# Effect of thyroid hormone on mtHsp70 expression, mitochondrial import and processing in cardiac muscle

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## Abstract

Mitochondrial heat shock protein 70 (mtHsp70), an important mitochondrial chaperone, is increased in cardiac muscle mitochondria of hyperthyroid rats. To determine the mechanism(s) underlying this increase, we used variations in thyroid status. In Series I, rats were made hyperthyroid by injecting them with 3,3′,5-triiodo-L-thyronine ($T_3$) for 5 days, or by treating them with vehicle. In Series II, animals were given 6-n-propyl-2-thiouracil in their drinking water (0·05% w/v) for a period of 32–42 days to make them hypothyroid. During the last 5 days of treatment these animals received injections of either $T_3$ or vehicle. $T_3$ treatment resulted in parallel increases in mtHsp70 protein and mRNA levels in a variety of tissues, suggesting transcriptional regulation. However, evidence of tissue-specific post-transcriptional regulation was also apparent. In isolated heart mitochondria, $T_3$ treatment resulted in a 1·8-fold increase in mtHsp70. This was due to the 1·6-fold greater import of mtHsp70 into mitochondria in $T_3$, compared with hypothyroid animals, and it could not be attributed to an altered rate of intramitochondrial mtHsp70 degradation. The rate of processing of mtHsp70 to its mature form, reflecting mitochondrial processing peptidase activity, was unaffected by $T_3$, but was more rapid than mtHsp70 import. These data indicate a novel mechanism by which $T_3$ modifies the mitochondrial phenotype via the adaptations in the protein import pathway.

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## Introduction

The mitochondrial protein import process is responsible for the recognition of newly synthesized precursor proteins, and the subsequent uptake of those precursors into the organelle. The pathway has been well studied in yeast and fungi (Schatz 1996, Jensen & Kinnally 1997, Pfanner *et al.* 1997), and is becoming increasingly well defined in higher animals (Hachiya *et al.* 1993, Armstrong *et al.* 1997, Iwahashi *et al.* 1997). Movement of the precursor protein from the cytosol to the matrix of the mitochondria begins with its recognition by cytosolic chaperones (Komiya *et al.* 1996). These guide the precursor to the mitochondrion, whereupon they interact with translocases of the outer membrane (Toms). From the outer membrane, the precursor protein moves to the inner membrane, where the presequence interacts with translocases of the inner membrane (Tim proteins). One of these (Tim44) is a peripheral membrane protein bound to the matrix side of the inner mitochondrial membrane. Tim44 serves to anchor the 70 kDa mitochondrial heat shock protein (mtHsp70), which pulls the presequence and the remainder of the protein into the matrix in an energy-dependent manner (Schneider *et al.* 1994). Subsequently, the presequence is cleaved by a mitochondrial processing peptidase (MPP) yielding a lower molecular weight, mature protein.

mtHsp70 is essential for cell viability in yeast, and it has been suggested that it may play a regulatory role in the import of matrix-destined precursor proteins (Dekker *et al.* 1997). Evidence that mtHsp70 is critically important in the protein import process is also suggested by its marked inducibility under conditions of chronic contractile activity in skeletal muscle (Ornatsky *et al.* 1995, Takahashi *et al.* 1998), and as a result of thyroid hormone treatment in cardiac muscle mitochondria (Craig *et al.* 1998), conditions in which mitochondrial biogenesis is enhanced. The increase in mtHsp70 concentration in mitochondria under those conditions could be due either to an increased import, or a decreased degradation of the protein within the matrix. While the role of mtHsp70 in lower eukaryotic cells is becoming well defined, its expression, import into mitochondria, processing and degradation in mammalian cells has never been evaluated. Furthermore, analysis of all of these important steps, which in general determine mitochondrial phenotypic adaptations, has not been performed in a tissue undergoing mitochondrial biogenesis. Thus, by using thyroid hormone ($T_3$; 3,3′,5-triiodo-L-thyronine) as an inducer of mitochondrial biogenesis, we examined the pathway of gene expression of the


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mitochondrial chaperone mtHsp70, and we have identified the protein import step as one which is adaptable under conditions of $T_3$-induced organelle synthesis.

## Materials and Methods

In this study we sought to examine the expression pathway of a single protein (mtHsp70) starting from its mRNA expression to its final destination within the organelle, in an attempt to identify some potential sites of regulation during mitochondrial biogenesis. In order to visualize the effect of $T_3$ most dramatically, two series of experiments were performed. In the first Series, normal animals were administered $T_3$ at a dose sufficient to make them hyperthyroid, as done previously (Paradies *et al.* 1994, Craig *et al.* 1998). In the second Series, animals were first made hypothyroid with 6-n-propyl-2-thiouracil (PTU) treatment for >30 days, after which they were injected with $T_3$ as in Series I.

