

Human Mitochondrial Transcription Factor A Reduction and Mitochondrial Dysfunction in Hashimoto's Hypothyroid Myopathy

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Abstract

Background: Mitochondrial changes have been described in muscle tissue in acquired hypothyroidism. Among the molecular mechanisms by which thyroid hormones regulate expression of nuclear genes encoding for regulatory proteins of mitochondrial respiratory function, the mitochondrial transcription factor A (h-mtTFA) has been proposed to be a target of thyroid hormone action. The aim of this study has been to relate h-mtTFA levels in the skeletal muscle of patients affected by Hashimoto's hypothyroidism and myopathy (HHM) to muscle disease and thyroid status. **Patients and Methods:** Eleven HHM patients underwent complete thyroid status and neurologic assessment, along with determination of exercise lactate anaerobic threshold (LT) and muscle biopsy in which h-mtTFA levels were measured and mtDNA was analyzed.

Results: Decreased exercise lactate threshold, presence of cytochrome c oxidase negative fibers, reduction of cytochrome c oxidase activity, and mitochondrial DNA copy number at muscle biopsy were indicative of mitochondrial involvement in these patients. Furthermore, muscle h-mtTFA levels were reduced to a variable extent in comparison with a group of euthyroid controls. The h-mtTFA levels were inversely correlated with TSH and LT lactate, and positively correlated with FT4.

Conclusions: These results indicate that low levels of the h-mtTFA occur in skeletal muscle of HHM and suggest that abnormal h-mtTFA turnover may be implicated in the pathogenesis of mitochondrial alterations in this disease.

Introduction

Involvement of skeletal muscle is among the most prevalent clinical consequences of hypothyroidism (1). Symptoms such as fatigue, exercise intolerance, exertional myalgias, and cramps are frequent hallmarks of such an involvement (2) and sometimes represent the prodromic signs of hypothyroidism (3,4). Investigation of the physiopathology of skeletal muscle involvement in hypothyroidism has so far failed to identify the mechanisms responsible for hypothyroid myopathy. Although rather disparate results have been reported (5–7), it is widely accepted that mitochondrial respiration represents one of the main targets of thyroid hormone deficiency (2,8).

Recently, attention has been focused on the molecular mechanisms by which thyroid hormones can regulate gene expression of regulatory or structural proteins of mitochondrial respiratory function (9). Among these proteins, mitochondrial transcription

factor A (mtTFA or Tfam) has been shown to be one of the putative targets of thyroid hormones (10). Human mitochondrial transcription factor A (h-mtTFA) is a nuclear encoded polypeptide with high regulatory effects on both replication and transcription of mitochondrial DNA (mtDNA) (10). A pathogenetic role of h-mtTFA deficiency has been reported in some primary mitochondrial disorders with defects of the mitochondrial respiratory chain such as those associated with mtDNA depletion (11). The aim of this work has been to evaluate the levels of h-mtTFA in skeletal muscle of patients affected by Hashimoto's hypothyroid myopathy (HHM) and to relate them to thyroid function as well as to clinical and pathologic aspects of this myopathy.

Patients and Methods

Patients

Eleven consecutive patients (7 women and 4 men, mean age \pm SD, 49.7 \pm 12.1 years) with HHM entered the study. The protocol was approved by the institutional committee on human experimentation. Informed, written consent was obtained from each subject prior to entry into the study.

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Thyroid Status

Serum thyroid stimulating hormone (TSH), free thyroid hormones (FT3, FT4), anti-thyroglobulin antibodies (TgAb), and anti-thyroid peroxidase antibodies (TPOAb) were assessed at 8 AM after an overnight fast. Serum levels of FT3 and FT4 were measured by specific RIAs (Techno-Genetics Recordati, Milan, Italy). Anti-TgAb were measured by a specific IRMA (TGAb IRMA, Biocode, Sclessin, Belgium); anti-TPOAb were measured with a specific RIA (AB-TPO; Sorin Biomedica, Saluggia, Italy). Normal ranges in our laboratory are as follows: FT3, 3.7–8.6 pmol/l; FT4, 7.2–19.3 pmol/l; TgAb, <50 IU/ml; and TPOAb, <10 IU/ml. TSH was determined with an ultrasensitive IRMA method (Cis Diagnostici, Tronzano Vercellese, Italy). The normal range in our laboratory is 0.3–3.6 mIU/l. At the time of the study, no patient was on levothyroxine therapy.

