta-hydroxyacyl-CoA dehydrogenase

OGTT = oral glucose tolerance test

OXPHOS = oxidative phosphorylation

Q_{10} = coenzyme Q_{10}

ROS = reactive oxygen species

VDAC = voltage-dependent anion channel

$\dot{V}O_{2MAX}$ = maximal oxygen uptake

The ethics committee of the municipality of Copenhagen and Frederiksberg in Denmark approved the study protocol (H-4-2009-095), and oral and written consent were obtained from each participant in accordance with the Helsinki Declaration.

Experimental protocol. Subjects reported to the laboratory at 8:00 AM after an overnight fast (10 to 12 h) on 2 separate days. The subjects were instructed to abstain from alcohol and strenuous exercise the day before each visit. On the first day, a medical history was obtained, followed by a standard 2-h OGTT with blood samples drawn at baseline (fasting) and every 30 min until 2 h for glucose and insulin measurements. A resting electrocardiogram was then recorded to exclude individuals with signs of coronary ischemia, followed by measurement of body composition using a dual-energy X-ray absorptiometry scan (Lunar Prodigy Advance, Lunar, Madison, Wisconsin). Finally, an incremental cycling test was performed to determine $\dot{V}O_{2MAX}$ (Jaeger ER 800, Erich Jaeger, Würzburg, Germany). Achievement of $\dot{V}O_{2MAX}$ was accepted when a respiratory exchange ratio >1.15 and/or maximal heart rate (220 − age) was present, or a leveling off or decline in $\dot{V}O_2$ was reached. For further characterization of fitness, the daily physical activity was evaluated with the International Physical Activity Questionnaire (24). On day 2, subjects were placed in a supine position; after 30 min, a blood sample was drawn, and a muscle biopsy was obtained from the *m. vastus lateralis*.

Analytical procedures. MUSCLE BIOPSIES. The muscle biopsies were divided into 3 parts; the first part was quickly frozen in liquid nitrogen within 15 s after sampling; the second part was mounted in a mounting medium (Tissue-Tek, Hounisen Laboratorieudstyr, Risskov, Denmark) and frozen in isopentane cooled by liquid nitrogen; the third

Table 1 Subject Characteristics

	Patients (n = 10)	Controls (n = 9)
Age, yrs	45 ± 6	45 ± 4
Treatment time, yrs	5 ± 5	—
Weight, kg	89 [86–101]	85 [82–102]
BMI, kg/m^2	27 ± 2	27 ± 2
Body fat, %	28 ± 5	27 ± 5
LBM, kg	63 ± 8	63 ± 6
Systolic BP, mm Hg	141 ± 13*	121 ± 10
Diastolic BP, mm Hg	86 ± 11	77 ± 6
Vo_{2MAX}, ml O_2/min/kg	38 ± 3	40 ± 6
HR_{MAX}, bpm	168 ± 9	164 ± 10
VAS, AU	2 ± 2*	0 ± 0
IPAQ, kcal/week	3,455 ± 2,017	3,128 ± 2,128
HbA1c, %	5.7 ± 0.2*	5.2 ± 0.2
NEFA, μmol/l	455 ± 186	412 ± 317
TG, mmol/l	1.4 ± 0.6	1.3 ± 0.7
Cholesterol, mmol/l	4.8 ± 1.0	4.3 ± 0.7
LDL, mmol/l	3.1 ± 1.0	2.7 ± 0.6
HDL, mmol/l	1.3 ± 0.3	1.2 ± 0.3
CK, U/l	180 [145–226]	176 [117–239]
Lactate, mmol/l	0.83 [0.72–1.03]	0.96 [0.81–1.16]
SI (mg · l^2)/(mmol · Mu · min)	33 [29–40]*	56 [44–92]

Values are mean ± SD or median [interquartile range]. *p ≤ 0.05 for patients versus control subjects.

AU = arbitrary units; BP = blood pressure; BPM = beats per min; CK = creatine kinase; HbA_{1c} = glycated hemoglobin; HDL = high-density lipoprotein; HR = heart rate; IPAQ = International Physical Activity Questionnaire; LBM = lean body mass; LDL = low-density lipoprotein; NEFA = nonesterified fatty acids; SI = peripheral insulin sensitivity (Cederholm index); TG = triglycerides; VAS = visual analog scale; Vo_{2MAX} = maximal oxygen uptake.

part was placed in a relaxing buffer (BIOPS) and immediately analyzed for mitochondrial respiration. The first and second parts of the muscle biopsy were stored at −80°C until further analysis.

ENZYME ACTIVITIES. CS and beta-hydroxyacyl-CoA dehydrogenase (HAD) activities were measured as previously described (25).

