Compensatory responses of protein import and transcription factor expression in mitochondrial DNA defects

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Joseph, Anna-Maria, Arne A. Rungi, Brian H. Robinson, and David A. Hood. Compensatory responses of protein import and transcription factor expression in mitochondrial DNA defects. Am J Physiol Cell Physiol 286: C867–C875, 2004.—Defects in mitochondrial DNA (mtDNA) evoke distinctive responses in the nuclear genome, leading to altered mitochondrial biogenesis. We used C2C12 cells depleted of mtDNA (rho− cells) and fibroblasts from a mitochondrial encephalopathy, lactic acidosis, and strokelike episodes (MELAS) patient to examine adaptations of the protein import machinery and transcription factors involved in mitochondrial biogenesis. In rho− cells, Tom20 and Tim23 protein levels were reduced by 25% and 59%, whereas mtHSP70 was induced by twofold relative to control cells. These changes were accompanied by a 21% increase in enhanced yellow fluorescent protein (EYFP) import into mitochondria in rho− cells (P < 0.05). In contrast, in MELAS cells mtHSP70 was elevated by 70%, whereas Tom20 and Tom34 protein levels were increased by 45% and 112% relative to control values. EYFP import was not altered in MELAS cells. In rho− cells, protein levels of the transcription factors nuclear respiratory factor-1 (NRF-1) and transcription factor A (Tfam) declined by 33% and 54%, whereas no change was observed for the coactivator peroxisome proliferator receptor-γ coactivator-1α (PGC-1α). In contrast, Tfam was increased by 40% in MELAS cells. Rho− cells displayed reduced oxygen consumption (VO2) and ATP levels, along with a twofold increase in lactate levels (P < 0.05). In electrically stimulated C2C12 cells, 109% of mitochondrial COX activity, and Tom34 levels, respectively (P < 0.05). Our findings suggest that compensatory adaptations occurred to maintain normal rates of protein import in response to mtDNA defects and support a role for contractile activity in reducing pathophysiology associated with mtDNA depletion. Because the expression of nuclear-encoded transcription factors and protein import machinery components was dependent on the type of mtDNA defect, these findings suggest involvement of distinct signaling cascades, each dependent on the type of mitochondrial defect, resulting in divergent changes in nuclear gene expression patterns.

mitochondrial biogenesis; skeletal muscle; rho− cells; mitochondrial disease; peroxisome proliferator-activated receptor-γ coactivator-1α

THE MITOCHALONAL PROTEINS required for the production of energy that is used to drive cellular processes are derived from the expression of both the mitochondrial and nuclear genomes. Although the vast majority of these proteins are encoded in the nucleus, the small contributions made by mitochondrial DNA (mtDNA) have significant consequences for mitochondrial function and integrity. Defects in the synthesis of one or more of the 13 mtDNA-encoded respiratory subunits can lead to respiratory chain dysfunction and a wide range of pathogenic conditions, many of which affect skeletal muscle (36). Patients diagnosed with these conditions often display reduced mtDNA content, resulting in lower oxidative capacity, decreased levels of intracellular ATP, and reduced exercise tolerance (21).

The development of cell lines depleted of mtDNA (rho− cells) has provided researchers with a suitable model to study some of the molecular mechanisms governing mitochondrial defects. These cells are usually established from long-term exposure to ethidium bromide (EtBr) (9). Interestingly, the mtDNA depletion brought about by this treatment has been shown to activate a retrograde signaling pathway leading to distinctive responses within the nuclear genome (2). Studies in mammalian cells have shown increased mRNA expression and synthesis of nuclear-encoded mitochondrial enzymes that are crucial for maintaining metabolic control (2, 26). In human cells, mtDNA depletion results in an upregulation in the mRNA levels of several respiratory chain subunits (25), and in HeLa cells there is an increased expression of the transcription factors transcription factor A (Tfam) and nuclear respiratory factor-1 (NRF-1) (27). Furthermore, mtDNA-depleted cells have been shown to display an enhanced expression of proteins involved in Ca2+ signaling (2), as well as activation of the CAMP response element-binding protein (CREB; Ref. 1).

