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Evidence for metabolic aberrations in asymptomatic persons with type 2 diabetes after initiation of simvastatin therapy

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Abstract

HMG-CoA reductase inhibitors (statins) prevent vascular events and are widely prescribed, particularly in persons with type 2 diabetes. However, intolerance due to myopathic symptoms often limits their use. We investigated the effects of simvastatin on parameters of mitochondrial function and muscle gene expression in 11 subjects with type 2 diabetes; none of whom had statin intolerance. After withdrawal of statins for two months, we obtained blood samples, performed vastus lateralis muscle biopsies, and assessed whole body resting energy expenditure (REE). We then re-initiated therapy using simvastatin, 20 mg/day, for one month before repeating these studies. As expected, simvastatin lowered LDL, but did not induce myalgias or significant elevations in serum creatine kinase. However, we found subtle but significant reductions in muscle citrate synthase activity and REE. In addition, quantitative PCR and gene set enrichment analysis of muscle samples revealed significantly repressed gene sets involved in mitochondrial function and induced gene sets involved in remodeling of the extracellular matrix. Further, the effects of simvastatin on muscle gene sets showed some similarities to previously described changes that occur in Duchenne muscular dystrophy, polymyositis, and dermatomyositis. Although statins inhibit an early step in coenzyme Q (CoQ) biosynthesis, we observed no differences in CoQ content within skeletal muscle mitochondria, muscle tissue, or circulating platelets. In summary, we report subtle changes in whole body energetics, mitochondrial citrate synthase activity, and

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microarray data consistent with subclinical myopathy. Although the benefits of statin therapy are clear, further understanding of muscular perturbations should help guide safety and tolerability.

INTRODUCTION

Hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have proven effective in preventing vascular events and are currently recommended for most persons with diabetes over the age of 40 (1). However, their use is often limited because of intolerance, most of which is due to muscle pain.

Some reports have suggested that statin-induced myopathy may be due to mitochondrial dysfunction (2, 3) which would lead to impaired oxidative phosphorylation. One possible reason might be reduced ubiquinone (coenzyme Q or CoQ) content. This is based on knowledge that HMG-CoA reductase is the first step in both cholesterol and CoQ biosynthesis (4). However, whether statin therapy reduces CoQ levels in humans is controversial (5-9) and CoQ may be reduced in diabetes as well (10, 11). On the other hand, there is very little information regarding CoQ levels in diabetic patients taking statins.

There is evidence that statins slightly increase incident diabetes (12-14) while effects on insulin sensitivity are debated (15). Insulin resistance is associated with mitochondrial dysfunction (16), so it is possible that statin induced hyperglycemia and diabetes risk may be mediated at the mitochondrial level.

Based on the above considerations, we hypothesized that statin treatment of subjects with type 2 diabetes might reduce CoQ content within skeletal muscle, impair markers of mitochondrial function, reduce resting whole body energy expenditure, and increase circulating glucose.

Here we measured skeletal muscle mitochondrial CoQ and platelet CoQ content in 11 subjects with type 2 diabetes before and after one month of simvastatin treatment. These subjects did not have myalgia and reported no symptoms temporally related to statin therapy. We also assessed phenotypic characteristics including resting indirect calorimetry, measured skeletal muscle citrate synthase activity, measured plasma glycated albumin as a short-term marker of glucose metabolism, and carried out pre- and post-statin mRNA microarray analysis and confirmatory quantitative PCR (qPCR) on muscle biopsy samples.

METHODS

Human subjects and study protocol

The research was carried out according to the principles of the Declaration of Helsinki and reviewed and approved by our institutional Human Subjects Committee. All subjects signed informed consent. We enrolled 12 subjects, age 56-67 with type 2 diabetes. We report data for 11 subjects as one subject was withdrawn after he experienced a non-ST elevation myocardial infarct before returning for the final study visit. The protocol is graphically depicted in figure 1.

Inclusion criteria consisted of type 2 diabetes diagnosed by an endocrinologist, absence of a history of ketoacidosis, and supported by C-peptide > 0.8 ng/ml; age 30-70 yr; HbA1c 6.5 to 10.0 % inclusive; calculated fasting LDL cholesterol < 130 inclusive before therapy with a statin and meeting guideline indications for statin therapy in diabetes as defined by the American Diabetes Association in 2009 (17).