PROTEIN CONTENT (WESTERN BLOTS). Approximately 25 mg of muscle tissue was homogenized in a buffer. From each sample, 20 to 30 μg of total protein were separated by SDS-PAGE on 12.5% Criterion gels (BioRad Laboratories, Hercules, California) and electrophoretically transferred to polyvinylidene difluoride membranes. The following primary antibodies were used against the following proteins: catalase (AF3398, R&D Systems, Minneapolis, Minnesota), coenzyme Q_{10} (ab41997, Abcam, Cambridge, United Kingdom), complexes I–V (MS601, MitoSciences, Eugene, Oregon), glutathione peroxidase 1 (3286, Cell Signaling Technology, Danvers, Massachusetts), beta-hydroxyacyl-CoA dehydrogenase (HADHA) (ab54477, Abcam), mitochondrial superoxide dismutase (MnSOD) (06-984, Millipore, Billerica, Massachusetts), uncoupling protein 3 (UCP3) (ab3477, Abcam), and VDAC (4661, Cell Signaling Technology). Control and statin-treated subjects were loaded in an alternating pattern to avoid regional variation in transfer efficiency. Protein expression in simvastatin-treated subjects was measured relative to the mean values of the control group; all analyses were performed twice.

MYOSIN HEAVY CHAIN ANALYSIS. Analysis of myosin heavy chain composition has been described in detail elsewhere (26).

QUANTIFICATION OF SKELETAL MUSCLE LIPIDS (CARDIOLIPIN AND CHOLESTEROL). Lipids were extracted from approximately 20 mg (wet weight) of muscle with chloroform/methanol (27) and redissolved in toluol (1 l/mg wet weight) for quantitative thin layer chromatography (28). The precision and accuracy of this assay have been reported elsewhere (27,29).

MITOCHONDRIAL RESPIRATION. Mitochondrial respiratory capacity was measured in permeabilized skeletal muscle fibers. The details of the procedure have been described previously (30). Briefly, all respiratory measurements were carried out in duplicate after hyperoxygenation to avoid any potential oxygen limitation to respiration. Five different substrate and inhibitor protocols were employed simultaneously (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria).

Protocol 1 (evaluating complex I and complex II OXPHOS capacity): State 2 respiration was assessed with malate (2 mmol/l), glutamate (10 mmol/l), and pyruvate (5 mmol/l), followed by state 3 respiration (5 mmol/l, adenosine diphosphate [ADP]). Rotenone (0.5 μmol/l) was added to inhibit complex I, followed by succinate (10 mmol/l) as a substrate for complex II, then cytochrome *c* (10 μmol/l) was added to control for outer mitochondrial membrane integrity. Malonate (5 mmol/l) was used to inhibit complex II, and finally, antimycin A (2.5 μmol/l) was added to inhibit complex III.

Protocol 2 (evaluating complex I and complex I + II OXPHOS capacity): State 2 respiration was assessed with malate (2 mmol/l), glutamate (10 mmol/l), and pyruvate (5 mmol/l), followed by state 3 respiration (5 mmol/l, ADP). Simultaneous electron input into complex I + II (maximal OXPHOS capacity) was assessed with succinate (10 mmol/l), followed by cytochrome *c* (10 μmol/l). Finally, oligomycin (2 μg/ml) was added to inhibit complex V.

Protocol 3 (evaluating complex IV OXPHOS capacity): Complex IV (cytochrome *c* oxidase) activity was measured as follows: ADP (5 mmol/l) was added followed by cytochrome *c* (10 μmol/l) and antimycin A (2.5 μmol/l) inhibiting complex III, finally ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) was added (2 mmol/l and 0.5 mmol/l, respectively).

Protocol 4 (evaluating complex I substrate sensitivity): State 2 respiration was assessed with malate (2 mmol/l), followed by state 3 respiration (5 mmol/l, ADP). Subsequently, glutamate (complex I linked substrate) was titrated in 11 steps (0.1 to 96.0 mmol/l), followed by cytochrome *c* (10 μmol/l).