A group of nuclear-encoded proteins that is vital for mitochondrial biogenesis includes those that comprise the protein import machinery of mitochondria. These are important for regulating the rate at which precursor proteins enter the organelle. It has long been suspected that defects in the import pathway could be responsible for some mitochondrial diseases; this has now been fortified by the recent discovery of the Mohr-Tranebjaerg syndrome, which is directly caused by defects in the inner membrane protein machinery (14, 20). In addition, patients diagnosed with encephalomyopathies exhibit reduced expression of heat shock protein (HSP)60, an inner matrix protein responsible for refolding of the mature protein into its native form (16). The significance of the protein import system is further confirmed by studies demonstrating that several stimuli, including contractile activity of skeletal muscle, thyroid hormone treatment, and muscle cell differentiation, can alter the expression of the import pathway components, ultimately leading to a change in protein import rate and mitochondrial phenotype (8, 13, 34).

Although there is direct evidence to support a role for the protein import machinery in mitochondrial dysfunction, little is known about the specific adaptations that occur in this system in response to impaired states of mitochondrial biogenesis.

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Thus we examined whether mitochondrial defects could alter the expression of nuclear genes encoding key components of the mitochondrial protein import machinery, resulting in a functional change in the rate of import. We compared rho- 
C2C12 cells and fibroblasts from a mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS) patient harboring a mutation (Ala2343Gly) in the mitochondria-encoded tRNAleuc(UUR) gene. In addition, we wanted to further characterize the response of the nuclear genome to mtDNA defects at the level of transcription factor protein expression. Finally, we also examined the functional metabolic consequences of mtDNA defects in muscle cells and the potential of contractile activity to ameliorate the observed mitochondrial dysfunction. Our results emphasize the divergent adaptive gene expression responses to various defects in mtDNA, demonstrate that protein import rates are not reduced under these conditions, and illustrate the potential of contractile activity to attenuate the metabolic dysfunction produced by mitochondrial disease.

METHODS

All procedures were performed in conformance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society.

Materials. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Dulbecco’s phosphate-buffered saline (DPBS), horse serum (HS), and penicillin (P/S) were purchased from Sigma Aldrich (Oakville, ON, Canada). Nitrocellulose and nylon membranes (Hybond N+) were obtained from Amersham Pharmacia Biotech (Bale D’Urfe, QP, Canada). LipofectAMINE Plus reagent and TRIZol were purchased from Invitrogen (Burlington, ON, Canada). Reverse transcriptase (RT) was purchased from New England Biolabs (Mississauga, ON, Canada) and Tsg DNA polymerase from Bio Basics (Markham, ON, Canada). Materials for metabolite measurements [lactate standard, lactate dehydrogenase (LDH), and luciferase-luciferin] as well as phosphate inhibitors and PCR primers were acquired from Sigma. For luciferase assays, a Renilla luciferase kit from Promega (Markham, ON, Canada) was used. MitoTracker Red (CM-H2XRos) was purchased from Molecular Probes (Eugene, OR). For immunoblotting experiments, the enhanced yellow fluorescent protein (EYFP) antibody was from Clontech (Palo Alto, CA), the peroxisome isolation of rho- 
myotube formation, myoblasts were induced to differentiate with DMEM supplemented with 10% FBS and 1% P/S and maintained at 37°C in 5% CO2. For myotube formation, myoblasts were induced to differentiate with DMEM supplemented with 5% heat-inactivated HS and 1% P/S. For isolation of rho- 
cells, C2C12 cells were plated in 100-mm culture dishes and cultured as above, with the exception of the addition of EtBr to the medium. After 6 wk, cells were washed with PBS containing 5 mM EDTA and colonies were selected with silicone cloning cylinders. Cells were trypsinized, diluted, and dispersed in DMEM containing 10% FBS, 1% P/S, and 100 ng/ml EtBr. Frozen stocks from individual clones were made, and the mtDNA content in these cells was determined by PCR analyses. Skin fibroblasts obtained from a MELAS patient carrying a 51% mutation level of the tRNA gene were grown in α-MEM supplemented with 10% FBS and 1% P/S.

Determination of mtDNA content. For quantification of mtDNA content in control C2C12 and rho- 
cells, total cellular DNA was extracted with a GenElute Mammalian Genomic DNA Kit (Sigma). Sequence-specific primers for mtDNA included 5′-GTACATT-AATCAATGG-3′ (upstream) and 5′-GCGGGGTGTGAGGTT-3′ (downstream; 218 bp; Ref. 18). PCR conditions included denaturing at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min. Bands were quantified with SigmaGel software (Jandel).