### *Animal care and treatments*

Male Sprague-Dawley rats ($n$=66, 200–350 g) were housed individually with a ratio of 12 h light:12 h darkness cycle. Food and water were given *ad libitum*. All procedures involving animals were approved by the York University Animal Care Committee, in accordance with the Canadian Council on Animal Care.

### *Series I: $T_3$ treatment*

$T_3$ was dissolved in vehicle (100% propylene glycol:0·9% NaCl (1·5:1, v:v)) to a final concentration of 0·4 mg/ml. Animals were injected with $T_3$ (i.p. 0·4 mg/kg) once per day for 5 days. Control animals were administered vehicle in the same manner and at the same time as $T_3$-injected animals. Twenty-four hours following the fifth injection, they were anesthetized with sodium pentobarbitol (60 mg/kg, i.p) and selected hindlimb muscles (soleus, red gastrocnemius (RG), white gastrocnemius (WG)) and liver were removed, quick frozen and stored in liquid $N_2$ for the subsequent assessment of tissue mtHsp70 mRNA and protein levels. Animals were killed by the removal of the heart for mitochondrial isolation.

### *Series II: PTU administration followed by $T_3$ treatment*

6-n-propyl-2-thiouracil (PTU) was dissolved in the drinking water to a concentration of 0·05% (w:v). Rats were administered PTU for a period of 32–42 days. During the last 5 days of treatment, animals received injections of either $T_3$ or vehicle as described above. Twenty-four hours later they were anesthetized and the tissues were removed as described above.

### *Cardiac mitochondrial isolation*

The heart was removed from the chest cavity and placed on ice. Approximately 50 mg of the left ventricle was quick frozen and stored in liquid $N_2$ for the assessment of mtHsp70 mRNA and protein levels. The remaining heart tissue was used in the mitochondrial isolation procedure, as described previously (Craig *et al.* 1998). Mitochondrial protein was assessed (Bradford 1976) and the concentration was adjusted to 1 mg/ml.

### In vitro *transcription, translation and import of mtHsp70*

The full-length cDNA clone for human mtHsp70 (Bhattacharyya *et al.* 1995) was a gift from Dr Richard Morimoto (Northwestern University, Evanston, IL, USA). The cDNA was linearized using Bam H1 and subsequently phenol extracted and ethanol precipitated. MtHsp70 was *in vitro* transcribed with T7 RNA polymerase, followed by *in vitro* translation in the presence of $^{35}$S-methionine and rabbit reticulocyte lysate, as described previously (Takahashi & Hood 1996, Craig & Hood 1997). Mitochondria were pre-incubated for 5 min at 30 °C prior to the import assay. Translation mix (10 μl per 25 μg of mitochondrial protein) was added and the import incubation was allowed to proceed at 30 °C for 5, 10, 15 or 20 min. Import was halted by adding an aliquot of the mitochondria-translation mix to an ice-cold sucrose cushion (600 mM sucrose, 100 mM KCl, 20 mM HEPES, 2 mM $MgCl_2$). Mitochondria were pelleted for 15 min at 18 000 ***g*** (4 °C) and resuspended in 25 μl of ice-cold breaking buffer (600 mM sorbitol, 20 mM HEPES, pH 7·4), denatured with β-mercaptoethanol at 95 °C for 5 min, quick cooled on ice and then electophoresed through an 8% SDS-polyacrylamide gel. Gels were processed and dried as described previously (Takahashi & Hood 1996, Craig & Hood 1997). Images and subsequent quantification were obtained with electronic autoradiography (Instantimager, Packard, Meriden, CT, USA). Imported, mature mtHsp70 was distinguished from precursor mtHsp70 because of its lower molecular weight. The percentage of available protein that was imported was calculated based on the intensity of a lane containing 4 μl of translation mixture in the absence of mitochondria.

### *Degradation of mtHsp70*

Mitochondria were pre-incubated for 5 min at 30 °C. Translation mix (10 μl per 25 μg of mitochondrial protein) was added, and the import incubation was allowed to proceed at 30 °C for 20 min. Import was halted by placing the mitochondria-translation mixture on ice, followed by the addition of valinomycin to a concentration of 0·5 μM. Preliminary experiments verified that this concentration of valinomycin was effective in inhibiting any further import of external precursor protein. After 5 min on ice, samples were placed at 30 °C. Aliquots corresponding to 25 μg of mitochondrial protein were removed after 0, 0·5, 1, 1·5, and 2 h and placed on ice-cold sucrose cushions and samples were processed as described in the import assay.