Exclusion criteria included any neurologic, muscular, genetic, or other condition known to affect muscle function or exercise tolerance; electrolyte abnormalities; untreated hypothyroidism; abnormalities in calcium, phosphate, or magnesium concentrations, or any other metabolic disturbance affecting muscle function; cigarette smoking in the past year; cardiac, pulmonary, or any other disorder affecting exercise tolerance or interfering with blood or tissue oxygenation; inconsistent or variable physical activity; pregnancy or planned pregnancy during the study; use of any medications known to inhibit statin metabolism by cytochrome P450 3A4; any other medical or psychological condition judged to limit compliance with the protocol or interpretation of results; existing vascular disease; history of rhabdomyolysis or severe muscle symptoms when on statins or creatine kinase > 10 x normal. We chose the inclusion pre-statin LDL value of 130 as cutoff to avoid the risk of too great an elevation of LDL cholesterol during the run-in.

Potential participants came to our Institution for Clinical and Translational Studies Clinical Research unit (CRU) for a screening visit after an overnight fast. Blood was obtained at screening to determine HbA1c, TSH, C-peptide, and a lipid panel including HDL cholesterol, total cholesterol, triglycerides, and calculated LDL cholesterol.

Eligible subjects met with a nutritionist after screening and were instructed in a weight maintenance diet consisting of 30% fat, 50% carbohydrate, and 20% protein with low cholesterol diet (< 300 mg daily). Subjects were advised to maintain a constant exercise routine. All subjects had been on statin therapy prior to screening and study entry but had met the inclusion criteria of documented LDL cholesterol < 130 inclusive before therapy had begun. Statin medication was discontinued for two months, following which participants returned for study visit 1 (SV1). At SV1 fasting blood (150 ml) was obtained for a plasma lipid panel and creatine kinase, and preparation of blood platelets. Indirect calorimetry was then performed in the resting state followed by a vastus lateralis muscle biopsy. Subjects were then asked to begin taking simvastatin 20 mg daily at bedtime. Participants returned one month later for study visit 2 (SV2), at which time the procedures carried out at SV1 were repeated. Anti-glycemic drug therapy was not changed in any of the subjects during the study.

Respiratory gas exchange (Indirect calorimetry)

VO₂ and VCO₂ were determined using a Carefusion Vmax 229 System located in our CRU. Resting energy expenditure (REE) was calculated using the modified Weir equation (18).

Muscle biopsy

Percutaneous muscle biopsies were taken from the vastus lateralis muscle using a Temno biopsy needle (T1420, Cardinal Health, Dublin, OH) with ultrasound guidance under local anesthesia to the skin and the fascia. Several passes (4 to 5) of the needle were used to

collect approximately 100-120 mg of muscle specimen with a wide sampling range within the muscle. Following harvest, muscle biopsy samples were immediately placed in RNALater (Ambion) for RNA extraction or flash frozen in liquid nitrogen for protein extraction and stored at -80°C until further use.

Platelet preparation

Buffy coats from 50 ml of blood (anticoagulated with Na citrate) were diluted as described (19) and centrifuged at $300 \times g$ for 10 min. The upper phase of platelet rich plasma was washed and centrifuged at $1000 \times g$ for 10 min to obtain a platelet pellet. Platelet concentrations were determined using a Coulter Counter (Beckman Coulter).

Coenzyme Q quantification

CoQ was determined by HPLC in isolated muscle mitochondria and platelets and by ELISA in muscle tissue. Muscle mitochondria were isolated as we previously described (20). Sonicated mitochondria or platelets were treated with EDTA which drives CoQ to the oxidized (quinone) form, mixed with cold methanol to further disintegrate the samples, and extracted with hexane as described (21). The extracts were evaporated and the residue redissolved in ethanol for injection into a high pressure liquid chromatography (HPLC) system using a Ascentis C18, $25 \times 4.6\text{mm}$, $5\mu\text{M}$ particle size column by an established method (21).

CoQ content was also determined per wet weight of muscle tissue. Biopsy tissue samples were placed in a cold 1mL Kontes Dual 20 ground glass homogenizer containing 0.2mL of Dulbecco's phosphate buffered saline lacking calcium and magnesium and containing 1% protease inhibitors (Halt Protease Inhibitor Cocktail, Thermo Scientific Pierce Protein Biology Products). Homogenization was carried out for 20-30 sec using a drill-mounted ground glass pestle and then subject to three freeze thaw cycles. Homogenates were diluted 1:200 and CoQ10 content determined using a Human coenzyme Q10 ELISA Kit (MyBioSource, Inc. # MBS701260).