DNA transfection. Cells were harvested and plated as described in Cell culture and isolation of EtBr-treated clones, and they were 70–90% confluent on the day of transfection. For protein import assessments, control and rho- 
C2C12 myoblasts were transfected with either 3 μg of empty vector (pcDNA 3.1) or COX VIII fused to a yellow fluorescent protein (pEYFP-mito, Clontech, Palo Alto, CA). Cells were seeded, overexpressed, and total protein was extracted with either 3 μg of empty vector (pCAGGS) or a Tom34 expression plasmid (pCAGGS/Tom34) along with pCMV/Renilla luciferase (5 ng/dish). Transfections were done with LipofectAMINE Plus reagent according to the manufacturer’s protocol. At the end of the culture period, cells were either scraped for protein or visualized for fluorescence.

Immunoblotting and protein import. Total protein was isolated with 1× lysis buffer (Promega) with protease and phosphatase inhibitors, 0.3 μg/ml leupeptin, 0.3 μg/ml aprotinin, 0.3 μg/ml pepstatin, 0.5 mM PMSF, 1 mM DTT, 250 mM sodium pyrophosphate, and 250 mM sodium orthovanadate. Proteins were size-separated by gel electrophoresis on 10–12% [cytosolic (c)HSP70, mtHSP70, HSP60, MSF-L, Tfam, NRF-1, PGC-1α, EYFP, and COX IV; 1:3,500 for MSF-L; 1:500 for cpn10, NRF-1, and PGC-1α] polyclonal antibodies and transferred to nitrocellulose membranes. Blots were blocked for 1 h with 5% or 10% (PGC-1α) skim milk in 1× Tris-buffered saline-Tween 20-Tris-HCl (TBST, pH 7.4) and probed with the appropriate primary antibody (1:1,000 for cHSP70, mtHSP70, HSP60, Tom20, Tom34, Tom23, Tfam, EYPF, and COX IV; 1:3,500 for MSF-L; 1:500 for cpn10, NRF-1, and PGC-1α). Blots were then incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase at a dilution of 1:1,000 (PGC-1α), 1:500 (MSF-L and Tom34), 1:2,000 (Tom20 and Tom23), or 1:3,500 (cpn10), anti-goat secondary antibody at a dilution of 1:10,000 (NRF-1), or anti-mouse secondary antibody at a dilution of 1:1,000 (cHSP70, mtHSP70, HSP60, Tom20, Tom34, Tom23, Tfam, EYFP, and COX IV). Blots were washed (3 × 5 min), and protein was subsequently visualized with an enhanced chemiluminescence kit (Amersham Pharmacia) and quantified with SigmaGel software.

To assess protein import, cells transfected with the pEYFP-mito construct were subjected to Western blot analyses and EYFP protein levels were detected with a monoclonal antibody. Protein import was calculated as the amount of EYFP imported (mature) over the total protein available (preprotein and mature).

RT-PCR analyses. To compare NRF-1 mRNA expression levels between control and rho- 
cells, RT-PCR was performed. Total RNA was isolated with TRZol reagent according to the manufacturer’s instructions. Reverse transcription was performed with 10 μg of total RNA and 5 μl of RT (New England Biolabs) in a final volume of 20 μl. PCR amplification of NRF-1 and S12 RNA cDNAs was accomplished by using 50 pmol of primer in a final volume of 40 μl. NRF-1 primers used were 5′-CCACGGTACAGGGCGGTT-3′ (upstream) and 5′-AGTTGCTCCTCCTGAGCCTC-3′ (downstream; 121 bp; Ref. 29). S12 rRNA primers were 5′-GGAAGGACGATGCT-
described by Glick et al. (11). Briefly, cells were trypsinized and
Triton X-100 and protein contents were normalized to total protein content.
All measurements were normalized to total protein content.

RESULTS

mtDNA depletion. To confirm the effectiveness of the EtBr treatment, the mtDNA content of rho– clones used in this study were determined by PCR analyses. Results showed that the rho– cells used here contained only 12% of the mtDNA content of control cells (Fig. 1A).

