Effect of thyroid hormone on mitochondrial properties and oxidative stress in cells from patients with mtDNA defects

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Menzies KJ, Robinson BH, Hood DA. Effect of thyroid hormone on mitochondrial properties and oxidative stress in cells from patients with mtDNA defects. Am J Physiol Cell Physiol 296: C355–C362, 2009; doi:10.1152/ajpcell.00415.2007.—Mitochondrial (mtDNA) mutations contribute to various disease states characterized by low ATP production. In contrast, thyroid hormone [3,3',5-triiodothyronine (T3)] induces mitochondrial biogenesis and enhances ATP generation within cells. To evaluate the role of T3-mediated mitochondrial biogenesis in patients with mtDNA mutations, three fibroblast cell lines with mtDNA mutations were evaluated, including two patients with Leigh’s syndrome and one with hypertrophic cardiomyopathy. Compared with control cells, patient fibroblasts displayed similar levels of mitochondrial mass, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), mitochondrial transcription factor A (Tfam), and uncoupling protein 2 (UCP2) protein expression. However, patient cells exhibited a 1.6-fold elevation in ROS production, a 1.7-fold elevation in cytoplasmic Ca2+ levels, a 1.2-fold elevation in mitochondrial membrane potential, and 30% less complex V activity compared with control cells. Patient cells also displayed 20–25% reductions in both cytochrome c oxidase (COX) activity and MnSOD protein levels compared with control cells. After T3 treatment of patient cells, ROS production was decreased by 40%, cytoplasmic Ca2+ was reduced by 20%, COX activity was increased by 1.3-fold, and ATP levels were elevated by 1.6-fold, despite the absence of a change in mitochondrial mass. There were no significant alterations in the protein expression of PGC-1α, Tfam, or UCP2 in either T3-treated patient or control cells. However, T3 restored the mitochondrial membrane potential, complex V activity, and levels of MnSOD to normal values in patient cells and elevated MnSOD levels by 21% in control cells. These results suggest that T3 acts to reduce cellular oxidative stress, which may help attenuate ROS-mediated damage, along with improving mitochondrial function and energy status in cells with mtDNA defects.

reactive oxygen species; mitochondrial biogenesis; cytoplasmic calcium; mitochondrial disease; 3,3',5-triiodothyronine; mitochondrial DNA

NUCLEAR DNA (nDNA) and mitochondrial DNA (mtDNA) are both responsible for encoding ~1,500 gene products that make up the mitochondrial proteome. A total of 37 of these genes are found in maternally inherited mtDNA, and they encode 22 tRNAs, 2 rRNAs, and 13 polypeptide subunits. Each of these 13 polypeptides are integral components of the oxidative phosphorylation system, whereas the remaining mitochondrial proteins, which are required for proper organelle function and energy generation, are imported from the cytosol.

Despite the fact that a majority of the mitochondrial components are nuclear encoded, there remains a large dependence on mitochondrially encoded subunits. This is evident in patients with mitochondrial diseases caused by mtDNA mutations. In adults, mtDNA mutations have an overall prevalence of 6.57/100,000 (6). This can result in myopathies or clinical pathologies, such as hypertrophic cardiomyopathy (HCM) (15, 43) and Leigh’s syndrome (LS) (45). These mutations arise more frequently in mtDNA since this genome is more susceptible to damage compared with nDNA. mtDNA lacks the protective effects of DNA-binding histones (38), has less adequate DNA repair mechanisms (24), and is in close proximity to respiration-induced reactive oxygen species (ROS). However, one protective quality of mtDNA is that there are several copies of the genome per mitochondria, which makes most inherited mutations heterogeneous in nature. In addition, depending on the type of mtDNA mutation, the cell can alter the expression of nuclear-encoded transcription factors and mitochondrial protein import machinery components to reduce the associated pathophysiology of the disease (17).