Neurologic Status

All patients had been referred to the neurologist because of symptoms and/or signs suggesting myopathy complicating thyroid disease. In one patient (# 4), a depressive syndrome treated with antidepressant was present. Exercise intolerance with exertional myalgias and cramps, especially in the legs, was the most common symptom. Duration of the muscle symptoms was 6.5 ± 2.7 years. Fixed weakness, mainly in limb-girdle muscles, was evident in three patients (1, 5, and 8) and in two (1 and 9), the clinical picture was suggestive of overt Hoffmann's syndrome. Blood CK levels were abnormally high in all patients (Table 1). The SF-36 Health Survey Questionnaire (12) gave a mean value of 72 (range, 63–84;

maximal value, 145), not statistically different from orthopedic controls (see below).

Exercise Lactate Anaerobic Threshold

To evaluate oxidative metabolism in contracting muscle, resting blood lactate concentration and the anaerobic lactate threshold (LT) during isometric incremental arm exercise were assessed, as previously described (3), and compared to 10 age- and sex-matched normal controls. After having established the maximal voluntary contraction (MVC) of forearm flexor muscles for each patient, by performing three brief maximal efforts on a hand-grip dynamometer at 3-min intervals, the test began with a first bout of 10% of MVC, then continued through successive 10% increments until exhaustion was achieved. Each bout consisted of 1-min intermittent (1/sec) contractions on the hand-grip dynamometer followed by a 2-min rest. LT was set at the exercise step at which the lactate curve took on an exponential trend (13), and was expressed as a fraction of MVC. At this level, the percent increment in lactate levels above baseline was also recorded (LT lactate).

Muscle Biopsy

A muscle biopsy was obtained from the left deltoid under local anesthesia. Muscle specimens were frozen in liquid nitrogen-chilled isopentane, and processed for histologic, histochemical, biochemical, and Southern and Western blot analysis, as described below. Routine stainings for hematoxylin-eosin, Gomori's trichrome, ATPase pH 4.6 and 9.4, as well as oxidative histochemistry for nicotinamide dehydrogenase tetrazolium reductase (NADH-TR), succinate

Table 1. Thyroid status and exercise blood lactate

Patient	Sex/Age	TSH (mIU/l)	FT4 (pmol/l)	FT3 (pmol/l)	Rest Lactate (mmol/l)	LT Lactate*/LT [†]	CK (U/l)
1	F/28	65.10	0.80	1.1	0.36	267/40	1052
2	F/56	62.10	1.70	1.5	0.56	140/40	1423
3	F/61	3.93	11.60	3.4	2.30	116/50	1339
4	F/36	5.24	9.80	4.0	1.97	127/50	232
5	F/66	6.90	8.70	3.8	0.72	190/40	312
6	F/58	5.80	8.80	4.2	1.34	102/50	473
7	F/54	6.30	9.60	4.0	2.70	142/50	582
8	M/60	>100.00	0.60	0.9	0.63	292/40	5645
9	M/42	>100.00	0.38	0.85	1.50	284/40	2017
10	M/40	8.26	7.80	3.7	0.90	201/40	380
11	M/46	4.12	11.00	3.4	2.70	135/50	487
Normal values	—	0.30–3.60	7.20–19.30	3.70–8.60	0.67–2.47	*	25–195

*Lactate value at LT, expressed as percentage of basal lactate (control value, $173 \pm 20.5\%$).