Effect of mitochondrial defects on protein import machinery components. To determine whether defects in mtDNA could lead to alterations in the expression of protein import machinery components, we examined the mRNA and protein levels of several key import machinery components in rho– and MELAS patient cells. mRNA transcripts encoding Tom40 and Tom20 and Tim23 were significantly reduced, whereas Tom20 mRNA tended (P = 0.06) to decrease, in rho– cells. Tim17 mRNA remained unaffected by mtDNA depletion (Fig. 1B). At the protein level of expression, no significant changes were observed for the intramitochondrial chaperonin HSP60 or the cytosolic chaperone MSF. However, levels of the cytosolic chaperone chS70, as well as Tom20, Tom34, and Tim23 membrane protein levels in rho– cells were reduced by 30%, 25%, 12%, and 59% relative to control, respectively. In contrast, both intramitochondrial chaperonin proteins hsp70 and cpn10 protein were induced by 2- to 2.5-fold in rho–

Fig. 1. A: total DNA was isolated from control and rho– C2C12 cells, and the content of mitochondrial DNA (mtDNA) as well as the nuclear-encoded S12 rRNA were determined by PCR. Equal amounts of the PCR reaction (20 μl/lane) were loaded on a 1.8% agarose gel, and the products were visualized after ethidium bromide (EtBr) staining. B: Northern blot (25 μg/lane) analyses of the mRNA expression of the nuclear-encoded protein import machinery components Tom40, Tom20, Tim23, and Tim17 in control and rho– cells. EtBr-stained gels are shown at bottom. A summary of repeated experiments is shown at right, expressed as % of control cells. *P < 0.05 (n = 3–4) vs. control. For quantification, all Northern blots were subsequently probed for 18S to correct for any loading inequalities between samples.
relative to control cells (Figs. 2A). Similarly, Western blot analysis of MELAS patient fibroblasts revealed an upregulation of mtHSP70 to 1.7-fold above control values. However, compared with the changes observed in mtDNA-depleted cells, Tom20 and Tom34 protein levels were increased by 45% and 112% above control values, respectively, whereas HSP60 was reduced by 25% of control cells (Fig. 2B). Thus the adaptability of the individual protein import machinery components is dependent on the type of mtDNA defect.

**Effects of mtDNA depletion on membrane potential and protein import.** We qualitatively assessed the mitochondrial membrane potential ($\Delta \psi_m$) with the cationic fluorescent dye MitoTracker because of the influence of the $\Delta \psi_m$ on the rate of protein import. Rho$^-$ cells exhibited reduced levels of fluorescence compared with control cells with a normal oxidative capacity (Fig. 3A, a and c). In addition, Fig. 3A shows that reduced mtDNA content in rho$^-$ cells resulted in subtle changes in the structural organization of the reticular network, leading to mitochondria that appeared round and swollen (Fig. 3A, a and c). Despite this, fluorescence microscopy of cells transfected with the pEYFP-mito vector showed specific fluorescence in mitochondria indicative of proper processing and import of EYFP to the mitochondrial matrix (Fig. 3A, b and d). To quantify the changes occurring in the protein import pathway, we measured the amount of EYFP imported into mitochondria by immunoblotting after transfection of control and rho$^-$ C2C12 cells with an empty vector or the pEYFP-mito construct. EYFP import, calculated as the fraction of total EYFP synthesized, was 21% greater in rho$^-$ cells than in control cells ($P < 0.05$; Fig. 2B). This result was further corroborated by experiments in which the cells were metabolically labeled with $^{35}$S-labeled methionine and radiolabeled malate dehydrogenase (MDH) was immunoprecipitated. The import of MDH was enhanced by 23% in mtDNA-depleted cells compared with control cells with normal levels of mtDNA and rho$^-$ C2C12 cells exhibiting reduced mtDNA content. The results of repeated experiments are depicted graphically on right and are expressed as % control.