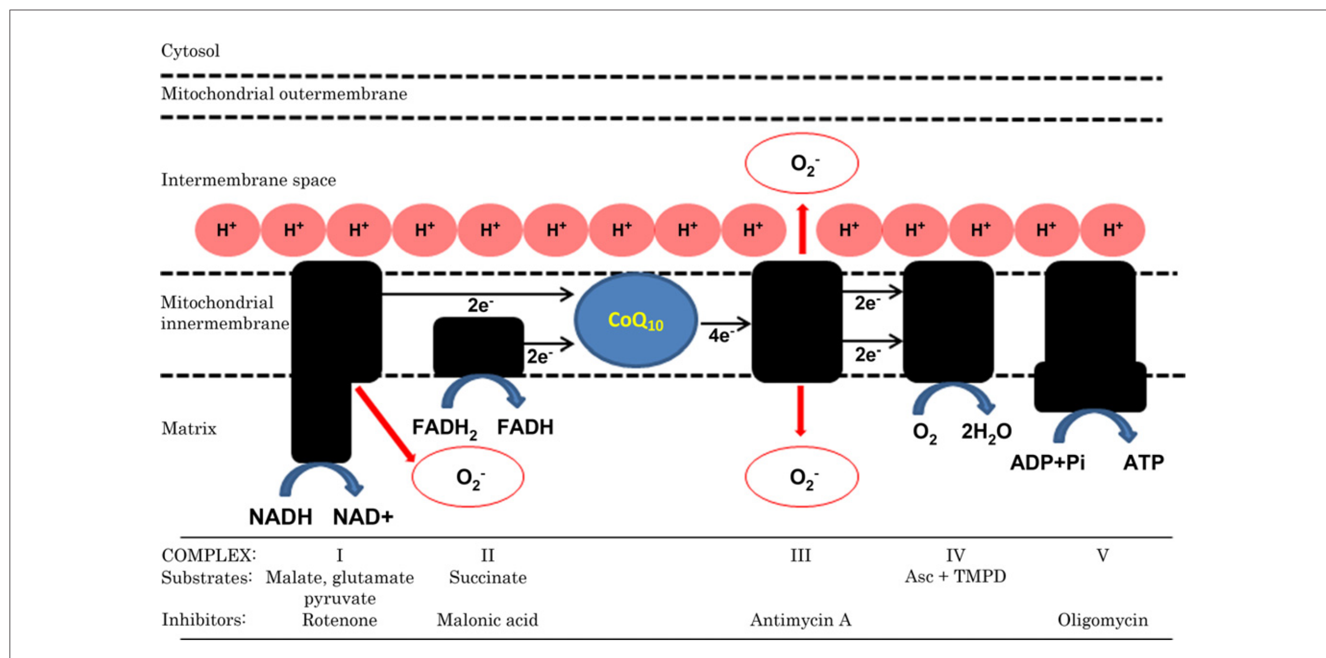

Figure 1 **Simplified View of the Electron Transport Chain Illustrating the Electron Carrier Function of Q_{10}**

Reactive oxygen species–producing complexes are emphasized. Below each complex is listed the substrates and inhibitors that were used. ADP = adenosine diphosphate; Asc = ascorbate; ATP = adenosine triphosphate; Pi = phosphate; Q_{10} = coenzyme Q_{10}; TMPD = *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

Protocol 5 (evaluating complex II substrate sensitivity): State 2 respiration was assessed with malate (2 mmol/l) followed by state 3 respiration (5 mmol/l, ADP). Glutamate (10 mmol/l) (complex I substrate) was added, and succinate (complex II linked substrate) was titrated in 10 steps (0.1 to 48.0 mmol/l), followed by cytochrome *c* ((10 μmol/l). See Figure 1 for an overview of the used substrates and inhibitors in relation to the mitochondrial electron transport chain.

Blood samples. Blood samples were collected in chilled tubes and immediately centrifuged (2,500 *g*; 4°C) for 10 min. Plasma was stored at −20°C or −80°C before analysis. The analysis of plasma glucose, insulin, nonesterified fatty acids, cholesterol, low-density lipoproteins, high-density lipoproteins, and glycated hemoglobin (HbA_{1c}) was done as previously described (31). Glucose and insulin concentrations during the OGTT were used to calculate the Cederholm index of peripheral insulin sensitivity (32), which has been validated against the hyperinsulinemic−euglycemic clamp (33). Lactate and CK levels were analyzed enzymatically on an automated clinical chemical analyzer (COBAS 501, Roche, Mannheim, Germany).

Statistics and calculations. Data are presented as mean ± SD in the text and in the tables, and presented in all figures as mean ± SE. $p < 0.05$ was considered significant. Statistical analysis of differences in mitochondrial OXPHOS capacity between the groups was carried out with a 2-way analysis of variance for repeated measures. The restrictive assumptions, normality and equal variance, were checked before the statistical analysis was conducted. Significant main effects or interactions were further analyzed by the Holm-Sidak post hoc test. If normality and equal variance test failed, the data were transformed and reanalyzed; this has been stated in the figure legend if this was the case. Differences between the 2 groups were evaluated using a 1-way analysis of variance. If normality of the data was not present, the data were analyzed using a nonparametric test, and further analyzed by the Dunn, Tukey, or Kruskal-Wallis test. In this situation, data are summarized with medians and quartiles (median [interquartile range]) (Tables 1 and 2, and in the Results section). Pearson's correlation analysis was performed to establish the presence of correlations. All statistical analysis was performed using the software program SigmaStat 3.1 (Systat Software, San Jose, California).

The calculation of substrate sensitivity (C_{50}) and OXPHOS capacity has been described previously (31).

Results

Fasting plasma glucose and insulin concentrations were comparable between groups (Fig. 2). During the OGTT, glucose concentration was significantly higher ($p < 0.05$) in patients compared with controls, resulting in an increased ($p < 0.05$) area under the glucose curve in the simvastatin-treated patients (Fig. 2). During the OGTT, insulin levels were similar between groups. Patients had impaired insulin sensitivity ($p < 0.05$) (Cederholm Index) compared with controls (Table 1). Four of the 10 patients reported muscle pain, whereas none of the controls did (Table 1). No difference was found in plasma lactate between the 2 groups.