[†]Percentage of MVC at which LT itself was achieved (control value, 60% of MVC).

dehydrogenase (SDH), and cytochrome c oxidase (COX) were performed. For each of five different biopsy fields, serial sections (8 μm) were studied for fiber-type characterization in a number of fibers ranging from 500 to 600 per case. Morphometry was performed by an automatic micrometric optical method at a magnification of 250 \times . The percentage of type 1 and 2 fibers, type 1 and 2 fiber diameters, and atrophy and hypertrophy factors (AF and HF) were calculated (14). Histochemical mitochondrial stain (COX, NADH-TR, and SDH) alterations were assessed by automated imaging analysis (Matlab, Mathworks, Natick, MA, USA) and expressed as percentage of affected fibers among type 1 fibers.

Skeletal muscle levels of h-mtTFA were assessed by Western blot analysis. Proteins were extracted from muscle specimens by boiling buffer (0.05 M DTT, 1 mM EDTA, 0.125 M Tris, 1% SDS, 0.1% bromophenol blue), and the protein concentration was determined with the BCA protein assay (Pierce Chemical, Rockford, IL, USA) using the Lowry method. Thirty micrograms of proteins were separated in SDS-polyacrylamide gels (resolving gel 12%, stacking gel 5%), and then electroblotted onto PVDF membranes (0.45 μm pore size, Millipore, Bedford, MA, USA) (15). Filters were incubated with a rabbit polyclonal antibody against h-mtTFA (a kind gift of Dr D. Clayton) (1:2500). Filters were also incubated with a monoclonal antibody against the stress-related protein Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:250), as internal standard. Bound antibodies were detected with ECL⁺ kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or with alkaline phosphatase methods (Biorad, Hercules, CA, USA). Quantitation was done by densitometric analysis of the h-mtTFA band and values were corrected for both unequal loading and the internal standard.

To study mtDNA, total DNA was extracted from 10–20 mg of frozen muscle homogenates by standard methods. Southern blots were performed with 4 μg of DNA digested with Pvu II, electrophoresed through a 0.8% agarose gel, then transferred to a nitrocellulose membrane (Biorad). Human skeletal muscle mtDNA and a probe to the nuclear 18S rRNA gene were labeled by random-primer incorporation of digoxigenin-labeled deoxyuridine triphosphate (Boehringer-Mannheim, Roche Diagnostics Italy, Milan, Italy) and used as hybridization probes (16). The membrane was hybridized with the two labeled probes overnight at 68°C, then washed, and exposed at film X-OMAT AR (Kodak, Rochester, NY, USA) for 30 min at room temperature.

For the quantitative determination of the mtDNA/18S ribosomal DNA (rDNA) ratio, equal amounts of the two ³²P-labelled probes were used. The autoradiograms were scanned on a phosphorimager and the results were densitometrically analyzed using the Image-Quant provided software.

In six cases (3,6,7,8,9, and 11), the activities of respiratory chain complex IV (COX; EC 1.9.3.1) and

citrate synthase (CS; EC 4.1.3.7) were determined spectrophotometrically following reported methodologies (17). All enzymatic activities were assessed at least in duplicate.

In these cases, to verify whether unspecific damage of mitochondria in Hashimoto's muscle samples was responsible for a decrease of mtDNA content, we compared the relative mtDNA content (expressed as mtDNA:18S rDNA ratio) with the amount of mitochondria (expressed as CS activity). The normalized ($\times 1000$) CS relative mtDNA content could be indicative of primary mtDNA reduction.

Results were compared to those of age- and sex-matched healthy control subjects recruited from the orthopedic clinics.

Statistical Analysis

Following the Kolmogorov-Smirnov test for non-Gaussian distribution, nonparametric analysis was used. In particular, the Mann-Whitney U test or, when necessary, the Kruskal-Wallis test was employed to estimate differences between groups. Associations were tested by the Spearman rank correlation test and regression analysis.