**Fig. 2.** Effect of mtDNA defects on the levels of protein import machinery components. A: representative Western blots of Tom20, Tom34, Tim23, mitochondrial heat shock protein (mtHSP)70, HSP60, Tim23, cHSP70, mitochondrial import stimulation factor, L subunit (MSF-L), and chaperonin 10 (cpn10) in control cells with normal levels of mtDNA and rho$^-$/C2C12 cells exhibiting reduced mtDNA content. The results of repeated experiments are depicted graphically on right and are expressed as % control. B: representative Western blots of Tom20, Tom34, mitochondrial heat shock protein, HSP60 levels in control skin fibroblasts lacking any mtDNA mutations and mitochondrial encephalopathy, lactic acidosis, and strokelike episodes (MELAS) patient cells exhibiting a mutation in the tRNA$^\text{Leu(UUR)}$ gene. Protein levels of Tim23 were not detectable in MELAS cells with the antibody used. Protein expression was quantified, and a summary of results is shown at right.

**Fig. 3.** A: fluorescence microscopy of control (a) and rho$^-$ (c) cells stained with MitoTracker (400 nM) and enhanced yellow fluorescent protein (EYFP) import in control C2C12 myoblasts (b) and rho$^-$ (d) cells transfected with a pEYFP-mito construct. B: Western blot of total protein (80–100 µg protein/lane) from rho$^-$ cells and MELAS cells compared with their respective C2C12 and fibroblast controls, transfected with pEYFP-mito and measured with a monoclonal antibody directed toward EYFP. Bottom: graphic representation of EYFP import, in which the amount of mature protein is expressed as % of total protein available. *$P < 0.05$ (n = 3) vs. control cells. Equal loading was determined with Ponceau staining.
cells (data not shown). These results suggest that during mtDNA depletion, changes in the expression of individual import proteins directly result in the accelerated import of precursor proteins into the inner mitochondrial compartment. Although an increased rate of protein import was observed in rho− cells, no difference existed between control and MELAS cells (Fig. 2C). However, we did note an overall greater accumulation of total EYFP (preprotein and mature) in patient cells, which cannot be attributed to transfection efficiency but may be explained by differences in transcriptional and/or translational processing events related to the expression of the EYFP protein.

Effect of mitochondrial defects on transcription factor protein expression. It was established previously that mtDNA-depleted HeLa cells display increased mRNA levels of the nuclear-encoded genes Tfam and NRF-1 (27). Therefore, we evaluated the expression of these genes in muscle cells at the protein level and included the important transcriptional coactivator PGC-1α, as well as an inner membrane marker, COX IV. RT-PCR analysis of NRF-1 confirmed a 64% increase in NRF-1 transcript levels in rho− cells compared with controls (P < 0.05; Fig. 4A). Interestingly, this was not reflected at the protein level. NRF-1 protein was 33% lower in rho− cells compared with control values (Fig. 4B). Although only a modest change was observed in PGC-1α protein between control and mtDNA-depleted cells, COX IV and Tfam protein levels were decreased by 34 and 54%, respectively, relative to control cells (P < 0.05; Fig. 4B). In contrast, in the MELAS patient cells, we observed a 40% induction of Tfam protein levels, a nonsignificant change in PGC-1α (−8%), and a 53% increase in COX IV protein (Fig. 4C). Thus the expression of important transcription factors involved in mitochondrial biogenesis is also dependent on the type of mtDNA defect.

Effect of Tom34 overexpression on protein import. Recently, studies have revealed an important role for Tom34 in the protein import pathway (5). To further characterize its function in muscle cells, we examined the effects of Tom34 overexpression on EYFP protein import in C2C12 myoblasts. Transfection with the Tom34 expression plasmid resulted in an ∼33% higher Tom34 protein level in C2C12 cells. This overexpression led to a modest 18% increase in EYFP import into mitochondria (P < 0.05; Fig. 5A).

Effect of contractile activity on Tom34 and Tom20 protein levels. In view of the effect of Tom34 overexpression, and the fact that previous work has shown that components of the protein import machinery are inducible in response to muscle chronic contractile activity (34), we evaluated the effect of 2 and 4 days of muscle cell stimulation on Tom34 and Tom20 protein expression in C2C12 cells. Both Tom34 and Tom20 protein levels progressively increased throughout the stimulation period, reaching values that were 1.7- and 2.9-fold above levels found in nonstimulated cells after 4 days of contractions (P < 0.05; Fig. 5, B and C).