Microarray analysis (mRNA expression)

Total RNA was prepared using an RNeasy Fibrous Tissue Kit (Qiagen) according to kit instructions using approximately 25 mg biopsy tissue. Tissues were homogenized with an Omni TH handheld homogenizer and hard tissue probe (Omni International). Purified RNA samples were submitted to the University of Iowa Institute of Human Genetics Genomics Division for quality assessment and gene expression array analysis. RNA quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). 100 nanograms of total RNA was converted to amplified Biotin-aRNA using the Epicentre TargetAmp-Nano Labeling Kit for Illumina Expression BeadChip (Illumina, Inc., San Diego, CA, Cat. #TAN07924) according to the manufacturer's protocol. The amplified product was purified through a QIAGEN RNeasy MinElute Cleanup column (QIAGEN Cat #74204). 750 ng purified product was mixed with Illumina hybridization buffer, placed onto Illumina Human-HT12 v4 BeadChips (Part No. BD-103-0204), and incubated at 58°C for 17h with rocking in an Illumina Hybridization Oven. Following hybridization, the arrays were washed, blocked, and stained with streptavidin-Cy3 (Amersham/GE Healthcare,

Piscataway, NJ) according to the Illumina Whole-Genome Gene Expression Direct Hybridization Assay protocol. BeadChips were scanned with the Illumina iScan System (ID #N0534) and data collected using the GenomeStudio software v2011.1.

mRNA expression data were examined by pathway analyses using Gene Set Enrichment Analysis (GSEA) available online through the Broad Institute. Data were analyzed using the KEGG and Reactome databases. In addition, we compared our pathway results to expression data collected for other muscle disorders. Specifically, we used the Gene Expression Omnibus (GEO) to access previously reported expression results for Duchenne muscular dystrophy (GEO Accession Number: GSE6011) (22), polymyositis and dermatomyositis (GEO Accession Number GSE48280) (23), and for skeletal muscle atrophy (GEO Accession Number: GSE28016) (24) and examined these data using GSEA.

Further we used the HGMC (HUGO Gene Nomenclature Committee) resource (<http://www.genenames.org/>) to identify and examine the expression of specific nuclear genes of interest including 82 within the mitochondrial respiratory chain complex gene family and 25 within the extracellular matrix (ECM) proteoglycan family.

Quantitative PCR

To confirm microarray-detected changes in expression of selected genes, we carried out qPCR using reverse transcribed cDNA from the quadriceps muscle mRNA isolated from the biopsy specimens. Probe and primer sequences (supplemental table 1) were obtained from Integrated DNA Technologies (IDT, Coralville, IA) and PrimeTime® 5' nuclease probe assays were carried out as described by IDT. Single-stranded cDNA was synthesized from 200 ng of total RNA in a 20 uL reaction volume using an Applied Biosystems High Capacity cDNA kit (# 4368814). Real-time qPCR (RT-PCR) was carried out in 96 well PCR plates using an Applied Biosystems 7500 RTPCR System. Reaction volumes (20 µL) included Applied Biosystems TaqMan® Gene Expression Master Mix (#4369016), 4 ng of cDNA template, 250 nM probe, and 500 nM primers. Cycling parameters were: one cycle of 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Cycle threshold values were determined automatically for each plate using the Applied Biosystems Model 7500 instrument software (SDS v1.4) set to auto baseline and auto threshold functions.

Relative expression (post-statin/pre-statin therapy) was determined for each transcript using the 2^{-C_T} method (25) normalizing to individual pre- and post-statin C_T values determined as the mean for three control probes including ubiquitin C (UBC), beta-2 microglobulin (B2M), and beta-actin (ACTB) whose expression did not differ as an effect of statin therapy (mean post/pre relative expression of the three probes 0.985).

Serum and plasma analyses

C-peptide was determined by radioimmunoassay using a Human C-Peptide RIA kit (EMD Millipore). Hemoglobin A1c, thyroid stimulating hormone (TSH), and lipid panel (HDL cholesterol, total cholesterol, triglycerides, and LDL cholesterol) were determined by the clinical chemistry laboratory at our institution using standard methodology as applied in the clinical setting.