The most common molecule of ROS produced by the mitochondria is superoxide, which has not only been proven to contribute to oxidative damage but also acts as a cell signaling molecule in redox signaling pathways such as the cell cycle (40), apoptosis (27), and cell proliferation (20). Superoxide is reduced by SOD to hydrogen peroxide, which may then react with free iron or copper to produce damaging hydroxyl radical. There are two SOD enzymes: MnSOD and CuZnSOD, which are located in the mitochondria and cytosol, respectively (3). Under physiological conditions, the mitochondrial respiratory chain is considered to be a key site for cellular ROS production (23), which can lead to the progressive damage of mitochondrial macromolecules, including mtDNA. These defects lead to reduced mitochondrial energy production, a situation commonly found in mitochondrial disorders. Thus, one way in which it may be possible to improve the state of these cells is by increasing their ability for aerobic ATP production. In this respect, thyroid hormone [3,3',5-triiodothyronine (T3)] has been shown to induce mitochondrial biogenesis, thus enhancing the ability of the cell to generate the energy required for biological functions (48). The action of T3-mediated mitochondrial biogenesis is first initiated when T3 binds to thyroid receptors (TRs) found in the nucleus. This leads to the upregulation of multiple nuclear-encoded respiratory genes, thereby altering the activity of the mitochondrial electron transport chain (42). The mechanisms of mitochondrial biogenesis involve the upregulation of nuclear-encoded mitochondrial transcription factor A (Tfam), a protein that binds mtDNA to...
regulate its transcription (33), as well as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis. T3 has been shown to increase the expression of both Tfam and PGC-1α in a tissue-specific manner (14, 16).

Therefore, the purposes of this work were to determine the unique phenotypic character of cells derived from patients with heterogeneous mtDNA defects and to evaluate the ability of T3 to attenuate the pathological features of these cells through the process of mitochondrial biogenesis. Due to the heterogeneous nature of cells with mtDNA defects, the possibility exists that both functional and aberrant mitochondrial proteins would be amplified with T3-induced mitochondrial biogenesis. We suspect that any increase in functional mitochondrial proteins would result in an overall increase in the cellular capacity for oxidative ATP synthesis. Three primary cultured fibroblast cell lines with mtDNA mutations were evaluated, including a patient with an ATPase 6 mutation (LS), one with a tRNA<sub>Leu(UUR)</sub> mutation (LS), and another with a ND5 subunit mutation (HCM), which were then compared with three normal control cell lines. We hypothesized that T3, via the stimulation of mitochondrial biogenesis, would enhance mitochondrial function in patients with mtDNA defects.

METHODS

Cell culture. Human skin fibroblasts taken from patients diagnosed with G13513A, T9185C, and A3260G mutations (Table 1) along with three age-matched control subjects were grown in α-MEM (Wisent, St-Bruno, QC, Canada) containing l-glutamine (2 mM), glucose (1 g/l), and sodium pyruvate (110 mg/l) and supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO2. Despite the differences in mtDNA mutations for each of the patient fibroblast cell lines used in this study, they all showed similar biochemical afflictions. Therefore, samples were limited to these three patients, which was sufficient to indicate statistically significant biochemical trends after T3 treatment. Cell lines used in this study were kept at low-passage numbers, and they maintained good cell growth rates through 12 days of T3 treatment. Cell lines used in this study were kept in α-MEM (Wisent, St-Bruno, QC, Canada) containing l-glutamine (2 mM), glucose (1 g/l), and sodium pyruvate (110 mg/l) and supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO2. Despite the differences in mtDNA mutations for each of the patient fibroblast cell lines used in this study, they all showed similar biochemical afflictions. Therefore, samples were limited to these three patients, which was sufficient to indicate statistically significant biochemical trends after T3 treatment. Cell lines used in this study were kept at low-passage numbers, and they maintained good cell growth rates throughout the study. All cells were treated with 1 μM T3 or vehicle (DMSO) for 12 days. Lower doses (10<sup>−7</sup> M) or treatments for shorter periods of time (4 days) did not result in significant effects of T3 on the characteristics of these fibroblast cells (data not shown).

