Muscle Phenotype and Mutation Load in 51 Persons With the 3243A>G Mitochondrial DNA Mutation

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**Background:** Mitochondrial disorders are generally not associated with a clear phenotype-genotype relationship, which complicates the understanding of the disease and genetic counseling.

**Objective:** To investigate the relationship between the muscle and blood mitochondrial DNA mutation load and phenotype.

**Design:** Survey.

**Setting:** The Neuromuscular Research Unit, Rigshospitalet, Copenhagen, Denmark.

**Participants:** Fifty-one persons with the 3243A>G point mutation of mitochondrial DNA, and 20 healthy control subjects.

**Methods:** We recorded the maximal oxygen uptake (V\textsubscript{O\textsubscript{2}}\text{max}), maximal workload, resting and peak-exercise plasma lactate levels, muscle and blood mutation load, muscle morphology, and presence of diabetes mellitus and hearing impairment in all subjects.

**Results:** Muscle mutation load (mean±SE, 50%±5%; range, 2%-95%) correlated with V\textsubscript{O\textsubscript{2}}\text{max} and resting plasma lactate level (P<.001; R=0.64). All persons except 5 with a muscle mutation load above 50% had abnormal V\textsubscript{O\textsubscript{2}}\text{max} and morphology on muscle biopsy findings. Persons with hearing impairment and diabetes mellitus had a muscle mutation load above 65%. The mutation load in blood (mean±SE, 18%±3%; range, 0%-61%) did not correlate with V\textsubscript{O\textsubscript{2}}\text{max}, resting plasma lactate levels, or presence of hearing impairment or diabetes mellitus.

**Conclusions:** This study demonstrates a close relationship between the muscle mutation load and phenotype in persons carrying the 3243A>G mutation. The lack of correlation between the mutation load in blood and symptoms from other tissues emphasizes the importance of assessing phenotype-genotype correlations in the same tissue in mitochondrial disease. The results indicate that the threshold of muscle mutation load at which oxidative impairment occurs can be as low as 50%, which is as much as 40% lower than that suggested by in vitro studies.

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Since the discovery of the first mitochondrial DNA (mtDNA) mutations in 1988,1,2 more than 200 disease-causing mutations in the mtDNA have been discovered. The spectrum of phenotypes associated with these mutations is extensive, although these mutations generally lead to the same cellular dysfunction, ie, impaired oxidative phosphorylation. Pathogenic mtDNA mutations usually coexist with wild-type (normal) mtDNA in cells, even within the same mitochondrion: a condition called heteroplasmy. The wide spectrum of phenotypes associated with mitochondrial disease can partly be explained by the high variability of the mtDNA mutation load in different tissues. It is generally believed that symptoms emerge in a tissue when the level of wild-type mtDNA can no longer support a sufficient production of respiratory chain enzymes and, thus, adenosine triphosphate production. Based on findings in vitro, the threshold level for mutation load at which symptoms emerge has been set at 90% in tissues carrying the 3243A>G point mutations of mtDNA.3

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The high variation in mutation load among the tissues in mitochondrial diseases suggests that the assessment of phenotype and genotype must be performed in the same tissue. It is therefore not surprising that previous attempts in persons with point mutations of mtDNA to correlate the mutation load in one tissue with the phenotype of another tissue have generally failed.4-20

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Skeletal muscle is known to carry a relatively high mutation load in mitochondrial diseases caused by mtDNA mutations. During exercise, skeletal muscle can increase its oxygen demand up to 100-fold, which is unmatched by any other tissue. Mitochondrial myopathy with exercise intolerance, premature exertional dyspnea, and lactic acid buildup is therefore one of the most frequent symptoms in patients with mtDNA mutations. In this study, we tested 51 persons, all harboring the common 3243A>G point mutation of mtDNA, to investigate the mutation type–specific correlation between the skeletal muscle mutation load and indices of oxidative capacity, ie, maximal oxygen uptake (VO₂max), maximal workload (Wmax), plasma lactate levels, muscle morphology, and mitochondrial enzyme activity levels. Muscle mutation load was also correlated with symptoms of hearing impairment and diabetes mellitus that arise in 2 other postmitotic tissues. To investigate the importance of assessing the phenotype and mutation load in the same tissue, we also studied the relationship between the blood mutation load and the symptoms from skeletal muscle and 2 other postmitotic tissues in the 51 subjects.