Citrate synthase

Enzyme activity was quantified using a Human Citrate Synthase Activity Assay Kit (Abcam). Citrate synthase activity was determined in the same whole biopsy homogenates used for the CoQ ELISA. These homogenates were further diluted 1:5 with extraction buffer and cleared by centrifugation prior to loading in assay wells.

Glycated albumin

Glycated albumin was measured in undiluted plasma using a Human Glycated Albumin (GA) ELISA kit (MyBioSource, Inc. # MBS729387).

Statistics

Data were analyzed by paired t-test or one way ANOVA with repeated measures within individual participants as described in the text or figure legends. A 1-tailed t-test was used for hypothesized reductions in citrate synthase, resting energy expenditure, VO_2 and VCO_2 ; otherwise a 2-tailed test was used. Chi square analysis was performed to assess the proportion of gene expression values as repressed or induced as described in the text or table legends.

RESULTS

Baseline characteristics and effects on plasma lipid concentrations

As was true prior to enrollment, all subjects tolerated statin therapy without muscular or other symptoms; thereby, enabling assessment of study parameters in the absence of clinical myopathy. Baseline characteristics and the effects of simvastatin on body mass, lipids, and creatine kinase are depicted in table 1. As expected, simvastatin therapy markedly lowered both LDL and total cholesterol (table 1). No significant changes were seen in HDL cholesterol, triglycerides or creatine kinase. Glycated albumin, which depends on glucose control for the prior two weeks, was not significantly altered by simvastatin therapy.

Bioenergetics

Citrate synthase, a marker of mitochondrial function and/or content, was measured in muscle tissue samples obtained at biopsy. Enzyme activity was modestly reduced after simvastatin treatment compared to pre-therapy (figure 2).

Whole body REE was mildly reduced after simvastatin therapy (figure 3A). VO_2 , VCO_2 , and respiratory quotient (RQ) were not significantly changed (figures 3B-3D).

Gene expression

Gene expression microarray analyses were carried out on skeletal muscle mRNA from six of the eleven subjects pre- and post-statin therapy (4 male, 2 female). None of these patients experienced myalgias or weakness while on simvastatin, and creatine kinase values in this subset of subjects did not significantly differ (mean $\pm$ SE, 122 ± 33 and 160 ± 60 units/L pre- and post-statin therapy, $p = 0.24$). We used these data to perform gene set enrichment analysis (GSEA), a method for detecting changes in cellular pathways.

Consistent with the above bioenergetic data, GSEA revealed that statins repressed 15 gene sets, all involved in metabolism (table 2). Of particular interest and consistent with our study hypotheses, most of these gene sets promote mitochondrial function. In addition, simvastatin repressed gene sets for steroid hormone biosynthesis and terpenoid backbone synthesis, consistent with on target inhibition HMG-CoA reductase.

Interestingly, statins also induced 24 gene sets, many of which promote inflammation and remodeling of the extracellular matrix (table 3). Because similar changes may be seen in conditions that involve muscle degeneration, we obtained microarray data from previously published studies that investigated effects of Duchenne muscular dystrophy (DMD), polymyositis, dermatomyositis, and fasting on human skeletal muscle, and then used these microarray data to perform GSEA and compare the effects of these muscle disorders to the effects of simvastatin. We found that many of the changes induced by statins also occur in DMD, polymyositis, and dermatomyositis (figure 4). In contrast, there was no overlap with muscle gene sets up or down regulated by fasting (figure 4).

Based on the gene sets representing mitochondrial function and ECM remodeling, we further examined specific transcripts within the microarray results and carried out confirmatory qPCR. We focused on all the transcripts within the gene families encompassing mitochondrial oxidative phosphorylation complexes and ECM proteoglycans as defined by the HGMC website. Our microarray data included 81 of 82 mitochondrial respiratory complex genes and all 25 ECM proteoglycans. By microarray, 77 of 81 of the respiratory complex (oxphos) gene transcripts were repressed while 20 ECM transcripts were induced and 5 minimally repressed (figures 5A and 5B and supplementary table 2). We then carried out qPCR for selected oxphos or ECM proteoglycan gene transcripts, the results of which were highly consistent with our microarray findings showing changes in expression in the same directions (figure 5C, table 4).