Table 2 Skeletal Muscle Analysis

	Patients (n = 10)	Controls (n = 9)
Protein content		
Coenzyme Q_{10}, AU	0.84 ± 0.15*	1.00 ± 0.18
Catalase, AU	0.58 ± 0.33*	1.00 ± 0.51
MnSOD, AU	0.74 ± 0.20*	1.00 ± 0.18
Glutathione peroxidase, AU	0.40 ± 0.21*	1.00 ± 0.81
UCP3, AU	0.20 [0.11–0.45]*	1.04 [0.64–1.46]
HAD, AU	0.83 ± 0.19	1.00 ± 0.19
VDAC, AU	1.19 ± 0.39	1.00 ± 0.26
Complex I, AU	0.81 ± 0.24	1.00 ± 0.31
Complex II, AU	0.84 ± 0.15	1.00 ± 0.19
Complex III, AU	1.00 ± 0.22	1.00 ± 0.20
Complex IV, AU	0.81 ± 0.19*	1.00 ± 0.18
Complex V, AU	0.82 [0.77–0.96]	0.98 [0.89–1.13]
Enzyme activities		
CS activity, μmol/g dw/min	101 ± 18	116 ± 25
HAD activity, μmol/g dw/min	89 ± 16	109 ± 27
Skeletal muscle lipids		
Cardiolipin, μg/mg ww	0.49 ± 0.08	0.55 ± 0.10
Free cholesterol, nmol/mg ww	0.96 ± 0.15	1.05 ± 0.16
Skeletal muscle fiber types		
MHC I, %	42 ± 10*	58 ± 16
MHC IIA, %	39 ± 10	29 ± 12
MHC IIX, %	19 ± 7	13 ± 5

Values are mean ± SD or median [interquartile range]. Skeletal muscle characteristics in hypercholesterolemic patients in long-term treatment with simvastatin and in healthy control subjects are shown. Representative Western blots are presented in Figure 6. *p ≤ 0.05 for patients versus control subjects.

AU = arbitrary units; CS = citrate synthase; dw = dry weight; GPX = glutathione peroxidase; HAD = beta-hydroxyacyl-CoA dehydrogenase; MHC = myosin heavy chain; MnSOD = manganese superoxide dismutase; UCP3 = uncoupling protein 3; VDAC = voltage-dependent anion channel; ww = wet weight.

CK levels were also comparable between patients and controls (180 [145 to 226] U/l vs. 176 [117 to 239] U/l, respectively) (Table 1). Characteristics and blood data of the patients and control subjects are provided in Table 1 and Figure 2.

Muscle characteristics. Q_{10} protein content was reduced (p = 0.05) in patients compared with control subjects (Table 2). The antioxidant (catalase [p < 0.05], MnSOD [p < 0.01], and glutathione peroxidase [p < 0.05]) as well as UCP3 (p < 0.01) protein content was significantly reduced in patients compared with control subjects (Table 2). Complex IV content was significantly reduced (p < 0.05), whereas complex V showed a tendency (p = 0.07) to be reduced in patients compared with control subjects, but complex I to III protein content was similar between the groups (Table 2). CS activity, VDAC protein content, and cardiolipin content were similar between groups. When VDAC protein content was expressed per CS activity (VDAC/CS activity) or cardiolipin content (VDAC/cardiolipin content), patients had a significantly (p < 0.05) higher ratio compared with control subjects (data not shown); this could indicate that VDAC protein content is increased due to simvastatin treatment. A tendency (p = 0.06) towards lower HAD activity was seen in patients compared with control subjects (Table 2); the same was seen in HAD protein content (p = 0.07) in patients compared with control subjects. Skeletal muscle free cholesterol was similar in the 2 groups (Table 2). Skeletal muscle fiber type composition was different between the groups (Table 2).

Mitochondrial respiration. OXPHOS capacity with complex I–linked substrates was not different between control subjects and patients treated with simvastatin (26 ± 6 pmol/mg/s vs. 24 ± 8 pmol/mg/s, respectively) (Figs. 3 and 4A). However, substrate sensitivity was significantly higher (p < 0.05) in the patients compared with control subjects when glutamate (complex I–linked substrate) was used as substrate (Fig. 4B). Thus, C_{50} was lower in the patients (0.98 ± 0.23 mmol/l) compared with the control subjects (1.37 ± 0.51 mmol/l) (p < 0.05) (Fig. 4B). A tendency (p = 0.076) for a drug dose effect was seen in substrate sensitivity when glutamate was used as substrate; the patients that were treated with 40 mg of simvastatin per day (n = 5) had a higher mitochondrial substrate sensitivity (complex I–linked substrate) compared with patients treated