METHODS

SUBJECTS

We included in the study 51 persons (22 men and 29 women) from 13 families with the 3243A>G point mutation of mtDNA. The mean±SD demographic data of the patients were age of 40±15 (range, 13-74) years, weight of 66±15 (range, 43-96) kg, and height of 169±8 (range, 158-186) cm. To study a wide range of mutation load in muscle, we also investigated relatives from 9 of the 13 families. Genetic investigation of muscle from 42 family members revealed that 38 relatives carried at least 2% of the 3243A>G mtDNA mutation in muscle. These 38 relatives were included in the study, along with the 13 probands. The findings in the persons with the 3243A>G mutation were compared with findings in 20 healthy sedentary control subjects (10 men and 10 women) with a mean±SD age of 39±13 years, weight of 74±14 kg, and height of 174±8 cm. Fourteen of the 51 persons with the 3243A>G mutation in muscle had diabetes mellitus and were treated with insulin or diet. An oral glucose tolerance test was performed in 29 of the remaining 37 persons. A neurological examination was performed in the 51 persons carrying the 3243A>G mutation, and they were interviewed about clinical symptoms related to hearing impairment, stroke-like episodes, ataxia, and the presence of exercise intolerance. The presence of exercise intolerance was based on self-reported symptoms of impaired work capacity (dyspnea during mild exercise, premature fatigue, or muscle pain or cramps) or on clear evidence in the medical history of problems in running, cycling, walking stairs, or participating in school gym lessons. Apart from the insulin and antiepileptic treatments in 4 persons, no subject took any medication.

The study was approved by the scientific ethical committee of Copenhagen, Denmark. The subjects were all informed about the nature and risks of the study and gave written consent to participate.

PREEXPERIMENTAL PREPARATIONS

All subjects reported to the laboratory at 9 AM after an overnight fast and ingestion of a breakfast that contained approximately 100 g of carbohydrates 2 hours before the experiment. The subjects did not consume any alcohol or caffeine for 12 hours and did not perform any exercise for 24 hours before the experiment. Resting lactate levels were evaluated after 45 minutes of rest in the supine position.

EXPERIMENTAL PROTOCOL

All subjects underwent studies on a cycle ergometer (Medi- Graphics CPE 2000; Medical Graphics Corp, St Paul, Minn) operated via a cardiopulmonary exercise test system (Med- Graphics CPX/D; Medical Graphics Corp) that measured gas exchanges, workload, and heart rate. We determined VO₂max and Wmax in all subjects by means of an incremental exercise test to exhaustion. We applied 2- to 3-W increments every other minute in the maximum test for patients with mitochondrial disease and a medical history of severe exercise intolerance, 5- to 15-W increments every other minute for patients with a medical history of moderate exercise intolerance, and 15- to 25-W increments every other minute in unaffected relatives and healthy controls. These increments in workload were applied to reach a similar duration of the cycle test in all subjects. Blood samples were collected from the median cubital vein at rest and at exhaustion.

MUSCLE BIOPSY

A needle biopsy of the left lateral vastus muscle was performed in the 13 probands, the 38 relatives, and in all healthy controls. The biopsy specimen was frozen immediately after sampling in isopentane, cooled in liquid nitrogen, and stored at −80°C until genetic, biochemical, and morphological analyses.

The muscle biopsy specimen was stained with trichrome, succinate dehydrogenase, and cytochrome oxidase (COX), and we assessed the number of ragged red/blue fibers and COX-negative fibers.

MOLECULAR GENETIC INVESTIGATIONS

Total genomic DNA from muscle and blood was isolated using a DNA kit (QIaAMP DNA Mini Kit; QIAGEN GmbH, Hilden, Germany) and following the manufacturer’s protocol. An allele-specific polymerase chain reaction (PCR) assay was designed, allowing the detection of the 3243A>G mutation by a direct PCR reaction. Two sets of primers were used in 1 reaction tube: 3014L (forward) GTCGACGGCTATTAAAGGT and 3262HM17 (reverse with 2 mismatches) TTTATGGATTACCCGCAGC (PCR product, 249 base pairs). The control product was coamplified using the following primers: 3289-5309 (forward) and 5903-3884 (reverse) (PCR product, 614 base pairs).