Results

Thyroid Status

All patients showed elevated serum anti-TgAb and/or anti-TPOAb levels. Among them, four patients (1,2,8, and 9) suffered from overt hypothyroidism (reduced free thyroid hormone and elevated TSH level), seven patients (3,4,5,6,7,10, and 11) were affected by subclinical hypothyroidism (elevated serum TSH levels in the face of normal FT3 and FT4 levels) to different extents (Table 1). Notably, patient 10 had suffered from transient Hashitoxicosis 3 months before our observation when he became hypothyroid.

Neurologic Status

In all patients, electromyography showed myopathic changes in the form of small amplitude and duration motor unit (MU) potentials, increased percentage of multiphasic, low amplitude MU potentials, and low amplitude interference pattern with precocious MU recruitment at MVC. In one patient (5), mild impairment of motor and sensitive nerve conduction was detected by nerve conduction studies.

Exercise Lactate Anaerobic Threshold

Resting blood lactate concentration was slightly increased in two HHM patients (7 and 11; Table 1). In HHM patients, LT was achieved at exercise levels as high as 40% or 50% of MVC, whereas it corresponded to 60% of the MVC in normal controls; at those levels of exercise, the mean values of lactate (LT lactate) were $181.5 \pm 70.4\%$ (percentage of the baseline) in HHM patients and $173 \pm 20.5\%$ in controls, respectively. Starting from that inward,

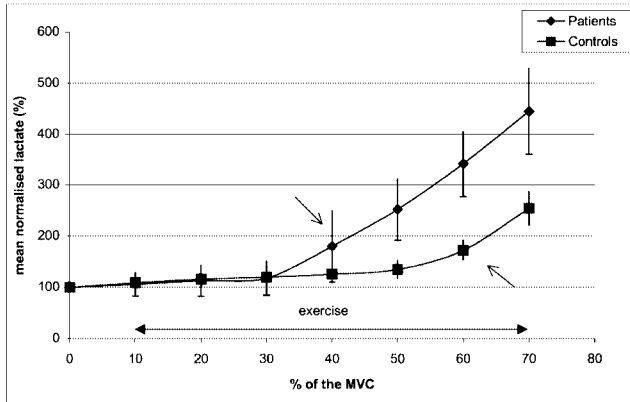


Fig. 1. Lactate values (normalized mean values) in HHM patients and controls. Arrows indicate lactate threshold in both groups.

lactate value in HHM patients was significantly higher ($p < 0.01$) than in controls (Table 1 and Fig. 1). Accordingly, the recovery after exercise was slower in HHM patients than in controls.

Muscle Biopsy

A prevalence of type 1 fibers was found in two patients. Nonspecific findings, such as sporadic round-shaped atrophic fibers and/or increased variability of muscle fiber diameter and internal nuclei, were found in all HHM cases. Fiber degeneration in the form of fiber splitting and opaque fibers was detected in patients 1 and 10. Scattered

fiber necrosis and endophagocytosis were present in patients 1, 10, and 11; in one of them it was associated with increased endomysial connective tissue. In case 5, the presence of group atrophy and type grouping indicated a neurogenic damage. Atrophy and hypertrophy factors for type 2 fibers as well as hypertrophy factors for type 1 fibers were within the normal range, whereas atrophy factors for type 1 fibers were abnormally increased in all HHM patients but one (Table 2).

Histochemical analysis showed the presence of scattered COX negative fibers in eight cases (1,2,3,4,6,8,9, and 11), and hyperintense subsarcolemmal rims for SDH enzyme activity in all patients (Fig. 2A and 2B) but one. Further mitochondrial staining abnormalities in the form of central core or core-like staining defects for NADH and COX enzyme activity were observed in all patients and ranged from 2–65% of type 1 fibers (Table 2).

Muscle h-mtTFA levels were reduced to a variable extent by comparison with the control mean: below 2 SD in nine patients, between 1 and 2 SD in one, and within 1 SD in another (Table 3, Fig. 3A).