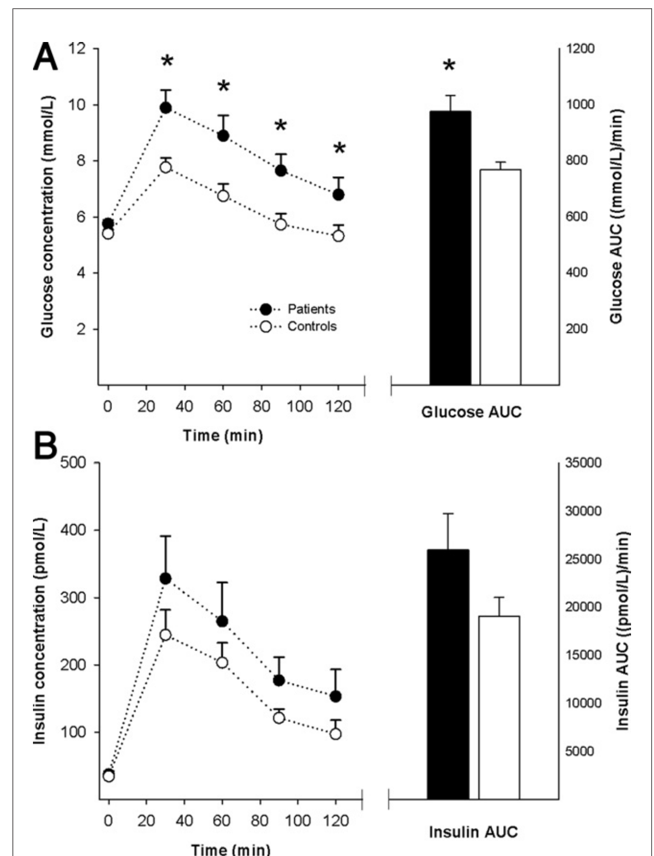

Figure 2 Plasma Glucose and Insulin Concentrations During the 120-min OGTT

Plasma glucose **(A)** and insulin **(B)** concentrations in patients with hypercholesterolemia **(soild circles and soild bars)** and in control subjects **(open circles and open bars)** are shown. Data are mean ± SE. *p < 0.05 for patients versus control subjects. AUC = area under the curve; OGTT = oral glucose tolerance test.

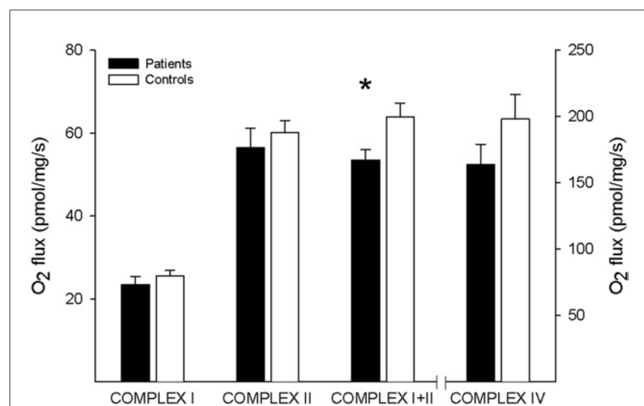

Figure 3 **Mitochondrial OXPHOS Capacity for Complexes I, II, I + II, and IV**

Oxidative phosphorylation (OXPHOS) capacity (measured as O_2 flux rates per mg tissue) in skeletal muscle from patients with hypercholesterolemia **(solid bars)** and control subjects **(open bars)** is shown. The following substrates were used: complex I: malate, glutamate, pyruvate, ADP; complex II: malate, glutamate, pyruvate, ADP, rotenone (inhibiting complex I), succinate; complex I + II: malate, glutamate, pyruvate, ADP, succinate; and complex IV: ADP, cytochrome *c*, antimycin A, TMPD + Asc. The y-axis to the right refers to the O_2 flux rates for complex IV. Data are mean ± SE. *p < 0.05 for patients versus control subjects. Abbreviations as in Figure 1.

with 10 to 20 mg of simvastatin per day (n = 5) and with the control subjects (0.84 ± 0.06 mmol/l vs. 1.12 ± 0.26 mmol/l vs. 1.37 ± 0.51 mmol/l). When succinate (complex II–linked substrate) was used as a substrate, no differences were seen in OXPHOS capacity (Fig. 3) or in substrate sensitivity (Fig. 5B) between the groups. However, maximal OXPHOS capacity with dual electron input to complex I and II (feeding electrons into the Q intersection) was significantly higher ($p < 0.01$) in control subjects compared with patients treated with simvastatin (64 ± 10 pmol/mg/s vs. 54 ± 8 pmol/mg/s, respectively) (Figs. 3 and 5A). When complex I was inhibited (protocol 1, inhibition with rotenone), no difference was seen in complex II OXPHOS capacity (Fig. 3) between control subjects and patients treated with simvastatin (60 ± 9 pmol/mg/s vs. 57 ± 15 pmol/mg/s, respectively). No difference was seen in complex IV OXPHOS capacity between control subjects and patients treated with simvastatin (198 ± 56 pmol/mg/s vs. 164 ± 49 pmol/mg/s, respectively) (Fig. 3). No cytochrome *c* response was observed in any of the protocols (data not shown), implying that the outer mitochondrial membrane was intact after the experimental procedure.

A weak positive correlation ($r = 0.46$, $p < 0.05$) was observed between maximal OXPHOS capacity (complex I + II–linked substrates) and Q_{10} content. This correlation was not observed between OXPHOS capacity with complex I or complex II–linked substrates alone and Q_{10} content.

Discussion

A major and novel finding is the impaired glucose tolerance found in the patients in treatment with simvastatin, which was shown by a simple OGTT. Furthermore, a decrease is found in Q_{10} protein content in skeletal muscle, accompanied by a limitation in maximal OXPHOS capacity in the patients treated with simvastatin. This OXPHOS limitation was only apparent at high respiratory flux rates when the coupled respiration was measured during experiments with convergent electron input from both complex I and II to the Q intersection in the respiratory chain. We suggest that these observations may be part of the explanation of the most common side effects of statin treatment: muscle pain and weakness.