Regarding the microarray data, although genes within the above mentioned groups were generally induced or repressed, there was no marked trend for mRNA transcripts to be altered in either direction. Overall 17,039 transcripts were induced and 17,562 repressed (complete data for all transcripts listed in supplemental Excel file).

Coenzyme Q content

CoQ content was determined by HPLC in extracts from vastus lateralis muscle mitochondria and circulating platelets. CoQ content was also measured in extracts of intact vastus lateralis muscle using the enzymatic technique described above. Simvastatin therapy did not alter muscle mitochondrial or tissue CoQ content or platelet content (figure 6).

DISCUSSION

The greater risk of vascular events among persons with diabetes (26) supports recent American College of Cardiology/American Heart Association guidelines listing diabetes as one of four clinical categories wherein statin therapy appears particularly important (27). On the other hand, their widespread use implies that we understand even subtle metabolic anomalies that might be associated with their use.

One explanation for the effect of statins to increase glucose may be that these agents impair mitochondrial function. This has been reported to manifest as decreased TCA enzyme content (7), impaired electron transport involving both complexes I and II (8, 28), increased lactate to pyruvate ratios (29), impaired skeletal muscle mitochondrial calcium signaling (28, 30), and mitochondrial apoptosis (2). Evidence for a statin-induced reduction in human muscle CoQ content are controversial with tissue levels reportedly increased (31), decreased (7), possibly decreased (9), or unchanged (32). Another study reported a decrease in platelet CoQ as well as a decrease in plasma CoQ (33). At the whole body level statins prevented the beneficial effect of aerobic exercise to increase peak oxygen consumption (VO_2) (34) and were reported to increase the respiratory exchange ratio (RQ) interpreted by the authors as impaired fat metabolism (35). Further, post exercise phosphocreatine recovery time was increased in hypercholesterolemic subjects after a 4-week regimen of statin therapy compared to pre-treatment (36).

Here we assessed certain bioenergetic markers of whole body and mitochondrial metabolism as well as gene expression in subjects pre- and post-treatment for one month with simvastatin 20 mg day. Our studies are unique in that we focused on asymptomatic individuals with reasonably well controlled type 2 diabetes and included microarray studies of global gene expression. All subjects were treated in like fashion with a moderate dose of simvastatin. Creatine kinase was not elevated, as is commonly the case even in subjects reporting myalgia (37).

In spite of the lack of symptoms or creatine kinase elevations, our metabolic data is suggestive of subclinical myopathy as evidenced by the decrease in muscle citrate synthase (figure 2) and the pattern of statin-induced gene pathway repression (table 2). In addition, we observed a reduction in whole body REE (figure 3A), a finding consistent with mitochondrial dysfunction.

Although our metabolic results were modest in magnitude, they are in agreement with other reports. Paiva, et. al. (7) reported that citrate synthase activity was reduced after treatment of 12 subjects with atorvastatin or simvastatin. In another study, Mikus, et. al. (34) described a differential effect of exercise to increase citrate synthase activity and peak VO_2 in 18 subjects in the absence of statin therapy, as opposed to a decrease in these parameters in subjects randomly assigned to simvastatin, 40 mg/day. Hence, our results together with other studies, also involving small numbers of subjects, imply subtle statin-induced metabolic abnormalities.

Our microarray data for specific gene transcripts (figures 5A and B and supplemental table 2) data are consistent with this concept demonstrating a consistent reduction in the expression of transcripts encoding mitochondrial respiratory complexes II through IV. Moreover, these data are supported by our qPCR studies (figure 5C and table 4).

We also observed an interesting pattern of statin-induced up regulation of several pathways (figure 4 and table 3) and specific genes (figures 5B and 5C, table 4, and supplemental table 2) that have been implicated in tissue remodeling. These include ECM receptor interaction, focal adhesion, actin cytoskeleton, complement and coagulation cascades, and calcium

signaling as defined by the Kegg database; as well as integrin cell surface interactions, collagen formation, and extracellular matrix organization as defined by the Reactome database. We can only speculate as to the reason for this pattern, but this could occur secondary to metabolic or inflammatory insult (38, 39).

In addition to the above, we observed an interesting pattern of statin-induced up regulation of pathways also involved in certain muscular disorders (figure 4), in particular, DMD. Of course, the implications of this are vague at present. The major defect in DMD involves dysfunction of a mutant dystrophin protein and DMD is generally associated with severe myopathy as opposed to what can only be considered subclinical effects of simvastatin. Nonetheless, the shared gene pattern is intriguing and consistent with the overall concept of subtle statin-induced myopathy. Interestingly, there was no signature overlap between statin therapy and fasting; the latter representing a condition associated with muscle atrophy without inflammation or myalgia.