No large-scale rearrangement (deletions or duplications) of mtDNA was found in HHM patients. Their mtDNA/18S rDNA ratio was also reduced, although to a lower extent with respect to h-mtTFA decrease: below in one patient (9) and within 1 SD of control mean in nine. Contrarily, in one case (10), this value was increased (Table 3, Fig. 3B).

In all six cases where biochemical enzymatic analysis was performed, COX activity and COX/CS

Table 2. Muscle biopsy

Patient	Type 1 Fiber (%)	Type 1 Fiber AF/HF	Type 2 Fiber AF/HF	NADH/COX Abnormalities*	SDH+ Fibers [†]	COX- Fibers
1	58	113/0	144/0	59	Yes	Yes
2	56	124/30	100/140	51	Yes	Yes
3	61	120/48	118/64	23	Yes	Yes
4	58	31/74	95/5	47	Yes	Yes
5	65	132/4	140/11	2	No	No
6	48	144/56	132/44	39	Yes	Yes
7	52	138/42	86/60	12	Yes	No
8	59	174/32	62/84	58	Yes	Yes
9	72	186/68	56/72	65	Yes	Yes
10	51	168/75	41/42	37	Yes	No
11	63	124/52	82/124	42	Yes	Yes
Controls (n = 8)	45 ± 9 [¶]	F < 100/280 [¶] M < 150/200	F < 165/150 [¶] M < 150/400	0.40 ± 0.35	No	No

*Mean value between NADH and COX core-like hyporeactive type 1 fibers.

[†]Subsarcolemmally hyperreactive fibers;

[¶]Values calculated according to Dubowitz et al. (14).

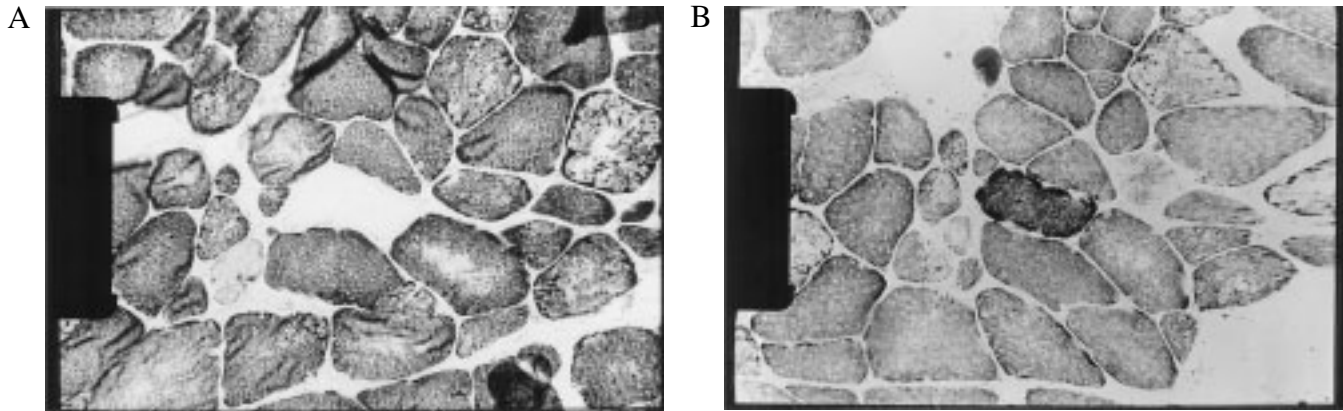


Fig. 2. (A and B) Muscle biopsy for patient 6. A COX-negative and SDH positive fiber (serial sections, original magnification 250 \times).

activity ratio were reduced, whereas the (mtDNA/18S)/CS ratio was reduced in four of them, unchanged in one, and slightly increased in one (Table 3).