QUANTIFICATION OF MUTANT mtDNA

The ratio between the wild-type mtDNA (3243A) and mutant mtDNA (3243G) was determined using solid-phase minisequencing.21,22 The PCR products spanning the position in question were generated by the 5'-biotinylated primer (3014-3033) and the primer (3376-3337). The PCR products were immobilized on a streptavidin-coated solid support (96-well plate) and denatured by sodium hydroxide. To quantify the ratio of 3243A and 3243G in the sample, the sequencing primer (3263-3244) was designed to anneal adjacent (downstream) to nucleotide 3243. The nucleotide at the site of the mutation was analyzed by primer extension reaction, in which a tritium-labeled deoxyribonucleotide triphosphate corresponding to the 3243A (deoxythymidine triphosphate) or the 3243G (deoxy- cytidine triphosphate) nucleotide was added to 2 parallel re-
actions. After washing, the elongated primers were eluted by sodium hydroxide, and the amount of incorporated tritium-labeled deoxyribonucleoside monophosphate was determined using a liquid scintillation counter. The ratio of T to C incorporated into each sequencing primer was determined and compared with a standard curve constructed using known proportions of cloned mitochondrial 3243A and 3243G.

**BIOCHEMICAL ANALYSES**

Venous blood was sampled in syringes containing 50 µL of 0.33M EDTA solution, immediately spun at 4°C, and studied for lactate levels on a commercially available analyzer (YSI model 2300 STAT Plus; Bie & Berntsen, Rødovre, Denmark).

Mitochondrial enzyme activities of citrate synthase and complexes I to IV were measured in postnuclear supernatants of 30 µg of frozen muscle in 36 of the 51 persons carrying the 3243A>G mutation as previously described.23

**STATISTICAL ANALYSES**

Differences between persons carrying the 3243A>G mtDNA mutation and healthy controls were evaluated by a t test. We considered P<.05 (2-tailed testing) statistically significant. Correlation between the muscle mutation load and indices of oxidative capacity, i.e., VO₂, Wmax, exercise-induced increases in plasma lactate levels, and biochemical and morphological findings from the muscle biopsy specimens, were evaluated by the Pearson product moment correlation test using Sigma Stat software (SPSS Inc, Chicago, Ill). Unless otherwise indicated, all values are expressed as mean ± SE.

**RESULTS**

**CORRELATION BETWEEN VO₂max AND Wmax AND MUTATION LOAD**

The mean VO₂max was 1905±117 (range, 702-3124) mL/min and the mean Wmax was 121±9 (range, 21-284) W in the 51 persons carrying the 3243A>G mtDNA mutation in muscle (Figure 1). These values were 40% and 60% lower, respectively, than in healthy controls (2731±162 mL/min and 202±11 W, respectively; P<.001).

The percentage of mutation in the skeletal muscle ranged from 2% to 95% (50%±5%). The VO₂max correlated inversely with the percentage of mtDNA mutation load in skeletal muscle (R=0.64; P<.001) (Figure 1). The Wmax also correlated with the percentage of heteroplasmy in patients with mitochondrial myopathy (R=0.61; P<.001). Besides training status and mitochondrial function, the VO₂max depends on sex and age. Owing to these possible influences, we adjusted the VO₂max for age and sex, but this correction did not improve the unadjusted correlation between the muscle mutation load and muscle oxidative capacity (R=0.61; P<.001).

**CORRELATION BETWEEN INDICES OF OXIDATIVE CAPACITY AND MUTATION LOAD IN MUSCLE AND BLOOD**

The resting plasma lactate level was higher in persons with the 3243A>G mutation (18.9±1.8 mg/dL [2.1±0.2 mmol/L]; range, 7.2-46.0 mg/dL [0.8-5.1 mmol/L]) than in healthy controls (9.9±0.9 mg/dL [1.1±0.1 mmol/L]; P<.001) (Figure 2), but at peak exercise, plasma lactate levels rose to similar levels in both groups (3243A>G subjects, 70.3±3.6 mg/dL [7.8±0.4 mmol/L]; healthy controls, 75.7±4.5 mg/dL [8.4±0.5 mmol/L]). In line with the VO₂max, resting plasma lactate levels correlated with the mutation load in skeletal muscle (R=0.81; P<.001) by a first polynomial order (Figure 2).