The impaired glucose tolerance found in the simvastatin-treated patients is in agreement with previous findings of

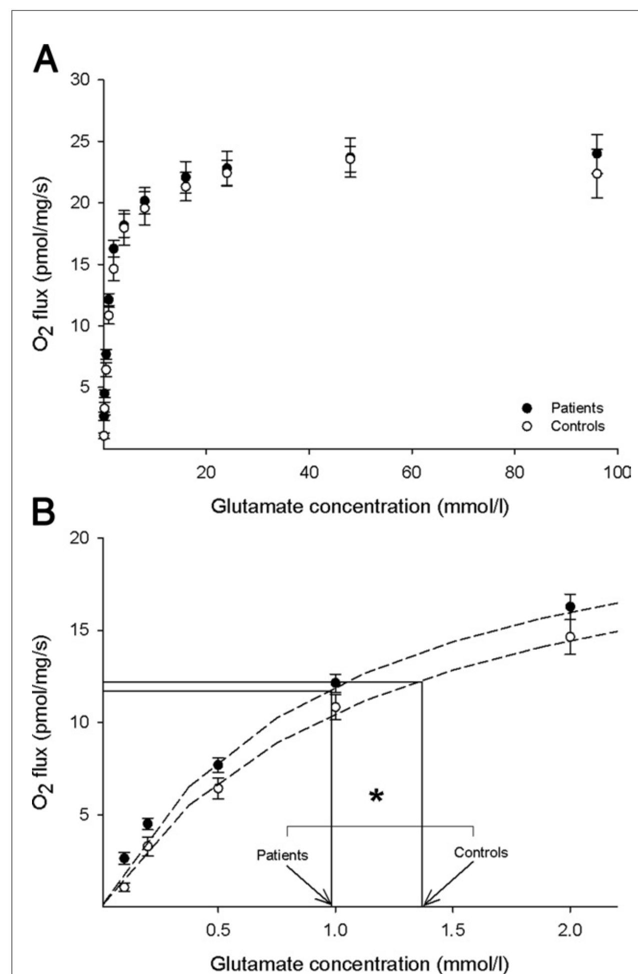

Figure 4 **Mitochondrial OXPHOS Capacity With Glutamate**

(A and B) O_2 flux rates per mg tissue in skeletal muscle from patients with hypercholesterolemia **(solid circles)** and control subjects **(open circles)** are shown for the following protocol: malate, ADP, and glutamate titration (0.1 to 96.0 mmol/l). **(A)** Glutamate titration where Michaelis-Menten kinetics can be seen (entire titration protocol). **(B)** Extract of the respiration curve highlighting respiration for glutamate concentration (0.1 to 2.5 mmol/l), which makes it possible to appreciate the substrate kinetics (C_{50}). **Drop lines (B)** indicate C_{50} (x-axis) corresponding to half maximum OXPHOS capacity (y-axis), respectively. In order to perform the appropriate statistical testing, the data were transformed before they were analyzed. Data are mean ± SE. *p < 0.05 for patients versus control subjects. Abbreviations as in Figures 1 and 3.

