Functional consequences of thyroid hormone-induced changes in the mitochondrial protein import pathway

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Colavecchia, Marco, Loraine N. Christie, Yashpal S. Kanwar, and David A. Hood. Functional consequences of thyroid hormone-induced changes in the mitochondrial protein import pathway. Am J Physiol Endocrinol Metab 284: E29–E35, 2003. First published September 17, 2002; 10.1152/ajpendo.00294.2002.—Thyroid hormone [3,5,3'-triiodo-L-thyronine (T3)] induces phenotypic alterations in cardiac mitochondria, in part by influencing protein import and the expression of the import motor mitochondrial heat shock protein (mtHsp70). Here we examined the adaptability of translocases of the inner membrane (Tim) proteins, as well as the outer membrane receptor Tom34, to T3. Administration of T3 to rats for 5 days increased cardiac Tim23 and Tim44 mRNA levels by 55 and 50%, respectively, but had no effect on Tim17. T3 treatment also induced a 45% increase in Tom34 mRNA, with no accompanying changes at the protein level, suggesting regulation at the posttranscriptional level. In H9c2 cardiac cells, Tim17 mRNA was elevated by 114% by 9 days of differentiation, whereas Tim23 and Tim44 declined by 25 and 29%, respectively. To determine the functional consequences of these T3-induced changes, malate dehydrogenase (MDH) import rates were measured in H9c2 cells stably overexpressing Tim44 and mtHsp70, either alone or in combination. MDH import remained unaltered in cells overexpressing Tim44 or in cells overexpressing both Tim44 and mtHsp70. However, when mtHsp70 was overexpressed alone, a 13% (P < 0.05) increase in MDH import rate was observed. These findings indicate that import machinery components are differentially regulated in response to stimuli that induce mitochondrial biogenesis, like T3 and differentiation. In addition, the induction of an import machinery component in response to T3 may not necessarily result in functional changes in protein import in mitochondrial biogenesis. Finally, mtHsp70 may play a regulatory role in the import process that is independent of its interaction with Tim44.

mitochondrial biogenesis; heart; translocases of the inner membrane; mitochondrial heat shock protein 70; muscle differentiation

MITOCHONDRIAL BIOGENESIS is a process that involves the expansion of the mitochondrial reticulum via the synthesis of lipids, and the insertion of nascent proteins in the lipid bilayer. This complex process has been well characterized in striated muscle, and it involves the coordinated expression of mitochondrial proteins encoded by both the nuclear and mitochondrial genomes (8). It is well established that the induction of mitochondrial biogenesis in striated muscle can be enhanced in response to various physiological stimuli. One stimulus that has been shown to have a profound effect on mitochondrial function is thyroid hormone (3,5,3'-triiodo-L-thyronine, or T3; see Refs. 24 and 27). Studies in vivo have demonstrated that T3 elicits a number of adaptations in the mitochondrial compartment, including changes in mitochondrial composition and enzyme activities (20, 27). Such alterations in mitochondrial phenotype during conditions of T3-induced mitochondrial biogenesis are mediated, in part, via adaptations in the mitochondrial protein import pathway.

The majority of proteins required for mitochondrial structure and function are encoded on nuclear genes and synthesized as precursor proteins on cytosolic polysomes (19). These precursors harbor mitochondrial-targeting sequences, which allow cytosolic chaperones to identify the precursor as a mitochondrial protein and escort it to its proper cellular location (13). Once at the mitochondrion, the precursor interacts with import receptors located in both the outer and inner mitochondrial membranes. These import molecules, collectively referred to as translocases of the outer and inner membrane (Toms and Tims, respectively), help guide the precursor in the mitochondrion, whereupon its targeting signal is proteolytically cleaved to form a mature mitochondrial protein (2).

Previous work in our laboratory has demonstrated that the kinetics of protein import in mitochondria are influenced by T3. The outer membrane receptor Tom20 and the matrix chaperone mitochondrial heat shock protein 70 (mtHsp70) are upregulated in response to T3 (4, 22). In addition to increased expression levels of these import machinery components, T3 also stimulates the import of precursor proteins in the mitochondria.
drial matrix. The increase in Tom20 expression was accompanied by a parallel increase in the rate of import of the mitochondrial enzymes malate dehydrogenase (MDH) and ornithine carboxytransferase (4). Similarly, the increase in mtHsp70 levels within the matrix of the mitochondrion was attributable to an accelerated rate of import of this chaperone in the mitochondrial (22).