Cytochrome c oxidase assay. Cells were washed and scraped with ice-cold Dulbecco’s PBS (Sigma-Aldrich, St. Louis, MO) after 12 days of T3 treatment. After centrifugation, pellets were resuspended in 100 mM Na<sub>2</sub>HPO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> and 2 mM EDTA (pH 7.2), sonicated on ice (3 × 30 s), frozen in liquid N<sub>2</sub>, and then thawed. Samples were then centrifuged for 5 min at 4°C, and supernates were mixed with a test solution (10 mM K<sub>2</sub>HPO<sub>4</sub>) containing reduced cytochrome c (Sigma-Aldrich, St. Louis, MO). Enzyme activity was determined by measuring the maximal rate of oxidation of reduced cytochrome c using the change in absorbance at 550 nm on a Bio-Tek Synergy HT microplate reader.

Complex I and V activity. Complex I activity, also described as rotenone-sensitive NADH-CoQ reductase activity, was determined using previously described techniques (21). This assay measured the oxidation rate of NADH in the presence of fibroblast homogenate. The decrease in absorbance was monitored using an Ultrospec 2100 pro (Biochrom, Cambridge, UK) with and without 40 μmol/l rotenone. Complex I rotenone-sensitive enzyme activity was calculated from the difference between total and rotenone-insensitive activity rates. Complex V activity was measured with a modified version of the protocol used by Krieger et al. (22). This assay measured complex V activity through an oligomycin-sensitive ATPase-linked enzyme assay (22). This assay measures the reduction of NADH to NAD with ATPase being the limiting factor. Oligomycin was used to inhibit the activity of the F<sub>1</sub>-ATPase enzyme of complex V. Therefore, nonmitochondrial ATPase activity within the fibroblast homogenate was subtracted from the total activity by performing the assay in the presence and absence of oligomycin.

ATP levels and ADP-to-ATP ratios. Mitochondria were extracted from fibroblast cells using a perchloric acid extraction procedure (11). ATP content was measured using a Berthold luminometer after a 1-min incubation of the cell extract with luciferase-luciferin. ATP content (in nM/μg protein) was obtained by extrapolation using a standard curve of known ATP concentrations. The ADP-to-ATP ratio was measured using a bioluminescent ApoSENSOR ADP-to-ATP ratio assay kit (Biovision Reaserach Products, Mountain View, CA). Enzyme extracts were used in this assay to determine the ADP-to-ATP ratio and were expressed per microgram of protein.

Protein extraction and immunoblot analysis. Fibroblasts were washed with PBS and then scraped with passive lysis buffer (Promega, Madison, WI) supplemented with protease inhibitors. Protein quantitation was accomplished using the Bradford method. Equal amounts of total protein (20 μg) were mixed with loading buffer and separated on 12% SDS polyacrylamide gels. Gels were then electroblotted onto nitrocellulose membranes (Hybond ECL, GE Healthcare BioSciences, Piscataway, NJ) and incubated with antibodies raised against Tfam (1:1,000), cytochrome c oxidase (COX)-I (1:500, Invitrogen Canada, Burlington, ON, Canada), GAPDH (1:30,000, Abcam, Cambridge, MA), MnSOD (1:2,000, Upstate, Charlottesville, VA), anti-phospho H2A (1:1,000, Sigma-Aldrich, St. Louis, MO), and anti-H2A antibodies raised in rabbit. Blots were washed with PBS and then incubated with antibodies raised against Tfam (1:1,000), cytochrome c oxidase (COX)-I (1:500, Invitrogen Canada, Burlington, ON, Canada), GAPDH (1:30,000, Abcam, Cambridge, MA), MnSOD (1:2,000, Upstate, Charlottesville, VA), anti-phospho H2A (1:1,000, Sigma-Aldrich, St. Louis, MO), and anti-H2A antibodies raised in rabbit.