Correlation Analysis

TSH serum levels were directly and FT3 and FT4 were inversely correlated to the number of type I fibers with NADH and COX core-like histochemical alterations, with significance ranging from $p < 0.05$ to $p < 0.01$. Muscle h-mtTFA levels showed a positive correlation with FT4 ($r = 0.67$, $p = 0.02$) (Fig. 4), and an inverse correlation with both LT lactate ($r = -0.70$, $p < 0.02$) (Fig. 4) and TSH ($r = -0.59$, $p = 0.05$). The regression coefficient approached statistical significance ($r = 0.61$, $p = 0.06$) between

h-mtTFA and FT3 (data not shown). No correlation was found with muscle mtDNA/18S rDNA ratio.

Discussion

We report here the occurrence, in patients with hypothyroid myopathy, of h-mtTFA reduction in skeletal muscle, together with a number of mitochondrial alterations, as indicated by increased lactate production during incremental workload exercise, defects in respiratory chain enzymes, and reduction of mtDNA copy number in muscle biopsy. Hypothyroid myopathy is a heterogeneous clinical entity in which mitochondria involvement does not invariably represent the consequence of thyroid hormone deficiency (18–20). In fact, diverse abnormalities in

Table 3. Muscle biopsy

Patients	CS	COX	COX/CS	h-mtTFA*	mtDNA/18S DNA ratio	(mtDNA/18S)/CS \times 1000
1	n.e.	n.e.	n.e.	0.034	1.26	n.e.
2	n.e.	n.e.	n.e.	0.074	1.39	n.e.
3	193	94	0.49	0.193	1.68	8.74
4	n.e.	n.e.	n.e.	0.060	1.94	n.e.
5	n.e.	n.e.	n.e.	0.064	1.27	n.e.
6	185	254	1.37	0.127	1.55	8.38
7	128	175	1.37	0.125	1.84	14.38
8	121	188	1.55	0.036	1.56	12.81
9	79	169	2.14	0.052	1.08	13.67
10	n.e.	n.e.	n.e.	0.081	3.54	n.e.
11	173	296	1.71	0.156	1.81	10.46
Controls	166 \pm 33.67 (n = 35)	449 \pm 101.4 (n = 35)	2.7 \pm 0.04 (n = 35)	0.211 \pm 0.038 (n = 20)	2.27 \pm 1.12 (n = 20)	13.67 \pm 3.14 (n = 20)

*Densitometric value of h-mtTFA Western blot band.

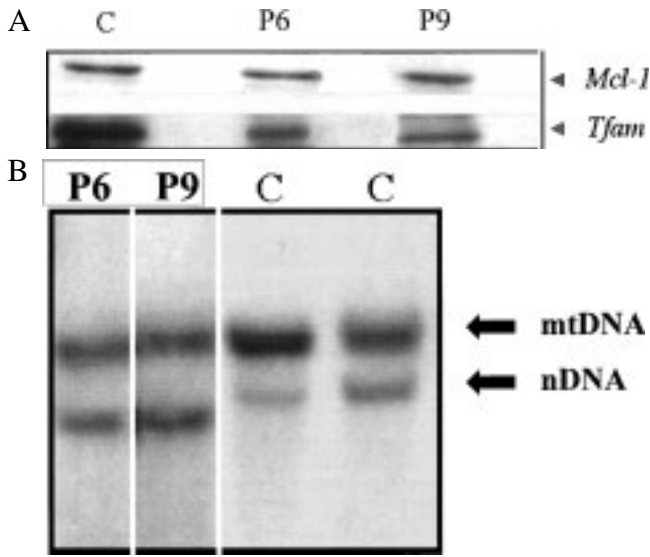


Fig. 3. Muscle biopsy. (A) H-mtTFA and Mcl-1 western blot of patient 6, patient 9, and a control. (B) MtDNA and 18S rDNA southern blot of patient 6, patient 9, and two controls.

muscle function have been reported in hypothyroidism (1). Biochemical changes such as glycogen accumulation and decreased activity of enzymes involved in different energy production pathways have been described in hypothyroid red (type 1) fibers (1,21,22). This is not surprising; skeletal muscle is a target organ for thyroid hormones (2). However, the presence of T3 receptors on the mitochondrial membrane of skeletal muscle fibers (23) implies a direct impact of thyroid hormones on mitochondrial oxidative metabolism.