Any interpretation of our results must keep in mind that all our subjects had type 2 diabetes, which in itself is associated with mitochondrial dysfunction (40, 41). However, we point out that our studies involved each subject as their own control, so the perturbations we observed cannot be due to diabetes *per se*; although, it is possible that underlying diabetes created a degree of susceptibility to mitochondrial dysfunction.

Currently, there is only limited data regarding statin-induced myopathy in persons with diabetes and mechanisms are not clear. In the Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) trial, 633 participants with diabetes assigned to 80 mg daily of simvastatin were found to have a relative risk for myopathy of 1.7 compared to 5398 subjects without diabetes (42). There is also evidence that the risk of statin-induced rhabdomyolysis, although rare, is greater in persons with diabetes (43). It is prudent to note that statin intolerance may be more problematic in diabetes because of co-morbid conditions such as obesity and vascular disease and the need for potentially interacting additional drugs. Moreover, LDL targets and current guidelines commonly lead physicians to prescribe higher doses of statin drugs to persons with diabetes compared to those without the disorder.

We did not find differences in muscle tissue or mitochondrial CoQ content after statin therapy compared to pre-treatment. It has been argued that inhibition of HMGCoA reductase is of greater consequence towards blocking cholesterol synthesis than that of CoQ because squalene synthetase (in the cholesterol but not CoQ pathway) has a lower affinity for farnesyl-PP (which is proximal to both pathways) (44). Nonetheless, blood CoQ levels were found lower after statin intervention in 21 of 24 studies reviewed and tabulated (6). On the other hand, the effect of statin therapy on skeletal muscle CoQ content is quite controversial. For example, CoQ content was reportedly increased (31), decreased (7, 8), possibly decreased (9), or unchanged (32) while the effect of CoQ treatment on myopathy has generated contradictory results (45). We are aware of no reports of CoQ levels in mitochondria *per se* of muscle from statin treated subjects or of reports of muscle tissue CoQ content from diabetic subjects treated with statins. Interestingly, there is some data

suggesting that CoQ content is reduced in diabetes; although, this refers only to circulating, as opposed to tissue concentrations (10, 11).

Our study is limited in that we examined a small number of subjects and examined pre- and post-treatment global gene expression in only six. Another limitation is that we cannot conclude that mitochondrial function is limited because of decreased organelle numbers per unit muscle mass or because of dysfunction of individual mitochondria. Citrate synthase is generally considered a marker for mitochondrial content, although it could also reflect the status of individual mitochondria. If CoQ is unchanged and citrate synthase is reduced and we use citrate synthase as a marker of mitochondrial mass, we are led to conclude that CoQ content is increased. However, this does seem plausible since HMG-CoA reductase inhibition should, if anything, reduce CoQ. More likely there is too much variation in the values (particularly CoQ) for the ratio of CoQ to citrate synthase to be meaningful. An additional limitation is that although our metabolic data and finding of ECM gene induction raise the possibility of a subclinical myopathy, we do not have histologic data demonstrating this. A further limitation is that we cannot be sure that our baseline (pre-statin) findings are, in fact, representative of baseline data since the studies were done after discontinuing statins for two months. It is conceivable that different results might have been obtained with a longer time off treatment or in subjects who had never received statin therapy. Likewise, we cannot be sure that different results might have been obtained if statin therapy were continued for longer than one month. Finally, our results are limited to subjects with type 2 diabetes and cannot be extended to non-diabetic individuals.

In summary, we provide intriguing metabolic data and gene expression analyses derived from a small number of asymptomatic subjects with type 2 diabetes suggesting that statin treatment induces mild metabolic perturbations at the mitochondrial level associated with up regulation of genes involved in the ECM; changes that raise the possibility of a subtle subclinical myopathy. On the other hand, we emphasize that the overall benefits of statin drugs in type 2 diabetes are clear. Nonetheless, there is a need to further clarify statin effects on muscle tissue which should contribute to their tolerability and safe use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CoQ	Coenzyme Q
HMG-CoA	Hydroxy-methylglutaryl coenzyme A

HbA1c	Hemoglobin A1c
LDL	low density lipoprotein
HDL	High density lipoprotein
REE	Resting energy expenditure
CRU	Clinical Research unit
SV1	Study visit 1
SV2	Study visit 2
HPLC	High pressure liquid chromatography
GSEA	Gene Set Enrichment Analysis
GEO	Gene Expression Omnibus
DMD	Duchenne muscular dystrophy
TSH	Thyroid stimulating hormone
RQ	respiratory quotient
qPCR	quantitative polymerase chain reaction
ECM	extracellular matrix

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Figure 1.
Schematic depiction of study protocol.