Effect of contractile activity on mtDNA, COX activity, and metabolism in C2C12 cells. To evaluate the potential of contractile activity to reverse the metabolic defects in rho− cells, we first examined the effect of contractile activity on mtDNA levels. After 4 days of stimulation, mtDNA was increased by 2.1-fold in stimulated C2C12 cells compared with nonstimulated control values (Fig. 6A). In rho− cells, VO2 was reduced by ∼40% (P < 0.05) relative to control (19.8 natoms·mg protein−1·min−1) values. Cellular lactate levels were increased twofold above control (0.05 μmol/mg protein; P < 0.05) values, and ATP levels were 65% lower in rho− cells than in control cells (P < 0.05; Fig. 6B). In contrast to the changes
observed above, 4 days of contractile activity resulted in a 78% higher \((P < 0.05)\) \(V_\dot{O}_2\) in stimulated C\(_{2}\)C\(_{12}\) cells compared with control levels. This corresponded well with the observed stimulation-induced 60% increase in COX activity (Fig. 6C). Lactate and ATP levels were not significantly different between stimulated and nonstimulated cells (Fig. 6B).

**DISCUSSION**

A large number of studies implicate mitochondrial defects and impaired respiratory phenotype in the pathology of human disease. In the present study, we used muscle cells with chemically depleted mtDNA and cultured fibroblasts derived from a MELAS patient to evaluate the responses evoked by genetic and metabolic stress signals on key aspects of the pathway leading to mitochondrial biogenesis. These include the mitochondrial import machinery, as well as primary transcriptional regulators involved in nuclear and mitochondrial DNA transcription.
Mitochondria import the majority of proteins that are required for mtDNA transcription and replication, as well as the enzymes that comprise its metabolic pathways via the mitochondrial protein import machinery. Previous studies showed that the import pathway is inducible under physiological conditions such as increased contractile activity and thyroid hormone treatment (8, 34). Thus we wanted to determine whether mitochondrial dysfunction based on a mtDNA defect could elicit cellular signals that could also result in compensatory adaptations. We reasoned that these adaptations could ultimately be reflected by a change in the rate of precursor protein import into the organelle and presumably be mediated by alterations in the stoichiometry of protein import machinery components. In addition, because the mechanisms governing the expression of these nuclear-encoded translocating components (i.e., Tom and Tim complexes) are not known, we also sought to evaluate the expression of transcription factors that are currently recognized to be important in regulating the transcription of nuclear genes encoding mitochondrial proteins.

Our results show that severe mtDNA depletion in muscle ( rho− ) cells may modestly impair the capacity of the cells to direct precursor proteins to the outer membrane. This is reflected by a decrease in the level of the cytosolic chaperone chHSP70 but no change in the large subunit of MSF, an important and specific chaperone that binds to proteins possessing mitochondrial presequences. However, this did not have a functional consequence, because import rates were not decreased. In addition, mtDNA depletion led to reduced levels of the mRNA and protein levels of the outer membrane receptor Tom20, as well as the inner membrane protein Tim23. In addition, Tom40 mRNA levels were reduced in rho− cells. No changes were detected in HSP60, but rho− cells exhibited significant increases in the intramitochondrial chaperonin cpn10 and mtHSP70. Given that we previously documented (13) the importance of Tom20 in protein import, we assumed that this alteration in the protein import machinery would influence the rate of import. Surprisingly, we found that protein import was not attenuated in rho− myoblasts but rather was modestly increased. This occurred despite a reduced V_o2, lower levels of ATP, and a diminished Δψ_m, factors that are known to play a role in mediating protein translocation across the inner membrane. Evidently, either the extent of change of each of these factors was not great enough to impair the protein import process or sufficient compensation via the increased expression of alternative protein import machinery components (e.g., mtHSP70) occurred. It is of interest to note that the differential expression of these import machinery proteins led to a concomitant disruption in the distribution of EYFP in mitochondria, which was apparent by the focal accumulation of EYFP in rho− cells. Notably, overexpression of human Tom20 in mammalian cells has been reported to result in a significant change in mitochondrial structure (40). Together, these findings suggest that metabolic state-induced changes in the expression of protein import machinery components lead to alterations in organelle morphology (10). Thus the protein import machinery appears to be involved in regulating mitochondrial structure, perhaps via its association with cytoskeletal proteins (15).