In line with this, hyperthyroidism is characterized by an increased, and hypothyroidism by a reduced, rate of oxygen consumption (10). Stimulation of mitochondrial respiratory chain function by thyroid hormones is mediated by several molecular mechanisms that include both direct and indirect effects on mitochondrial structure, function, and biogenesis. T3 is known to directly increase the expression of some nuclear-encoded respiratory genes. This stimulation is mediated by thyroid hormone receptor, which binds to consensus sequences located in the promoter region of these genes, such as cytochrome c1 and b-F1-ATPase subunit genes (24,25). Recently the possibility that T3 indirectly stimulates respiratory function has been suggested. This effect can be mediated by transacting factors such as nuclear respiratory factor-1 (NRF-1) (26). Consensus sequences for NRF-1 have been found in some nuclear encoded respiratory genes, such as rat cytochrome c oxidase subunits VIc and VIIa genes (6,27), human ubiquinone binding protein gene (6), and human mitochondrial transcription factor A gene (h-mtTFA) (28).

H-mtTFA, a 25-kDa nuclear encoded protein belonging to the high mobility protein family (29), is

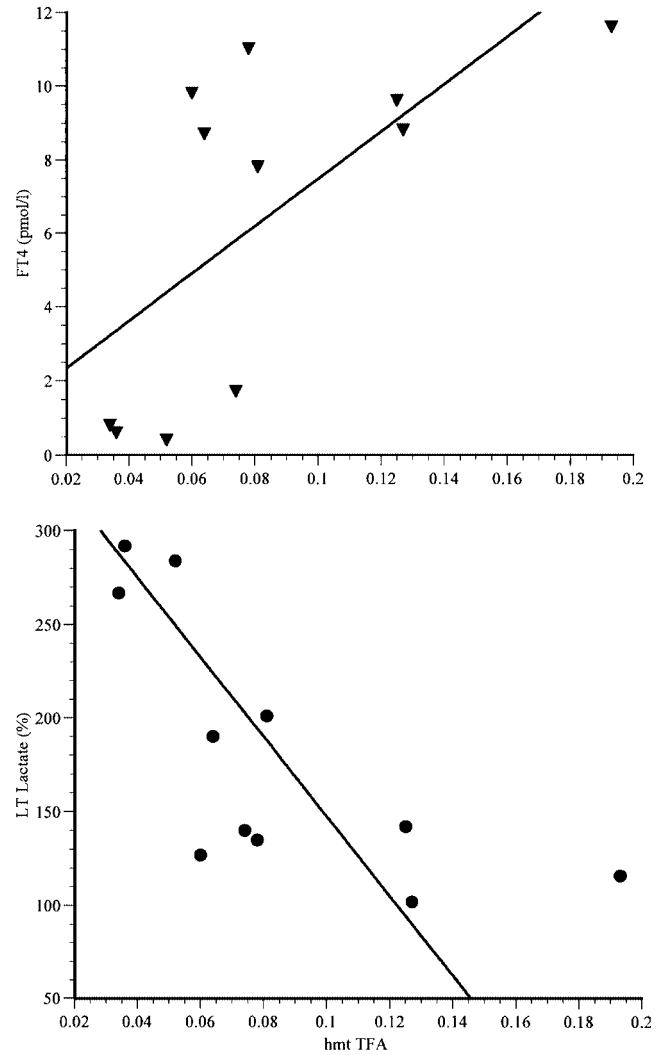


Fig. 4. Regression analysis between skeletal muscle h-mtTFA and serum FT4 (top) or exercise LT lactate (percentage of baseline) (bottom).

regarded as one of the major regulatory factors of mitochondria biogenesis, as concluded on the basis of its regulatory activity on mitochondrial genome transcription and replication in vertebrates (30). This protein binds to promoter sequence of heavy- and light-strand in the D-loop region (31). Expression of the mtTFA gene is under the regulatory effect of NRF 1 and 2 and is modulated by a number of factors including possible feedback signals originating in the mitochondrial environment itself (32).