Figure 2.

Vastus lateralis muscle tissue citrate synthase activity pre-and post-treatment with simvastatin. p value depicts significance pre vs post by one tailed, paired t-test, n=9.

Figure 3.

Whole body resting energy expenditure (REE) and parameters of gas exchange pre-and post-treatment with simvastatin. Panel A) REE depicted in individual subjects. Panel B) VO_2 . Panel C) VCO_2 . Panel D) RQ. Data represent mean $\pm$ S, n = 10. p values depict significance pre vs post by one tailed, paired t-test, n = 10.

Figure 4.

Overlap of mRNA pathway expression signatures between statin effects in our subjects (n=6) and signatures reported for the states indicated as listed in the Gene Expression Omnibus. Numbers up and down refer to pathways altered at $p < 0.05$. Area of individual circles is proportional to number of pathways altered. DMD = Duchenne Muscular Dystrophy.

Figure 5.

Fold change (post-statin/pre-statin) in mRNA transcripts encoding genes classified as within families defined by the HGNC (HUGO Gene Nomenclature Committee). Panel A) Changes by microarray analysis in expression of 81 of the 82 genes (one gene not in our array) defined by HGNC as members of mitochondrial respiratory chain (MRC) complexes. Panel B) Changes by microarray analysis in expression of 25 genes defined by HGNC as extracellular matrix (ECM) proteoglycans. Panel C) Independent qPCR verified changes in expression of selected genes within the MRC or ECM families of panels A and B. All gene transcripts analyzed were chosen *a priori* and data for all selected genes are shown. Each dot in panels A and B represent mean values determined using mRNA from 6 subjects (post-statin/pre-statin). Specific values and gene symbols are listed in supplemental table 2. Data in panel C represent mean values from 5 subjects (post-statin/pre-statin). Specific values and gene symbols are listed in table 4. The p values refer to the probability of the distribution of these genes as above or below the expected chance distribution (half above and half below 1.0) by chi square analysis.

Figure 6.

CoQ content within vastus lateralis muscle tissue, isolated vastus lateralis muscle mitochondria, and circulating platelets pre-and post-treatment with simvastatin. Panel A) CoQ expressed per unit mass of vastus lateralis muscle. Panel B) CoQ per unit mitochondrial protein. Panel C) CoQ per unit platelet protein. Data represent mean $\pm$ SE, n = 10-11. No significant differences were observed.

Table 1

Baseline characteristics of study participants and parameters determined pre- and post-simvastatin therapy.
Data represent mean $\pm$ SE.

Parameter	Baseline Value	Pre-Treatment	Post-Treatment
Total cholesterol (mg/100 ml)	152 $\pm$ 10 *	195 $\pm$ 8	141 $\pm$ 8 *
LDL cholesterol (mg/100 ml)	76 $\pm$ 6 *	113 $\pm$ 8	63 $\pm$ 5 *
HDL cholesterol (mg/100 ml)	44 $\pm$ 2	44 $\pm$ 2	45 $\pm$ 2
Triglycerides (mg/100 ml)	154 $\pm$ 30	193 $\pm$ 35	177 $\pm$ 41
Creatine kinase (Units/L)†	ND	120 $\pm$ 20	150 $\pm$ 34
Hemoglobin A1c (%)	7.12 $\pm$ 0.18	ND	ND
Glycated Albumin (ng/ml)	ND	184 $\pm$ 37	192 $\pm$ 37
Age	63 $\pm$ 1, range 56-67	NA	NA
Weight (kg)	99.0 $\pm$ 5.7	99.2 $\pm$ 5.78	98.9 $\pm$ 5.7
BMI	34.1 $\pm$ 1.9	ND	ND
Gender	5 female, 6 male	NA	NA
Thyroid Stimulating Hormone (μ IU/ml)	1.63 $\pm$ 0.33	ND	ND

Data were analyzed by one-way ANOVA with repeated measures or by paired t-test for pre-versus post-therapy, n = 11.