Table 1. Phenotypic and biochemical characteristics of the three patients

<table>
<thead>
<tr>
<th>Patient Cell Lines</th>
<th>Location of Mitochondrial DNA Mutation</th>
<th>Percent Heteroplasmy</th>
<th>Phenotypic Characteristics</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>A3260G [tRNA&lt;sub&gt;Leu(UUR)&lt;/sub&gt; gene]</td>
<td>80</td>
<td>Hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Skeletal muscle myopathy, seizures, and lactic acidosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MRI was normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heart transplant recipient</td>
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<td></td>
<td></td>
<td></td>
<td>13 yr old at sampling</td>
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<tr>
<td>Patient 2</td>
<td>T9185C (ATPase 6 gene)</td>
<td>90</td>
<td>Leigh’s syndrome</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Lethargy, ataxia, ptosis, and lactic acidosis</td>
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<td></td>
<td></td>
<td></td>
<td>MRI indicated bilateral density changes in brain stem and cerebral atrophy</td>
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<td></td>
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<td></td>
<td>Family history of Leigh’s syndrome</td>
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<td>9 yr old at sampling</td>
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<tr>
<td>Patient 3</td>
<td>G13513A (ND5 gene)</td>
<td>70</td>
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<td>Lactic acidosis</td>
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<td>MRI indicated optic atrophy and basal ganglia abnormalities</td>
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<tr>
<td></td>
<td></td>
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<td>5 mo old at sampling</td>
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Flow cytometric analysis of mitochondrial membrane potential. Changes in mitochondrial membrane potential (ΔΨm) were analyzed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1; Invitrogen, Carlsbad, CA). This cationic dye accumulates in mitochondria in a membrane potential-dependent manner, as indicated by a fluorescence emission shift from green (~525 nm) to red (~590 nm) (37). Cells were incubated in the dark with 0.3 μg/ml JC-1 for 1 h at 37°C and 5% CO2 in cell culture media, washed, and then trypsinized before being resuspended in supplemented phenol red-free DMEM (Sigma-Aldrich). Samples were then examined using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). JC-1 monomer fluorescence was collected in the green FL-1 channel, whereas JC-1 orange fluorescence, caused by the formation of j-aggregates, was measured using the FL-2 channel. The sensitivity of this technique was verified using the K+ ionophore valinomycin (10 μM) to depolarize the mitochondrial membrane for each of the samples. All analyzed data were derived from the geometric mean fluorescence of at least 25,000 gated events.

Flow cytometric analysis of mitochondrial mass, ROS, and cytoplasmic calcium. Mitochondrial mass was examined with MitoTracker green FM (MTGFM; Invitrogen Canada). This probe becomes fluorescent after incorporation into the lipid environment of mitochondria, where it interacts with peptide or protein thiol groups (19). Cells were stained with 20 nM MTGFM for 45 min at 37°C in cell culture media and then analyzed by flow cytometry. ROS were monitored using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Sigma-Aldrich). This cell-permeant nonfluorescent probe is deesterified within the cell, creating fluorescent 2′,7′-dichlorofluorescein after oxidation. Cells were preincubated with 5 μM H2DCFDA for 15 min at 37°C in supplemented phenol red-free DMEM before analysis on the flow cytometer. Cytoplasmic calcium was monitored by incubating the cells with 1 μM of the plasma membrane-permeable AM form of the fluo-3 probe (Invitrogen Canada) for 1 h at 37°C in cell culture media. Cells were then washed and resuspended in supplemented phenol red-free DMEM and analyzed by flow cytometry. Data analyzed from samples containing the previous set of probes were derived from the geometric mean fluorescence of at least 25,000 gated events.

Statistics. Each of the individual patient and control cell cultures was examined in triplicate for each measurement made. Comparisons between patient and control cells were evaluated using two-way ANOVA on the experimental measurements made for each cell line, resulting in an overall n = 3 for both patients and control subjects. Statistical comparisons were made between the grouped average of control and patient cell lines when the effect of T3 or the difference between patient and control cells was similar for all patient cell lines. Main effects of cell type or T3 treatment were considered significant if P < 0.05. A Bonferroni post hoc test was applied to identify the location of the significant difference where necessary. Data are expressed as means ± SE.

RESULTS

TR (α1 and β) proteins. To examine the potential responsiveness of fibroblast cells to T3, TR-α1 and TR-β protein were quantified in patient and control cells before and after T3 treatment. After T3 treatment, TR-α1 expression was augmented in both patient and control cells, whereas TR-β expression remained the same. In addition, patient cells demonstrated an elevated constitutive expression of TR-α1 compared with control cells. (Fig. 1A, top and bottom).
COX activity, complex V and I activity, ATP levels, and ADP-to-ATP ratios. Patient fibroblasts had 23% less constitutive COX activity (82 nmol·min⁻¹·mg protein⁻¹) than control cells (106 nmol·min⁻¹·mg protein⁻¹, P < 0.05). After 12 days of T₃ treatment, ANOVA indicated that T₃ produced an overall increase in COX activity in both patient and control cells (P < 0.05; Fig. 2A). A 1.3-fold increase was evident in treated patient cells, which compensated for the original deficiency in COX activity compared with control cells. These increases in holoenzyme activity were not due to changes in the expression of the catalytic subunit COX-I (Fig. 2B), since T₃ treatment did not alter COX-I protein levels in either cell type.