On muscle biopsy findings, 25 of the 51 persons with the 3243A>G mutation had more than 2% ragged red/blue and/or COX-negative fibers, 10 had ragged red/blue and COX-negative fibers, 11 had only ragged red/blue fibers, and 4 had only COX-negative fibers.

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**Figure 1.** Correlation between absolute maximal oxygen uptake (VO₂max) and the percentage of the mutation load in skeletal muscle of 51 persons with the 3243A>G mutation of mitochondrial DNA. Triangles each represent a person carrying the 3243A>G mutation. The cross at 0% mutation load represents the mean±SE of 20 healthy control subjects. The individual VO₂max in the persons with the 3243A>G mutation was compared with that found in sex- and age-matched healthy controls.

**Figure 2.** Correlation between the muscle mutation load and resting venous plasma lactate concentration in 51 persons (triangles) with the 3243A>G mutation of mitochondrial DNA. The cross at 0% mutation load represents the plasma lactate concentration (mean±SE) in 20 healthy control subjects. The dotted line represents the mean value ± 2 SDs found in the 20 healthy controls. To convert lactate levels to millimoles per liter, multiply by 0.111.
The numbers of COX-negative and/or ragged red/blue fibers in muscle did not correlate with the muscle mutation load, but there was a clear cutoff mutation level at which morphological abnormalities occurred (Figure 3). Thus, all 25 persons with COX-negative and/or ragged red/blue fibers on muscle biopsy findings had mutation loads above 50% in muscle.

Overall, citrate synthase–corrected complex I to IV activities were normal in persons with the 3243A>G mtDNA mutation. Only 4 patients with myopathic symptoms showed impairment of complex I activity, and complex IV activity was impaired in only 2 (data not shown). The absolute or citrate synthase–corrected complex I to IV activities did not correlate with the muscle mutation load.

The percentage of mutant mtDNA in blood ranged from 0% to 61% (mean, 18%±3%). In contrast to the mutation load in muscle, the mutation load in blood did not correlate with the VO₂max (R=0.23; P<.01), the resting plasma lactate levels (R=0.81; P<.001), the level of ragged red/blue and/or COX-negative fibers on muscle biopsy findings, or the mitochondrial enzyme activity.

**HEARING IMPAIRMENT, DIABETES-IMPAIRED GLUCOSE TOLERANCE, AND CLINICAL EVIDENCE OF EXERCISE INTOLERANCE**

Fourteen of the 51 persons carrying the 3243A>G mutation had diabetes mellitus and hearing loss. No other person had diabetes, but an additional 5 persons had abnormal oral glucose tolerance test results (Figure 4). In total, 23 persons had hearing loss, and 13 used hearing aids. Exercise intolerance was diagnosed in 25 of the 51 persons carrying the 3243A>G mutation (Figure 4). All persons with diabetes mellitus carried more than a 65% mutation load in muscle, whereas persons with impaired glucose tolerance had a wider range of mutation load (4%-90%) (Figure 4). Similar to persons with diabetes mellitus, all persons with hearing loss carried more than a 65% mutation load in skeletal muscle (Figure 4).

The 25 persons with exercise intolerance had more than 55% mutant mtDNA in muscle (Figure 4). Persons with hearing impairment, exercise intolerance, or diabetes mellitus had a higher mutation load in muscle compared with asymptomatic persons (P<.001). In contrast to the tight coupling between the mutation load in muscle and symptoms from postmitotic tissues, there was no correlation between the blood mutation load and diabetes mellitus and only a weak association between the blood mutation load and hearing loss and myopathic symptoms (P<.05).

**COMMENT**

It has previously been shown that the type of mtDNA mutation in muscle is important for phenotypic expression of muscle symptoms. However, the functional consequences of variable mutant loads of the same mutation in skeletal muscle have never been investigated in patients with mitochondrial disease carrying a wide range of this mutant load in muscle. This study demonstrates a close relationship between the muscle phenotype and muscle mutation load in persons carrying the 3243A>G point mutation of mtDNA. We also found a relationship between mutation load in skeletal muscle and symptoms from 2 other postmitotic tissues (hearing impairment and diabetes mellitus). In contrast to this, we found no correlation or, at the very best, a weak correlation when symptoms from postmitotic tissues were compared with...
the mutation load in blood, which stresses the importance of comparing the phenotype and genetic abnormalities in the same tissues in disorders caused by mtDNA defects. The study also suggests that the threshold for mutant load in muscle at which symptoms emerge in persons carrying the 3243A→G mutation may be much lower than previously believed. Thus, based on histological and physiological measurements, our findings suggest that symptoms may occur at mutation loads as low as 50%, a figure that is 40% lower than that found in vitro.3