Patients with MELAS possess a tRNALeu(UUR) gene mutation in mtDNA (12) that prevents the proper synthesis of all proteins encoded by the mitochondrial genome (3). In MELAS fibroblasts we found that HSP60 levels were reduced, a finding that was reported previously in patients with encephalomyopathies (16). HSP60 is involved in refolding of the mature protein after its import into the organelle. In distinct contrast to the decrease observed in rho− cells, the import receptors Tom20 and Tom34 were markedly elevated in MELAS cells, along with mtHSP70 levels. Under these conditions, the rate of protein import was not impaired, as EYFP was imported with equal efficiency in both control and MELAS cells. Thus neither model of mtDNA defects displayed reduced rates of protein import. A compensatory feature common to both conditions was the increase in mtHSP70 expression, which may serve to counteract the reduced expression of other components of the protein import pathway. This mitochondrial matrix chaperone directly interacts with preproteins in an ATP-dependent reaction to facilitate their translocation across the inner membrane (8). Therefore, higher levels of mtHSP70 could enhance the import of preproteins into the matrix, subsequently contributing to a greater rate of subunit assembly within the respiratory chain.

The compensatory changes in gene expression evoked with the two mtDNA defects investigated in this study differ from each other and, understandably, from those that occur as a result of nuclear genome mutations. The divergent adaptive responses of the MELAS and rho− cells could be due to the fact that they are different cell types (muscle vs. fibroblast) or that the difference in mtDNA defect (mtDNA depletion vs. mutation) has a real impact on the mitochondrial-to-nuclear signaling events that produce the compensatory changes in gene expression. Physiologically, this indicates that we cannot predict the gene expression outcome of a mtDNA defect without knowing the specifics of the defect in question. In our previous work with cells obtained from a patient with multiple mitochondrial disease (MMD) of nuclear origin, we also reported (30) that there was no impairment in the efficiency of protein import. However, this was not due to a compensatory adaptation in mtHSP70 expression, because levels of this chaperone were markedly reduced. It seems more likely that the lack of change of Tom20, an outer membrane protein that is known to influence the rate of protein import when either over- or underexpressed (13), played a role in preventing a decrease in import in MMD. However, the possibility that other protein import machinery components (e.g., Tom34) not measured in that study could have been influential cannot be ruled out. The differences in gene expression and protein import responses observed under these three mitochondrial disease conditions are summarized in Table 1, relative to their appropriate control cells.

Tom34 is a recently characterized outer membrane protein with no apparent yeast or fungal homolog. Its function is currently uncertain; however, antibody inhibition experiments have implicated it as an important component in the protein import process (5). We show here that forced overexpression...
of Tom34 in skeletal muscle cells stimulates an increase in EYFP import into the mitochondrial matrix. In addition, an increase in Tom34 protein was observed after a period of contractile activity, demonstrating the adaptability of this protein to the increased energy demands imposed by a physiological stimulus such as exercise. Indeed, contractile activity also presents a cellular metabolic disturbance in which compensatory changes in the protein import machinery components are evident. We showed previously (34) that mtHSP70, Tom20, and Tom34 in skeletal muscle by a program of increased contractile activity in vivo. These changes resulted in a coincident increase in precursor protein import, as measured in isolated mitochondria (34). Thus the data suggest 1) that sufficient levels of Tom34, as observed in both rho− and MELAS cells, may have been partly responsible for preventing a decline in the protein import rates of these cells and 2) that contractile activity can serve to counteract an observed decrement in protein import rate via its induction of protein import machinery components. The lack of decrease in import in the MELAS cells, despite a substantial change in Tom34, suggests that other, yet unidentified, import machinery components decrease in MELAS cells to prevent the effect observed by Tom34 overexpression alone (Fig. 5).