Complex V activity was also measured in patient and control cells. Patient cells showed similar declines in activity, with an average loss of 30% compared with control cells (Fig. 2C). After T₃ treatment, there were 1.3- and 1.4-fold increases in activity within control and patient cells, respectively. Complex I activity was also measured to assess whether reductions in activity could be predicted, in part, by the site of the patient’s mutation. All three patients demonstrated reduced complex I activity (Fig. 2D). As expected, the patient with the ND5 mutation (G13513A) exhibited the greatest percent reduction (42% less) compared with control subjects, whereas the reduction was more modest (29%) in the other two patients.

We also evaluated the levels of cellular ATP as an indicator of ATP production, largely in reflection of mitochondrial oxidative capacity. ATP levels were only modestly reduced (~20%) in patient cells compared with control cells (Fig. 2E). However, in response to T₃ treatment, both control and patient fibroblasts exhibited an elevation in ATP levels of 1.4- and 1.6-fold (P < 0.05), respectively. The elevation in ATP levels after T₃ treatment was mirrored by a reduction in ADP-to-ATP ratios for both control and patient cells (P < 0.05; Fig. 2F). These changes in adenine nucleotide ratios were independent of any alterations in adenine nucleotide translocator (ANT) content, as measured by Western blot analysis (data not shown).

ROS production and antioxidant proteins. Analysis of ROS production in each of the primary cell lines revealed that patient fibroblasts produced a 1.6-fold higher level of ROS than their control counterparts (P < 0.05; Fig. 3A). T₃ treatment resulted in substantial decreases in ROS production of 34% (P < 0.05) in control cells. This effect was significantly greater (43%) in patient cells (Fig. 3A), as revealed by a significant interaction of the effect of T₃ on ROS production in these two cell types (P < 0.05). Consequently, levels of ROS production in T₃-treated patient cell lines were reduced to approximately the same level produced by control cell lines.

MnSOD protein levels were examined to determine the role of this protein in the reduction of ROS production after T₃ treatment. Constitutive MnSOD protein expression was 1.4-fold greater in the control group compared with the patient group, as determined by Western blot analysis (P < 0.01; Fig. 3B). A main effect of T₃ was observed, resulting in a 1.2-fold increase in MnSOD expression in both cell types (P < 0.01; Fig. 3B). In contrast, the expression of the antioxidant UCP2 did not differ between control and patient cells, and no effect of T₃ was observed (Fig. 3B, top).

\[ \Delta \Psi_m \] was 1.2-fold higher in patient fibroblasts compared with control fibroblasts (P < 0.05; Fig. 4A). T₃ treatment induced a significant overall decline (P < 0.05) in \[ \Delta \Psi_m \] of both

Fig. 2. Mitochondrial function in fibroblast cells after T₃ treatment. A: cytochrome c oxidase (COX) activity in control and patient fibroblasts after 12 days of treatment with T₃ or vehicle shown graphically. B: Western blot showing COX subunit I protein. C: complex V activity in control and patient fibroblasts after the vehicle or T₃ treatment protocol shown graphically. D: constitutive complex I activity in control and patient fibroblasts shown graphically. E: ATP levels in control and patient fibroblasts after 12 days of treatment with T₃ or vehicle shown graphically. F: ADP-to-ATP ratios for control and patient fibroblasts after T₃ or vehicle treatment shown graphically. n = 3 control and 3 patient cell lines per analysis. *P < 0.05, overall effect of T₃ on both patient and control groups; †P < 0.05, overall effect of T₃ on both patient and control groups.
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**T3 AND MITOCHONDRIAL DISEASE**

**DISCUSSION**

The present study was undertaken with two main purposes in mind. The first was to confirm the molecular and physiological heterogeneity of diseases with origins in mutated mtDNA. The second was to examine the effect of a high dose of T3 on the cells of patients, in view of the ability of T3 to increase mitochondrial biogenesis and function (48). The dose of T3 employed (1 μM) is greater than that found under normal physiological conditions, and this represents a limitation of our study. Our goal was to investigate the potential of T3 to beneficially modify the properties of cells with mitochondrial defects, with possible implications for the development of T3 analogs that might prove useful for this purpose in the future.