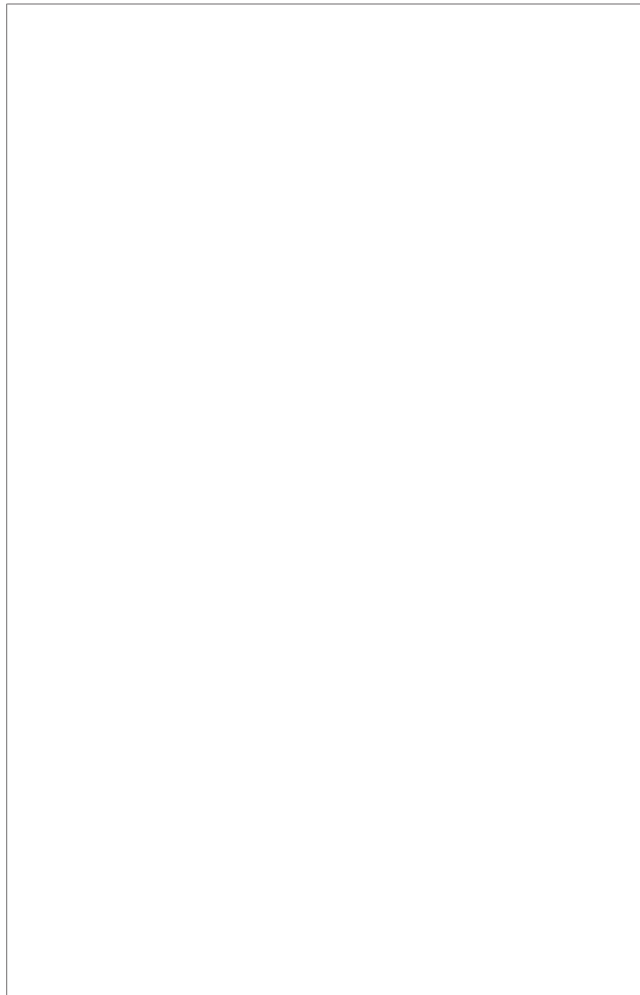

Figure 5 **Mitochondrial OXPHOS Capacity With Succinate**

(A and B) O_2 flux rates per mg tissue in skeletal muscle from patients with hypercholesterolemia **(solid circles)** and control subjects **(open circles)** are shown for the following protocol: malate, ADP, glutamate, and succinate titration (0.1 to 48.0 mmol/l). **(A)** Succinate titration where Michaelis-Menten kinetics can be seen (entire titration protocol). **(B)** Extract of the respiration curve highlighting respiration for succinate concentration (0.1 to 8.0 mmol/l), which makes it possible to appreciate the substrate kinetics (C_{50}). **Drop lines (B)** indicate C_{50} (x-axis) corresponding to half maximum OXPHOS capacity (y-axis). In order to perform the appropriate statistical testing, the data were transformed before they were analyzed. Data are mean ± SE. *$p < 0.05$ for patients versus control subjects. Abbreviations as in Figures 1 and 3.

impaired insulin sensitivity with simvastatin treatment (19–21), but the present study is to our knowledge the first to demonstrate a deleterious effect on the glucose tolerance in simvastatin-treated patients. The 3 studies by Koh et al. (19,20,22) from 2008, 2009, and 2010 used the Quantitative Insulin-Sensitivity Check Index as a surrogate measure for insulin sensitivity, whereas the study by Ohrvall et al. (21) from 1995 used the hyperinsulinemic–euglycemic clamp technique. The latter study was performed in patients with type 2 diabetes, which could be a confounding factor. The mechanism behind this unfavorable side effect on glucose tolerance is not known, and an explanation for the reduced glucose tolerance found in the patients can only be speculative, but 1 clue may be that it seems as if the effect on insulin sensitivity is more pronounced when the patients are treated with lipophilic statins (19,22,23). Insulin-resistant patients have been shown to have reduced UCP3 content in the skeletal muscle (34), and in the present study, we also found reduced UCP3 content in the simvastatin-treated patients. UCP3 has been suggested to protect the mitochondria against an accumulation of nonesterified fatty acids (35); an accumulation of these fatty acids and ceramides and diacylglycerol in the cytosolic compartment are linked to insulin resistance via an inhibition of the insulin signaling cascade (36,37). Thus, the observation of glucose intolerance in concert with reduced UCP3 levels supports the view that the simvastatin-treated patients were in a pre-insulin resistant state. Consensus about the role of intramyocellular lipids in insulin resistance is not reached in the literature, and further studies are clearly needed to elucidate this.

Q_{10} content and mitochondrial OXPHOS capacity. Other studies with high simvastatin doses (80 mg/day) have reported decreased Q_{10} protein content in muscle from simvastatin-treated patients (12,13), whereas studies using lower (20 mg/day) doses have been unable to demonstrate decreased muscle Q_{10} protein content (14,15), but no studies have so far combined this measurement of a key protein in the mitochondrial respiratory chain with a functional (high-resolution respirometry) mitochondrial test. In doing so, we can now show that OXPHOS capacity is not different between patients and control subjects, when substrates for complex I and complex II are used separately. Only when complex I and II substrates were combined, thus maximally taxing the electron transport system, did the effect of statin treatment become apparent (Figs. 1 and 3),

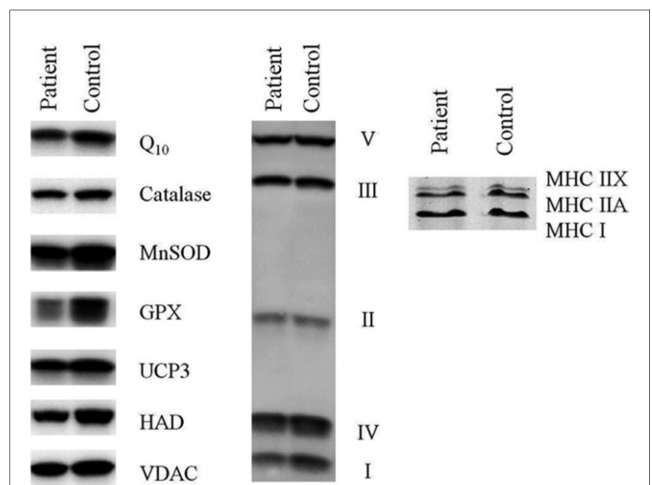

Figure 6 **Representative Western Blots Are Presented**

The bands for the different proteins are from the same 2 subjects (a patient and a control). GPX = glutathione peroxidase; HAD = beta-hydroxyacyl-CoA dehydrogenase; MHC = myosin heavy chain; MnSOD = manganese superoxide dismutase; Q_{10} = coenzyme Q_{10}; UCP3 = uncoupling protein 3; VDAC = voltage-dependent anion channel.