Although the important adaptive role of the mitochondrial protein import pathway in response to T₃ is well documented, there is limited information regarding the relative importance of individual import machinery components in the overall import process in mammalian cells. One study has examined the role of the outer membrane component Tom20 for mitochondrial protein import in C₂C₁₂ muscle cells (7). Overexpression of Tom20 resulted in an increase in MDH import rate in the mitochondrial matrix, an observation that paralleled the extent to which Tom20 was overexpressed. In the same study, inhibition of Tom20 expression using antisense oligonucleotides reduced the import rate of MDH. These findings suggest that this receptor plays a vital role in skeletal muscle mitochondrial protein import. However, studies have yet to examine the role of inner membrane machinery components in import and their adaptability during conditions of altered mitochondrial biogenesis. Thus the purpose of this study was to evaluate the mRNA expression of Tim44, Tim23, and Tim17 in response to T₃-induced mitochondrial biogenesis in cardiac tissue. In addition, we wished to determine the functional consequences of changes in the expression of selected import machinery components by overexpressing these in H9c2 cardiac cells and evaluating their impact on protein import rates. Finally, we determined the effect of altered thyroid status on cardiac levels of Tom34, a newly identified component of the mammalian import system that is imperative for protein import (3).

**MATERIALS AND METHODS**

**Materials.** The Tom34 antibody and the cDNA encoding human Tom34 were generously provided by Dr. M. Mori (Kumamoto University School of Medicine, Kumamoto, Japan). Geneticin, Lipofectamine, and Lipofectamine Plus reagents were purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada). T₃ and an Anti-Flag M2 monoclonal antibody for Tim44 detection (TIM-Flag) were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies for thyroid receptor-α₁ (TR-α₁) and mtHsp70 were purchased from Santa Cruz (Santa Cruz, CA) and Stressgen Biotechnologies (Victoria, BC, Canada), respectively.

**Animal care and treatments.** Male Sprague-Dawley rats received daily injections of either T₃ (0.4 mg/kg body wt) dissolved in vehicle [100% propylene glycol-0.9% NaCl (1:5:1, vol:vol)], or vehicle solution alone, for a period of 5 days. On the 6th day, animals were anesthetized with a intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt), and the hearts were removed and quick-frozen in liquid nitrogen. Heart weights were recorded and compared with body weight to assess the extent of cardiac hypertrophy. The tissues were then pulverized and stored in liquid nitrogen for subsequent mRNA and protein analyses.

**Culturing and treatment of H9c2 cardiac cells.** H9c2 cardiac myocytes were cultured in 100-mm dishes in 10 ml DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (PS). At a confluence of 70%, the cells were switched to low serum conditions (DMEM containing 5% horse serum and 1% PS) and were differentiated in the presence of either T₃ (100 nM) or vehicle for periods of 3, 6, and 9 days for mRNA determination and 3 and 9 days for TR-α₁ measurements (see below).

**Stable overexpression of selected import components in H9c2 cells.** H9c2 myocytes were cultured as described above. At 75–80% confluence, the cells were transfected with either a Tim44 [pcDNA 3.1/TIM-Flag (26)] or mtHsp70 (pcDNA 3.1/mtHsp70) expression construct or both. The DNA (3 μg) was diluted in 0.75 ml of serum- and antibiotic-free DMEM and precomplexed with Lipofectamine Plus reagent for 15 min at room temperature. A separate mixture containing Lipofectamine diluted in 0.75 ml of serum- and antibiotic-free DMEM was then prepared and added to the DNA-containing mixture (30 μg/Lipofectamine/ml, final concentration). The combined volumes were incubated for an additional 15 min at room temperature. During this time, the plates were washed three times with Dulbecco’s PBS (DPBS), and 5 ml of DMEM (no serum or antibiotics) were added to each plate. At the appropriate time, the DNA-Lipofectamine-Lipofectamine Plus solution was added to each plate, and the plates were incubated at 37°C for 3 h. The transfection medium was then discarded and replaced with 10 ml growth medium (DMEM supplemented with 10% FBS, no PS).

One day after transfection, the cells from each plate were trypsinized and dispersed in two separate dishes. Each group of two plates was labeled for identification purposes. An additional day later, geneticin was added to the growth medium at a concentration of 400 μg/ml. To generate stable cell lines expressing the protein(s) of interest, the cells were propagated for a period of 2 wk, with the growth medium and resistance antibiotic being replaced every 2 days. At the end of the selection period, one plate from each group was trypsinized, resuspended in freezing medium (90% FBS-10% DMSO), and stored in liquid nitrogen. Protein extracts were prepared from the remaining plates for purposes of verifying protein overexpression.