Because of the high dose employed, and because of the similar (or elevated) content of TR-α1 and TR-β in patient cells compared with control cells, we fully expected a robust T3 response in patient fibroblasts. Interestingly, T3 treatment increased TR-α1 expression levels in both patient and control cells. We believe that the responses of the cells as reported here represent genomically mediated adaptations as a result of hormone action. Although nongenomic effects likely occurred in response to this dose of T3, the time point for measurement of these would have to be much shorter than the 12-day period employed in the present study.

Our study included three human control fibroblast cell lines and three mitochondrial disease patient cell lines. One of these patient fibroblasts patient sample had a mtDNA G13513A mutation with 70% heteroplasmy at a highly conserved position in the ND5 subunit gene (36). This mutation resulted in LS; however, the mutation has also been reported in patients with symptoms of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke (MELAS) (7). A second patient had a T9185C mutation with 90% heteroplasmy in the mitochondrial ATPase 6 gene, as found in LS (44). The result of this mutation is a change from a leucine residue, located near the carboxyl terminus of the ATPase 6 subunit, to a proline residue, which affects proton translocation and ATP synthesis (5). The final patient sample had a A3260G mutation with 80% heteroplasmy. The mutation is located within the tRNA^Leu(UUR) gene, resulting in HCM (52). tRNA base substitutions cause an overall impairment of mitochondrial protein synthesis, resulting in a decrease in the oxidative phosphorylation capacity (39). Together, these mitochondrial diseases all exhibit reduced mitochondrial function, as reflected by COX and complex I activities. Thus, we hypothesized that beneficial phenotypic adaptations leading to improved mitochondrial biogenesis and function could result from T3 treatment.

The transcriptional coactivator PGC-1α is a known regulator of mitochondrial biogenesis, since it appears to coordinate the expression of genes involved in aerobic metabolism. The pleiotropic functions of this coactivator, and its importance during increased cellular energy demands, have been demonstrated in PGC-1α^−/− mice, which exhibit defects in oxidative phosphorylation, impaired cold resistance, and abnormal gluconeogenesis (28). These effects of PGC-1α are mediated through the transcriptional regulation of nuclear respiratory factor (NRF)-1 and -2 and through the coactivation of NRF-1-mediated transcription (35). NRFs are important for mitochondrial biogenesis, and they have binding sites located in the promoter region of various nuclear-encoded mitochondrial proteins such as Tfccr, which directly affects mitochondrial function by lowering COX and complex I activities. Thus, we hypothesized that beneficial phenotypic adaptations leading to improved mitochondrial biogenesis and function could result from T3 treatment.

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**Fig. 3.** ROS management in control and patient cells with T3 treatment. A: ROS production in control and patient cell lines before and after T3 treatment. Inset, overlay histogram for dichlorofluorescein (DCF) fluorescence shown for one patient subjected to vehicle and T3 treatment. B: Top: Western blot showing MnSOD and uncoupling protein 2 (UCP2) in patients and control cells treated with T3 or vehicle. Bottom: MnSOD protein expression in control and patient cells before and after T3 treatment shown graphically (in AU), n = 3 control and 3 patient cell lines per analysis. *P < 0.05, overall effect of T3 on both patient and control groups; ‡P < 0.05, overall effect of patient vs. control groups; §P < 0.05, interaction of the effect of T3 on patient and control groups.

**subject groups**, ranging from 13% to 18% below that of vehicle-treated cells (Fig. 4A).

**Cytoplasmic calcium levels.** Cytoplasmic calcium levels were 1.7-fold higher in the patient group compared with the control group (P < 0.05; Fig. 4B). T3 treatment resulted in a similar 23% decline in cytoplasmic calcium in both control and patient fibroblasts (P < 0.05; Fig. 4B).