The activation of genes that lead to the upregulation of proteins comprising the import pathway, and mitochondrial biogenesis in general, is in large part orchestrated by diverse transcription factors that include Tfam and NRF-1 (31). In addition, considerable evidence has shown the potential role of the transcriptional coactivator PGC-1α in the activation of nucleus-encoded mitochondrial proteins, leading to increased levels of mtDNA and an enhanced capacity for oxidative metabolism in both heart and skeletal muscle (23, 39). It is believed that the effects of PGC-1α on mitochondrial biogenesis are mediated by its strong coactivation of NRF-1, which binds to the promoter of multiple nuclear genes encoding mitochondrial respiratory chain proteins (31). The finding that PGC-1α protein is not altered in mtDNA-defective cells suggests that it is important for maintaining mitochondrial volume within the cell in the face of a reduced NRF-1 protein content. Consistent with previous findings, NRF-1 mRNA was upregulated in rho− cells (27) whereas NRF-1 protein levels were decreased, indicating that NRF-1 is posttranscriptionally modified in mtDNA-depleted cells. The level of Tfam, a downstream target of NRF-1, was also diminished in rho− cells. In addition, COX IV protein, used as an inner membrane protein marker, was decreased in mtDNA-depleted cells, coinciding with the findings observed in rho0 human fibroblasts (26). In contrast to these changes, both Tfam and COX IV proteins were elevated, whereas PGC-1α was unaffected in MELAS cells. Thus, whereas PGC-1α induction does not appear to play a role in these forms of metabolic stress, NRF-1 and Tfam seem to be directly involved. The importance of these genes is further supported in knockout studies in which a disruption of either NRF-1 or Tfam leads to severely reduced levels of mtDNA and lethality during the early stages of embryogenesis (17, 22). Interestingly, the metabolic stress imposed by chronic contractile activity appears to differ from that imposed by mtDNA defects, because PGC-1α protein is induced under contractile activity conditions, along with Tfam and NRF-1 (19). This may be related to the fact that contractile activity represents a cellular signal in which ATP demand is altered, whereas mtDNA defects affect cellular ATP supply.

Previous work strongly suggests the possibility that Ca2+ is involved in the transmission of the mitochondrial stress signal (1, 2, 37). This is based on studies conducted in mtDNA-depleted cells in which reduced ATP synthesis and Δψm led to elevated levels of steady-state cytosolic Ca2+ (2). These changes resulted in the increased levels of numerous nuclear genes, including ryanodine receptor-1 (RyR-1) and c-Jun NH2-terminal kinase (JNK) (2), as well as calcium/calmodulin-dependent protein kinase (CaMK) and CREB (1). Similarly, abnormal Ca2+ levels have also been documented in MELAS patient cells with reduced Δψm (28). Thus it is likely that the cells used in this study also display augmented intracellular Ca2+ levels, and this may activate signaling molecules and the transcription of nuclear genes. However, this is not likely to be the only signaling mechanism involved, because a differential adaptation was observed in rho− and MELAS cells.

Our findings on the effect of contractile activity on Tom34 and Tom20 (Fig. 5) and other import machinery components (34) suggest that exercise can serve to reverse a defect in the protein import pathway, if it exists. In addition, the effect of contractile activity on VO2, COX activity, and mtDNA levels (Fig. 6; Ref. 38) illustrates the potential of exercise in ameliorating mitochondrial disease brought about by mtDNA depletion. Consistent with this, it was shown recently that exercise can improve the oxidative capacity and reduce the severity of symptoms in patients with mitochondrial disease (32, 33). Thus future studies should continue to examine the potential role of exercise as a therapeutic strategy for the treatment of mitochondrial disorders as well to decipher the specific regulatory mechanisms of the intergenic signaling pathway involved in these adaptations.

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Table 1. Comparison of gene expression and protein import in rho−, MELAS, and MMD cells

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|-----------------|-----------------|-----------------|
| **Import rate** | **rho−** | **MELAS** | **MMD** |
| Tom20           | ↑↑           | ↔             | ↔       |
| Tom34           | ↑↓           | ↔             | ↔       |
| mtHSP70         | ↔            | ↔             | ↔       |
| HSP60           | ↔            | ↔             | ↔       |
| PGC-1           | ↔            | ↔             | ↔       |
| COX IV          | ↔            | ↔             | ↑       |
| Tfam            | ↑↓           | ↔             | ↔       |

Values are expressed relative to appropriate control cells. Multiple mitochondrial disease (MMD) data from Ref. 30. MELAS, mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes; mt, mitochondrial; HSP, heat shock protein; PGC-1, peroxisome proliferator-activated receptor-γ co-activator-1; COX IV, cytochrome-c oxidase subunit IV; Tfam, transcription factor A; NM, not measured.
REFERENCES