The variable distribution of mutant load among tissues in mitochondrial disease and the weak genotype-phenotype relationship that has previously been found in these conditions has made genetic counseling difficult. Many studies investigating genotype-phenotype relationships in mitochondrial disease have compared mtDNA mutation load in blood with symptoms from postmitotic tissues5,8,15,16,19,20 and found a weak or no correlation. This lack of correlation might be explained by the tissue-specific, differential, postnatal change in the mutation load, with time, as has been reported for most transfer RNA mtDNA mutations.26-30 Although there is evidence that the mutation load increases with time in postmitotic tissues,26-28,30 findings in mitotic tissues such as blood and epithelial cells31-34 indicate that the level of mutant mtDNA decreases with time.

Although the mutation load may differ considerably among mitotic and postmitotic tissues, postmortem findings indicate that the mutation load in postmitotic tissues (skeletal muscle and nervous and cochlear tissues) is similar.35-38 It is therefore surprising that studies correlating the mutation load in skeletal muscle with the phenotype in other postmitotic tissues have not been able to find a relationship. The lack of such an association may be explained by (1) comparison of old clinical information with new genetic findings11,19; (2) use of retrospective data for comparison13,18; (3) use of too small a sample size8,9,15,17,19; (4) investigation of phenotypes in patients carrying a narrow range of the mutation load in muscle (all >80%)12; and (5) the pooling of symptoms from different tissues for comparison.8,10,26 These problems, alone or in combination, seriously interfere with the interpretation of genotype-phenotype relationships in mitochondrial disease and have produced seemingly odd results, such as decreasing myopathy and exercise intolerance with increasing mutant load in muscle.8,10,11,13

In our study, we found a close correlation between the muscle mutation load and muscle phenotype; in addition, we found a close correlation between the muscle mutation load and symptoms from 2 other postmitotic tissues. Thus, all persons with hearing impairment and/or diabetes mellitus carried a mutation load above 65% in muscle (Figure 4). Although our findings on the correlation between the muscle mutation load and symptoms from other postmitotic tissues contradict a number of other studies, a correlation between hearing impairment and the muscle mutation load has been described in 8 and 38 persons carrying the 3243A→G mutation.39,40

Plasma lactate comes primarily from skeletal muscle.41 In adult patients with mitochondrial disease, the resting lactate level is always within reference limits after a night’s rest in a bed.42 Therefore, elevated resting plasma lactate levels in mitochondrial myopathy are always a result of previous exercise, and resting plasma lactate levels are therefore an index of oxidative capacity in line with the VO₂max. Similar to the VO₂max, resting plasma lactate levels in this study correlated inversely with the muscle mutation load in persons carrying the 3243A→G mtDNA mutation (Figure 2).

It is well known that persons carrying the 3243A→G mutation can have impaired complex I and, more rarely, complex IV activities.5,6,11 In this study, only 4 persons with the 3243A→G mutation had impaired complex I activity, and 2 of these persons also had decreased complex IV activity. Complex activities did not correlate with muscle mutation load, which is in agreement with other studies investigating other mtDNA mutations,12,19 whereas 1 study of the 3243A→G mutation found a correlation with complex I.11

Based on in vitro findings, it is generally believed that the mtDNA mutation load needs to exceed a certain threshold before symptoms emerge. This mtDNA mutation load threshold has been difficult to investigate in vivo because it requires a large number of persons carrying the same mtDNA mutation and a wide range of the mutation load. In this study, we recruited relatives of the probands with mitochondrial disease and thereby achieved a wide range of mtDNA mutation load in the studied cohort. Impaired VO₂max and ragged red/blue and COX-negative fibers were found in persons with a muscle mutation load of as low as 50% (Figures 1 and 3), and only 4 of 28 persons carrying a muscle mutation load above 50% had a VO₂max within reference limits and normal muscle morphological findings. These findings suggest that the mutation level at which oxidative impairment and muscle symptoms occur seems to be as low as 50% to 65% in persons carrying the 3243A→G mutation. This threshold is more than 30% lower than that suggested by cell culture studies and 10% to 20% lower than that suggested by in vivo studies.24,25

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