**RNA isolation and analysis.** Total RNA was isolated from cells and frozen tissue powders using TRIzol Reagent (Invitrogen), according to the manufacturer’s instructions. Total RNA (10–30 μg) was electrophoresed on formaldehyde gels and transferred to nylon membranes for Northern blot analyses. In some cases, total RNA (20–40 μg) was also applied directly to nylon membranes using a slot-blot apparatus. All membranes were exposed to ultraviolet light to fix the RNA to the membranes. Hybridization was carried out overnight at 42°C with 32P-labeled cDNA probes encoding Tim44, Tim23, Tim17, or Tom34, as done previously (5, 9, 25). To correct for any loading differences between samples, the membranes were hybridized with radiolabeled 18S rRNA probes. All signals were quantified using electronic autoradiography.

**Immunoblotting.** Cellular protein was isolated from H9c2 cells and frozen tissue powders, as done previously (5, 25). Protein extracts (10–50 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subsequently incubated overnight with the appropriate primary antibody (1:1,000 for Tom34, mtHsp70, and TR-α₁; 1:400 for TIM-Flag) followed by a 1-h incubation with an anti-mouse (TIM-Flag and mtHsp70) or anti-rabbit (Tom34 and TR-α₁) peroxidase-conjugated secondary antibody (1:1,000). Signals were detected using the enhanced chemiluminescence system (Amersham Biosciences, Baie
T3 induces cardiac hypertrophy. Table 1 summarizes basic anatomic modifications brought about by the in vivo administration of T3. The hearts of animals injected with T3 weighed 43% more than those from vehicle-treated animals (P < 0.01). To determine whether this difference was because of changes in body weight or the result of T3 treatment, cardiac hypertrophy was assessed by examining the heart-to-body weight ratios of the animals. This value was 58% higher (P < 0.01) in T3-treated animals, reflecting the effectiveness of the treatment.

Effect of T3 on cardiac Tim23, Tim44, and Tim17 mRNA levels. To assess the effect of T3 on the mRNA expression of Tim machinery components in cardiac muscle, Northern blots were performed (Fig. 1). T3 treatment resulted in a 55% increase in Tim23 mRNA levels compared with vehicle-treated animals (P < 0.01). A similar increase (50%) in Tim44 mRNA levels was observed. The Northern blot analyses using Sigma Gel software. The band intensities were quantified using Instant Imager or Sigma Gel software. The bands representing the precursor, intermediate, and mature forms of each mRNA were identified by image analysis using Sigma Gel software. The relative amounts of mature mRNA were expressed as a percentage of the total mRNA content. The data were analyzed using a two-way ANOVA, followed by Tukey’s post hoc test to determine the statistical significance of differences among the groups.

Fig. 1. Effect of 3,5,3'-triiodo-L-thyronine (T3) on translocase of the inner membrane (Tim) 23, 44, and 17 mRNA levels in cardiac muscle. Total RNA was isolated from heart tissues of vehicle (V)- and T3-treated animals (n = 3 for each condition), and Northern blot analyses (30 μg RNA/lane) were conducted to determine the mRNA levels of Tim machinery components. Examination of the ethidium bromide-stained gel indicated equal loading between samples. Five animals/condition were used for quantification.
occurred over the 5-day period ($P < 0.02$). However, there was no effect of T₃ on Tim17 mRNA levels in cardiac muscle.

**Effect of T₃ on cardiac Tom34 mRNA and protein levels.** We also examined the effect of T₃ on the mRNA expression of the outer membrane receptor Tom34 in cardiac muscle (Fig. 2A). Slot-blot analysis revealed that Tom34 mRNA was elevated by 45% in the hearts of T₃- compared with vehicle-treated animals ($P < 0.01$). Surprisingly, this increase in Tom34 transcript levels was not accompanied by any concomitant changes in the protein expression of this import component (Fig. 2B), whether examined in whole cell extracts (top) or in isolated mitochondria (bottom).

**Effect of T₃ and differentiation on Tim23, Tim44, and Tim17 levels in H9c2 cardiac cells.** To determine whether the changes observed in the mRNA expression of the Tim components were replicable in an in vitro environment, we measured the mRNA levels of Tim23, Tim44, and Tim17 in vehicle- and T₃-treated H9c2 cardiac cells. In contrast to the findings seen in vivo, T₃ had no detectable effect on the mRNA expression of the Tim components at any of the time points examined (Fig. 3A). In addition, no effect of T₃ was observed on COX activity (data not shown), suggesting that T₃ does not induce mitochondrial biogenesis in H9c2 cells. To determine whether this lack of response could be because of low levels of expression of the T₃ receptors (TR) in H9c2 cells, we assessed the levels of TR-α₁ by Western blotting. TR-α₁ levels were detectable, and there was no difference between 3- and 9-day differentiated cardiac cells. In addition, T₃ treatment had no effect on the expression of TR-α₁ (Fig. 3A). This lack of responsiveness of H9c2 cells to T₃, in contrast to our observations in the intact adult rat, is likely developmentally related, since H9c2 cells are derived from embryonic heart cells. As discussed previously, the response of the heart to thyroid hormone is known to be developmentally dependent (25).