**DISCUSSION**

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mitochondrial content but could be due to a decrease in COX-specific activity per unit of organellar mass. Since it has been previously demonstrated that PGC-1α expression increases after T3 treatment (16, 48, 49), we also examined the effect of T3 on patient and control cells. After T3 treatment, neither PGC-1α nor Tfam expression was altered in control or patient cell lines. This likely reflects the tissue-specific nature of the adaptive response to T3. In addition, PGC-1α and Tfam are not the only possible routes for T3 action on the mitochondria. T3 has also been shown to directly regulate mtDNA transcription in cardiomyocytes by binding to TR-α isoforms that localize to the mitochondria and bind to thyroid response elements on mtDNA (50). Despite the lack of PGC-1α or Tfam elevations, there was an increase in COX activity for both control and patient cell lines with T3 treatment. These increases indicate the possibility of an improved capacity for O2 consumption within patient and control cells, as also evidenced by the increase in ATP levels and the decrease in ADP-to-ATP ratios. In addition, a rise in COX activity, complex V activity, and ATP levels in conjunction with lower ADP-to-ATP ratios suggests that T3 can modify mitochondrial composition without necessarily influencing overall mitochondrial biogenesis, as reflected by mitochondrial mass.

$\Delta \Psi_m$ is strongly dependent on respiratory function as well as the composition of the inner membrane. In normal situations, $\Delta \Psi_m$ is maintained mainly through a balance between transmembrane proton pumping to the intermembrane space mediated by electron transfer and proton translocation to the matrix for ATP synthesis by the ATPase complex. A number of studies have examined $\Delta \Psi_m$ in mitochondrial disease, with various reports revealing lower values compared with controls and other reports describing conditions in which a higher $\Delta \Psi_m$ was found (2, 13, 41, 47). Alterations in $\Delta \Psi_m$ are variable and dependant on the type of mitochondrial mutation. In the present study, elevated $\Delta \Psi_m$ was found in each of the three patient cell lines compared with controls. This would be the result of a decrease in proton translocation into the matrix, as would be expected with the lower complex V activity in each of the patient cells. The T9185C LS patient with an ATPase 6 gene mutation resulted in a greater $\Delta \Psi_m$ due to the lower rate of proton pumping back into the mitochondrial matrix (5). Similarly, patient cells with A3260G tRNA$\text{Leu(UUR)}$ mutations demonstrated lower complex V activity, which is most likely a result of tRNA base substitutions at the mitochondrial encoded components of complex V. The final LS patient cells, containing a G13513A mutation at the ND5 gene, has most likely acquired further damage to the existing electron transport chain as a result of increased ROS generation. It is therefore apparent that this patient’s subsequent mutations have altered complex V activity. Thus, despite the presence of three distinct mtDNA mutations, all three patient cell lines examined in this study displayed increased $\Delta \Psi_m$ compared with the three control cell lines.

T3 treatment successfully decreased $\Delta \Psi_m$ in both control and patient cells. The effect observed in patient cells was the reduction of $\Delta \Psi_m$ toward the physiological range of the pretreatment control levels along with an increase in ATP generation. This is most likely due to the increase in complex V activity after T3 treatment, which has been previously shown to be regulated by thyroid hormone (8, 10, 34) and would therefore account for an increase in proton translocation to the matrix. However, this proton translocation appears to be independent of UCP2 and ANT (data not shown), since T3 did not alter the expression of these inner membrane proteins.

ROS production has been found to be higher in patient cells with various types of mitochondrial diseases (25, 31). It is likely that the elevated ROS production in our patient cells is due, in part, to their greater $\Delta \Psi_m$. This would inhibit proton pumping and stall electron flow through the electron transport chain (10), causing them to accumulate around complex I (12) and coenzyme Q (32), where they are proposed to increase ROS production. The elevated $\Delta \Psi_m$, as a result of the lower complex V activity, along with the lower levels of the antioxidant enzyme MnSOD, likely both contribute to the higher levels of ROS in patient cells. Notably, T3 treatment induced the expression of MnSOD in both control and patient cells.