In humans, alterations of h-mtTFA transcript or protein levels have been reported. In mtDNA depletion, a rare and fatal infantile syndrome, h-mtTFA depletion has been detected in liver, kidney, and skeletal muscle tissues (15,16), and h-mtTFA up-regulation has been observed in muscle fibers of patients affected by chronic progressive external ophthalmoplegia (33). Increased levels of h-mtTFA have been reported also in some physiologic conditions,

such as in skeletal muscle of subjects undergoing exercise training (34), indicating the existence of still unknown regulatory mechanisms in contractile activity-induced mitochondrial biogenesis (35). Regarding the thyroid hormone, h-mtTFA can be considered one of the putative targets of the thyroid hormone-thyroid hormone receptor complex, either directly or indirectly (i.e., through nuclear transcribing factors) (10,28,36). According to this, h-mtTFA deficiency has been proposed as a candidate mechanism to explain the large-scale mtDNA deletion in oxyphil Askanazy cells recently observed in Hashimoto's thyroiditis (37).

In the present study, we measured muscle h-mtTFA levels in 11 patients with Hashimoto's thyroiditis and myopathy in an attempt to investigate the relationships between thyroid hormone deficiency and mitochondrial function. A reduction of skeletal muscle h-mtTFA protein levels was detected in the hypothyroid patients, although to a variable extent. This reduction was related to the amount of lactate production during incremental exercise and associated with muscle histochemical and biochemical alterations of NADH, SDH, and COX. It is unlikely that these alterations, also in the form of COX negative and SDH hyperreactive fibers, are due to a neurogenic or inflammatory process (38) for which evidence was small in our patients, based on EMG and muscle pathology data. The occurrence of COX negative and SDH positive fibers is a highly specific hallmark of primary mitochondrial dysfunction, as in mitochondrial myopathies due to nuclear or mitochondrial genome alterations. The additional results of mtDNA and (mtDNA/18S)/CS ratio reduction in our patients suggest that decrement of mitochondrial DNA copy number, although slight in its entity, could be involved in the pathogenic mechanism of the above mentioned alterations, possibly relying on the effects of h-mtTFA on mitochondrial biogenesis (33). Only in one case (10) was this value above the control value, a result that could be explained by the recent history of Hashitoxicosis that preceded our observation in this patient. Whether or not the occurrence of mutational events or polymorphic variants in the D-loop region of mtDNA (16) can contribute to these findings in the presented patients remains to be determined.

It is worth mentioning that h-mtTFA reduction was evident not only in those patients with frank hypothyroidism but also in those with subclinical hypothyroidism. This finding may seem at odds with the definition of subclinical hypothyroidism, in which circulating thyroid hormone levels are still in the normal range. However, we have previously described a time-dependent impairment of mitochondrial oxidative metabolism in subclinical hypothyroid patients with symptoms of muscle dysfunction (3). Indeed, small decrements in hormone synthesis may over time lead to both biochemical signs and clinical symptoms qualitatively similar to those of overt hypothyroidism. However, in those patients with overt hypothyroidism the reduction of

h-mtTFA was more relevant, bolstering a dosage-type relationship between thyroid hormone decrement and h-mtTFA level in skeletal muscle.

In conclusion, our study supports the notion that thyroid hormones regulate muscle mitochondrial function in skeletal muscle, as previously suggested. The h-mtTFA gene can be involved in this action as a significant target of hypothyroidism, contributing to the occurrence of myopathy in such a condition. In this context, further studies including the evaluation of the effects of levothyroxine replacement therapy are necessary to assess the precise role of h-mtTFA in the cascade of events following thyroid hormone deficiency at the muscle level.

Acknowledgments

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