indicating a defect in the Q intersection when the electron transport system is challenged maximally. The fact that we were able to demonstrate this impairment in OXPHOS capacity (complex I and II substrates combined) in the patients even at these relatively low flux rates, emphasizes the potential of our finding (in vivo flux rates are much higher than these ex vivo flux rates [38]). It may be anticipated that the OXPHOS limitation observed ex vivo in the present study serves as a mechanism for the impaired exercise tolerance and fatigability seen in some patients using statins. The acute effect of statins has, however, been studied in isolated mitochondria from L6 cells (a rat skeletal muscle cell line) (39) and in skinned human and rat muscle (16) in which a toxic effect of simvastatin and depressed complex I respiration was found. Recently Kwak et al. (40) reported impaired ADP-stimulated respiration in primary human skeletal myotubes after 48 h of simvastatin treatment, and this was accompanied by increased mitochondrial oxidative stress (hydrogen peroxide emission). It may not be that surprising that a statin can produce an acute toxic effect in cells, but the fact that an impairment of function can be observed in human muscle fibers, obtained via a biopsy that is mechanically and chemically dissected and permeabilized, washed, and finally transferred to the respiration chamber, is truly remarkable. It shows that the impact of long-term treatment with the lipophilic statin simvastatin has profound functional (limitation of maximal OXPHOS capacity) and structural (decreased content of Q_{10}, complex IV, antioxidant proteins, and UCP3) impact. The present study differs from the aforementioned studies in that the patients are chronically treated with simvastatin, whereas the other studies are conducted in cell lines or the skeletal muscle is acutely treated with very high (thus not clinically relevant) concentrations of statins. Therefore, the clinical applicability of the present study is more obvious.

The decreased Q_{10} protein content in patients in long-term treatment with simvastatin offers a mechanistic explanation for the limitation in maximal OXPHOS capacity (Figs. 1 and 3). This hypothesis requires further testing, but is to some extent supported by the correlation between Q_{10} content and maximal OXPHOS capacity, even though this correlation was not strong and has to be confirmed in other studies.

Mitochondrial substrate sensitivity. Skeletal muscle from simvastatin-treated patients had increased mitochondrial substrate sensitivity (lower C_{50} value) with complex I–linked substrate (glutamate) compared with muscle from the control subjects, but no difference was seen with complex II–linked substrate (succinate). The reason for this disparity may be explained by the difference in muscle fiber type composition and/or mitochondrial membrane potential. First, higher substrate sensitivity for complex I–linked substrates has previously been reported in patients with type 2 diabetes compared with healthy control subjects (31). Second, differences between type I and type II muscle fibers have been reported previously in rats, with type II fibers being more sensitive than type I (41). Accordingly, the significantly lower proportion of type I fibers in the patients compared with the control subjects in the present study may contribute to the observed difference in substrate sensitivity between the groups. Third, it has previously been reported that statins, in a dose-dependent manner, decrease the mitochondrial membrane potential (16,39). The membrane potential is mainly created by virtue of the activity of complexes I, III, and IV, and a compromised function of 1 or more of these may lower the membrane potential. Therefore, a higher complex I substrate sensitivity may be regarded as a compensatory mechanism for upholding the mitochondrial membrane potential. In support of this notion is our observation that the patients treated with the highest simvastatin dose (40 mg/day) also showed a nonsignificant tendency to have the highest substrate sensitivity compared with both patients treated with the lowest simvastatin dose (10 to 20 mg/day) and the control subjects.

The difference between simvastatin-treated patients and the control subjects in complex I–linked substrate sensitivity is a true intrinsic event in the mitochondrion, because the derived sensitivity parameter (C_{50}) of the titration studies is independent of mitochondrial density.

Muscle characteristics. In the present study, ROS production was not measured, but other studies have shown increased ROS production (3,42) and a decreased antioxidant capacity (3) with statin treatment. The latter finding is confirmed by the present study in which we found decreased protein content of catalase, manganese superoxide dismutase, and glutathione peroxidase. Mitochondrial content evaluated by CS activity, cardiolipin content, or VDAC protein content was similar between the groups (Table 2). The ratio between VDAC and CS activity or cardiolipin was significantly higher in the patients compared with the control subjects, indicating that simvastatin-treated patients have a higher abundance of VDAC content per mitochondrion. It has been reported that VDAC located in the outer mitochondrial membrane is important for the in- and outflux of Ca^{2+} of the mitochondria (43), and the upregulated VDAC protein content in the simvastatin-treated patients could be because of the known statin-induced elevation of cytoplasmic Ca^{2+} concentration (44). In turn, an increased Ca^{2+} load in the mitochondrial matrix has been linked to increased ROS production and increased permeability transition pore opening, leading to apoptosis (40,45). Our findings of significantly reduced antioxidant capacity in combination with the known (3,40) elevated ROS production in the simvastatin-treated patients make it plausible that increased apoptosis could contribute to muscle pain in patients treated with statins. Furthermore, we observed a decreased protein content of complex IV in the patients compared with the control subjects; this was a somewhat unexpected finding.

Conclusions

In summary, we found that patients treated with simvastatin have impaired glucose tolerance compared with healthy control subjects. Furthermore, we found that skeletal muscle from patients treated long term with simvastatin displayed a decreased maximal OXPHOS capacity that may well be explained by a concomitant decrease in Q_{10} protein content without any changes in mitochondrial content. The result is a compromised energetic state within the skeletal muscle, possibly explaining side effects such as myalgia and exercise intolerance. Finally, we have reported that simvastatin-treated patients have an increased mitochondrial substrate sensitivity when complex I substrate was used, which is a true intrinsic mitochondrial event, and we hypothesize that this could be a compensatory mechanism for increased ROS production.

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