In normal H9c2 cells, the mRNA levels of Tim44 and Tim23 were reduced by 29% ($P < 0.01$) and 25% ($P <
Functional consequences of T₃-induced changes for protein import. To determine the functional consequences of increased in vivo mRNA expression of protein import machinery components, we overexpressed selected components in H9c2 cells and measured MDH import rates in intact cells. H9c2 cells transfected with a Tim44 mammalian expression construct had levels of Tim44 that were 2.7-fold higher than pcDNA-transfected control cells (Fig. 4A). Despite this high extent of overexpression, immunoprecipitation studies revealed no difference in MDH import rates between control- and Tim44-transfected samples (Fig. 4A; n = 2).

The function played by Tim44 in mitochondrial protein import involves cooperation with the matrix chaperone mtHsp70 (1,14). Because this protein is also inducible during conditions of T₃-induced mitochondrial biogenesis (22), we wanted to evaluate whether the level of mtHsp70 could limit the rate of protein import. Transfection of H9c2 cells with an mtHsp70 expression construct increased mtHsp70 levels by 3.3-fold compared with pcDNA-transfected cells (Fig. 4B). Unlike Tim44, mtHsp70 overexpression resulted in a small but significant increase (13%) in MDH import rates, as determined by immunoprecipitation analysis (P < 0.02; n = 3; Fig. 4B).

We next coexpressed both Tim44 and mtHsp70 in H9c2 cells, resulting in overexpression of both Tim44 and mtHsp70 (Fig. 4C). In contrast to what we observed with mtHsp70, overexpression of both Tim44 and mtHsp70 did not result in a significant increase in MDH import rates (n = 3; Fig. 4C).

DISCUSSION

It is well established that T₃ has profound effects on mitochondrial morphology and phenotype in cardiac muscle. These adaptations may result from T₃-mediated changes at the transcriptional or posttranscriptional level (18, 21) or via an acceleration of posttranslational import in mitochondria (4, 22). This latter change may, in turn, be because of T₃-induced changes in the expression of components of the protein import machinery. This machinery consists of a large number of components, only a few of which have been characterized in mammalian cells. One goal of our ongoing work is to characterize the biochemical and functional roles of these import components, their inducibility by physiological stimuli, and their roles in the etiology of
mitochondrial disorders. Protein import is an essential step in the overall process of mitochondrial biogenesis, and defects in mitochondrial protein import have been implicated in neurodegenerative disease and muscle myopathies (10, 11, 12). We have previously shown that T3 induces Tom20 (4) and mtHsp70 (22), coincident with increased import rates in mitochondria. The purpose of this study was to investigate the adaptability of other import machinery components in response to T3 and to determine whether some of these changes were of functional significance for protein import during conditions of mitochondrial biogenesis.

Our results show, for the first time, that T3 treatment can mediate changes in the mRNA expression of various components of the Tim machinery in vivo. Because T3 stimulates the import of precursors destined for the mitochondrial matrix (4, 22), and interactions of precursor proteins with Tim proteins are required for import in this compartment, these adaptations could play a key role in mediating changes in the mitochondrial phenotype during T3-induced organelle biogenesis. However, the responses we observed were not regulated coordinately. Cardiac mRNA levels of Tim44 and Tim23 were upregulated in response to T3, similar to the increase previously observed in mtHsp70 mRNA (22). In contrast, Tim17 mRNA levels remained unaltered. These changes, or lack thereof, may be because of the presence or absence of functional thyroid response elements (TREs) in the promoters of the genes encoding these import proteins. Currently, the promoter regions of the genes encoding the Tim proteins have yet to be characterized. However, transcriptional mechanisms may account for the T3-induced increase in mtHsp70 mRNA, since the murine mtHsp70 gene has been shown to contain TRE-related sites within its upstream regulatory region (15). The increases in Tim23 and Tim44 mRNA levels could also be in part because of T3-induced increases in mRNA stability, as observed with the cardiac α- and β-myosin heavy chain subunit mRNAs in response to T3 (18, 23). These possibilities remain to be investigated.