* p < 0.001 compared to pre-therapy.

Table 2

GSEA analysis of microarray data depicting gene sets repressed by one month of simvastatin therapy at $p < 0.05$.

Gene Set	Size	Database	p-value
Beta Alanine Metabolism	22	KEGG	0.026
Terpenoid Backbone Biosynthesis	15	KEGG	0.039
Valine Leucine and Isoleucine Degradation	43	KEGG	0.021
Oxidative Phosphorylation	116	KEGG	0.003
Steroid Hormone Biosynthesis	54	KEGG	0.042
TCA Cycle and Respiratory Electron Transport	119	REACTOME	0.000
Respiratory Electron Transport	64	REACTOME	0.004
Respiratory Electron Transport and ATP Synthesis by Chemiosmotic Coupling	80	REACTOME	0.007
Amine Derived Hormones	15	REACTOME	0.044
Pyruvate Metabolism and Citric Acid TCA Cycle	44	REACTOME	0.009
Lagging Strand Synthesis	17	REACTOME	0.045
Na Cl Dependent Neurotransmitter Transporters	17	REACTOME	0.044
Citric Acid Cycle TCA Cycle	23	REACTOME	0.046
Mitochondrial Protein Import	49	REACTOME	0.034
Class B 2 Secretin Family Receptors	85	REACTOME	0.046

Table 3

GSEA analysis of microarray data depicting gene sets induced by one month of simvastatin therapy at $p < 0.05$.

Gene Set	Size	Database	p-value
ECM Receptor Interaction	81	KEGG	< 0.001
Focal Adhesion	191	KEGG	< 0.001
Glycosaminoglycan Biosynthesis Chondroitin Sulfate	15	KEGG	0.002
Hematopoietic Cell Lineage	87	KEGG	0.002
Complement and Coagulation Cascades	43	KEGG	0.009
Regulation of Actin Cytoskeleton	202	KEGG	< 0.001
p53 Signaling Pathway	65	KEGG	0.009
Calcium Signaling Pathway	172	KEGG	< 0.001
Leukocyte Transendothelial Migration	110	KEGG	0.008
Chemokine Signaling Pathway	184	KEGG	0.038
Integrin Cell Surface Interactions	78	REACTOME	< 0.001
Collagen Formation	58	REACTOME	< 0.001
Generation of Second Messenger Molecules	27	REACTOME	0.005
Extracellular Matrix Organization	86	REACTOME	< 0.001
Cell Surface Interactions at the Vascular Wall	86	REACTOME	< 0.001
Semaphorin Interactions	64	REACTOME	0.002
EGRF Downregulation	24	REACTOME	0.011
Glucose Transport	38	REACTOME	0.005
Phosphorylation of CD3 and TCR Zeta Chains	16	REACTOME	0.026
NCAM1 Interactions	37	REACTOME	0.014
TCR Signaling	51	REACTOME	0.007
Immunoregulatory Interactions Between a Lymphoid and a Non Lymphoid Cell	65	REACTOME	0.005
Signaling by PDGF	112	REACTOME	0.017
Triglyceride Biosynthesis	32	REACTOME	0.049

Table 4

Listing and numerical values for the change in expression (pre-statin/post-statin therapy) by qPCR of the genes depicted in figure 5, panel C.

Mitochondrial Respiratory Chain	Relative expression [†]	p value
Complex I		
NDUFA12	0.978	
NDUFA9	0.926	
NDUFS2	0.945	
Complex II		
SDHB	0.885	*
SDHC	0.887	
Complex III		
UQCRC1	0.967	
UQCRCF1	1.014	
Complex IV		
COX5B	0.887	
COX6A2	0.909	*
COX6B1	0.952	
Complex V		
ATP5C1	0.878	**
ATP5G1	0.868	
ATP5G2	0.903	

Extracellular Matrix Proteoglycans	Relative expression [†]	p value
Small leucine-rich repeats		
DCN	1.413	**
LUM	1.631	*
Collagen proteoglycans		
COL12A1	1.614	**
COL15A1	1.135	
Hyalactans		
VCAN	1.572	

[†] relative to pre-statin values normalized to 1.0

* p < 0.1

** p < 0.05 by 2-tailed, paired t-test