Fig. 4. Mitochondrial membrane potential ($\Delta \Psi_m$) and cytoplasmic calcium levels in patient and control cells after T3 treatment. A, left: $\Delta \Psi_m$ in control and patient cell lines before and after T3 treatment. Right, overlay histogram for JC-1 fluorescence shown for one patient subjected to vehicle and T3 treatment. B, left: cytoplasmic calcium levels in control and patient fibroblasts after 12 days of treatment with vehicle or T3. Right, overlay histogram for fluo-3 fluorescence shown for one patient subjected to vehicle and T3 treatment. $n = 3$ control and 3 patient cell lines per analysis. *$P < 0.05$, overall effect of T3 on both patient and control groups; ‡$P < 0.05$, overall effect of patient vs. control groups.
Therefore, the lower ROS production in both patient and control cells could also be attributed, in part, to the enhancing effect of T3 on MnSOD levels.

Cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)],) is partially maintained by ATP-dependent Ca\(^{2+}\) pumps located at the plasma membrane and within intracellular organelles such as the endo-(sarcoplasmic reticulum (26). This process may be compromised in disease in which mitochondrial ATP synthesis is reduced, leading to elevated [Ca\(^{2+}\)], (4). In addition, each of the patients in this study was diagnosed with lactic acidosis (Table 1), which could also lead to an increase in [Ca\(^{2+}\)]. Intracellular acidification inhibits plasma membrane Na\(^{+}\)/H\(^{+}\) exchange, resulting in an elevation of cytoplasmic Na\(^{+}\) concentration and inhibition of Na\(^{+}\)/Ca\(^{2+}\) exchange at the plasma membrane (1). As predicted, we observed significantly elevated [Ca\(^{2+}\)], levels in each of our patients compared with control subjects. This elevation of [Ca\(^{2+}\)], has also been found by others (30) using cells from MELAS patients. A key consequence of slow or maintained elevated [Ca\(^{2+}\)], is that there is a corresponding progressive increase in resting mitochondrial Ca\(^{2+}\) concentration. When [Ca\(^{2+}\)], rises to 1–3 \(\mu\)M, a mitochondrial Ca\(^{2+}\) overload can occur, leading to permeability transition pore opening and the release of cytochrome c and apoptosis-inducing factor, resulting in apoptosis (9). It has been proposed that an increase in mitochondrial Ca\(^{2+}\) concentration results in the activation of Ca\(^{2+}\)-sensitive phosphatases, leading to the dephosphorylation of COX subunits. This leads to an increase in COX proton pumping and \(\Delta\psi_{m}\) (18). This would then result in elevated ROS production and could provide a fundamental explanation for the concurrent elevations in [Ca\(^{2+}\)],, ROS, and \(\Delta\psi_{m}\) in patient cells compared with control cells.

After T3 treatment, an elevation in complex V activity and a decrease in [Ca\(^{2+}\)], occurred. This could explain the observed effect of T3 on the reversal of \(\Delta\psi_{m}\) and ROS production within patient cells. Therefore, T3 treatment may decrease the propensity of patient cells to experience mitochondrial Ca\(^{2+}\) overload, permeability transition pore opening, and apoptosis (29, 51). With T3 treatment, there was also an increase in antioxidant MnSOD activity along with an increase in the capacity for O\(_2\) consumption, as demonstrated by the increase in COX and complex V activity, resulting in higher ATP levels. This adaptation provides a greater range of cellular respiratory rates and lessens the reliance of the cells on anaerobic metabolism. Thus, lactate formation is reduced, and the complete oxidation of lipid and carbohydrate is favored. As noted above, this may also contribute to a reduction in intracellular Ca\(^{2+}\) levels.

In conclusion, the results of the present study indicate that fibroblast cells from patients with mtDNA defects exhibit lower COX activities despite having similar mitochondrial masses as control cells. Furthermore, these cells have higher levels of ROS and intracellular calcium, along with lower complex V activity. T3 treatment reversed these abnormalities by improving mitochondrial function and cellular energy status. Therefore, it may be suggested that potent and specific T3 analogs may be useful to reverse the pathophysiology of cells from patients with mtDNA defects and impaired organelle function.

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