We also examined the effect of T3 treatment on the in vivo expression of the outer membrane receptor Tom34. This import component is unique in that yeast or fungal homologues have not yet been identified (17). The important role played by this molecule in mitochondrial protein import is underscored by the finding that its overexpression in COS-7 cells stimulates precursor import in vitro, whereas inhibition of its expression attenuates this process (3). Our preliminary results in skeletal muscle myocytes confirm this finding (A. M. Joseph and D. A. Hood). Interestingly, the intracellular localization of Tom34 is not exclusively restricted to the mitochondrion. In fact, the majority of the protein is found in the cytosolic fraction of rat tissues, suggesting that it may also be involved in protein targeting to the mitochondrion (3). Our results show that Tom34 mRNA levels were increased by 45% in response to T3 treatment. However, the observed changes in Tom34 mRNA expression were not accompanied by any concomitant increases in the protein level of this molecule, either in cardiac mitochondria or in whole tissue extracts. This indicates that posttranscriptional mechanisms are likely involved in regulating the expression of Tom34 in cardiac muscle. Specifically, detectable increases in the steady-state level of this protein may require a larger mRNA induction, or a latency period of longer duration may be necessary for T3-induced increases in Tom34 transcript concentration to be observed at the protein level.

The kinetics of protein import in mitochondria may differ depending on the conditions in which mitochondrial biogenesis is induced. For instance, protein levels of the outer membrane receptor Tom20 are increased in response to T3 (4) but remain unaltered during myocyte differentiation (7), another condition in which mitochondrial content is increased (7, 16). However, increases in mature levels of MDH within the mitochondrion are seen in both conditions, and, in the case of T3, this is because of an accelerated rate of import of MDH in the matrix (4). If increases in precursor import contribute to the differentiation-induced increase in mature MDH content, this would suggest that Tom20 may not play as integral a role in mediating changes in mitochondrial content and composition during myocyte differentiation. In our study, we wanted to determine whether differentiation of H9c2 cells could induce a pattern of expression of the Tim proteins that is distinct from that seen with T3 treatment. We found that differentiation results in a decline in Tim44 and Tim23 mRNA levels (Fig. 3, A and B). At the same time, an increase in the Tim17 mRNA level was observed. These results suggest that Tim17 may play a more important role in protein import than Tim44 or Tim23 during differentiation-induced biogenesis, whereas the opposite may be true during conditions of elevated thyroid hormone levels.

To determine the function of single components of the protein import machinery, selective expression of each, along with functional protein import measurements, is required. To date, only one study in myocytes has attempted to determine whether changes in the expression of import components during conditions of altered mitochondrial biogenesis could have functional consequences for protein import (7). When Tom20 was overexpressed in C2C12 skeletal muscle cells, parallel increases in protein import rates were observed. Conversely, inhibition of Tom20 expression using antisense oligonucleotides led to a similar decline in import rates. In this study, we wished to evaluate the role of Tim44 in import by stably overexpressing this protein and assessing the effects on MDH import via immunoprecipitation from intact cells. Because the function of Tim44 is integrally linked to mtHsp70 (1, 14), we also overexpressed this protein either alone or in combination with Tim44. When Tim44 was overexpressed on its own, no increase in MDH import was observed compared with control cells. On the other hand, mtHsp70 overexpression resulted in a modest increase in MDH import. However, this increase did not parallel the extent of overexpression. Surprisingly, no changes in MDH import rate were seen when Tim44 and
mtHsp70 were overexpressed together. These results suggest that at least a fraction of the role that mtHsp70 plays in pulling the precursor into the mitochondrial matrix can be performed independently of its interaction with Tim44. A recent study in yeast, using a temperature-sensitive Tim44 mutant that is impaired in its interaction with mtHsp70, found that precursor proteins with tightly folded domains were imported in the mitochondrial matrix with reduced efficiency (1). A subsequent study determined that those proteins that were imported in mitochondria with this mutation remained uncharacteristically bound to mtHsp70 for extended periods, resulting in a delay in their folding into the mature form within the matrix (6). Thus the interaction of mtHsp70 with Tim44 is likely very important but may not account for all mtHsp70 function during the import process.

In conclusion, proteins of the mitochondrial import apparatus are differentially regulated in response to conditions of altered mitochondrial biogenesis, such as myocyte differentiation and T3 treatment. More importantly, the inducibility of an import machinery component to a stimulus evoking mitochondrial biogenesis does not necessarily reflect the magnitude of its role in mediating precursor protein import during this time. Future studies must focus on measuring the adaptability of other protein import components, specifically those of the mitochondrial inner membrane, and evaluating their functional importance in the import process during conditions of mitochondrial biogenesis in mammalian cells. Such work will have clinical significance for the treatment of mitochondrial diseases that arise because of inherent defects in the mitochondrial protein import system (10, 11, 12).

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