*MPP activity*

Mitochondria previously stored at − 20 °C were thawed at 4 °C. Protease inhibitors were added to final concentrations of 2 μg/ml leupeptin, 2 μg/ml pepstatin A and 0·1 mM phenylmethylsuphonyl fluoride. Extracts were prepared by subjecting the mitochondria to 3 freeze/thaw cycles, alternating between liquid nitrogen and 30 °C. $MnCl_2$ was added to a final concentration of 0·5 mM. To measure MPP activity, the extracts were preincubated at 30 °C. Translation mix containing *in vitro* translated mtHsp70 (4 μl) was added to 25 μg of mitochondrial extract. Aliquots were removed at 0, 1, 2, 3, 5, and 10 min and placed onto ice-cold lysis buffer containing 5% β-mercaptoethanol. Samples were denatured and run on an 8% polyacrylamide gel as described above. The intensity of both the precursor and mature bands was quantified using electronic autoradiography, and MPP activity was expressed as the ratio of the intensity of mature band to the sum of the mature plus precursor band.

*RNA measurements*

Total RNA was isolated from tissue powders and quantified as described previously (Connor *et al.* 1996). Total RNA (10 μg per slot) was transferred onto a nitrocellulose membrane using a slot blot apparatus, as done previously (Hood 1990, Hood *et al.* 1992). RNA was fixed onto the membrane using UV light. The membrane was then hybridized with a random primer $^{32}$P-labeled 300 bp fragment of the mtHsp70 coding region obtained from the mtHsp70 cDNA cut with EcoRI. The intensity of the autoradiographic signal was used as the measure of mtHsp70 mRNA level. Variations in loading were corrected using the subsequent hybridization of a radiolabeled 18S rRNA probe (Takahashi *et al.* 1998).

*Immunoblotting*

Total tissue proteins were extracted from frozen powders as described previously (Takahashi *et al.* 1998). Mitochondrial proteins or total tissue proteins were then electrophoresed through a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). This membrane was subsequently probed with a monoclonal antibody (diluted 1:1000) directed against mtHsp70 (Stress-Gen Biotechnologies, Victoria, Canada). The secondary antibody used for detection of the final staining reaction was a goat anti-mouse IgG conjugated to alkaline phosphatase. Quantification of the colour intensity was obtained using laser densitometry (Ornatsky *et al.* 1995).

*Statistics*

Independent *t*-tests were employed for the comparison of mRNA and protein levels of mtHsp70 with $T_3$ treatment (Series I). Two-way analyses of variance with repeated measures on one factor (time) were used for import, degradation, and MPP activity data. A Tukey *post hoc* comparison was employed in the analysis of the import ANOVA. Data within the text and on all graphs are expressed as means ± S.E.M.

## Results

*Cardiac hypertrophy*

In Series I, the heart to body weight ratio was 3·42 ± 0·05 mg/g for $T_3$-treated rats and 2·46 ± 0·04 mg/g for vehicle-treated animals. Thus, $T_3$ treatment resulted in a 39% increase in heart weight ($n$=16). Similarly, in Series II, $T_3$ treatment following PTU administration resulted in a 42% increase in heart weight ($n$=9). The animals administered $T_3$ had a heart to body weight ratio of 3·21 ± 0·09 mg/g while the corresponding ratio for vehicle-treated animals was 2·27 ± 0·03 mg/g. Thus, the PTU treatment resulted in an 8% cardiac atrophy relative to vehicle control animals.

*Expression of mtHsp70*

Initially, we characterized the protein and mRNA expression of mtHsp70 in five different tissues which differ widely in mitochondrial content, as done previously (Hood 1990, Ornatsky *et al.* 1995). For Series I vehicle animals, slot blot analyses indicated that the highest level of mtHsp70 mRNA was found in heart, followed by RG, liver, soleus, and WG (Fig. 1A). The WG possessed mRNA values which were 3·5-fold lower than in heart. $T_3$ induced an increase in mtHsp70 mRNA by 49% in liver, 22% in heart, 40% in soleus and 85% in WG ($P$<0·05) in Series I animals. Immunoblot analyses of these same tissues indicated comparable levels of mtHsp70 protein in heart and liver. These were approximately 4-fold higher than those in the soleus and the RG, and 20-fold greater than in the WG. The induction of mtHsp70 protein levels by $T_3$ was particularly marked in liver and soleus, in which 61% and 82% increases were observed (Fig. 1B). In Series II, PTU treatment (reflecting the hypothyroid state) resulted in decreased ($P$<0·05) mRNA levels in heart, but not other tissues, when compared with vehicle treatment alone. $T_3$ induced a greater increase in mtHsp70 mRNA than in Series I, by 69% in liver, 47% in heart, 71% in soleus, 52% in RG and 169% in WG ($P$<0·05). At the protein level, PTU treatment resulted in a more widespread decrease in mtHsp70 level compared with vehicle-treated, euthyroid animals. $T_3$ also resulted in elevations ($P$<0·05) of mtHsp70 protein in liver (2·3-fold), soleus (14·2-fold) and RG (3·2-fold). These increases were greater than those observed in Series I.

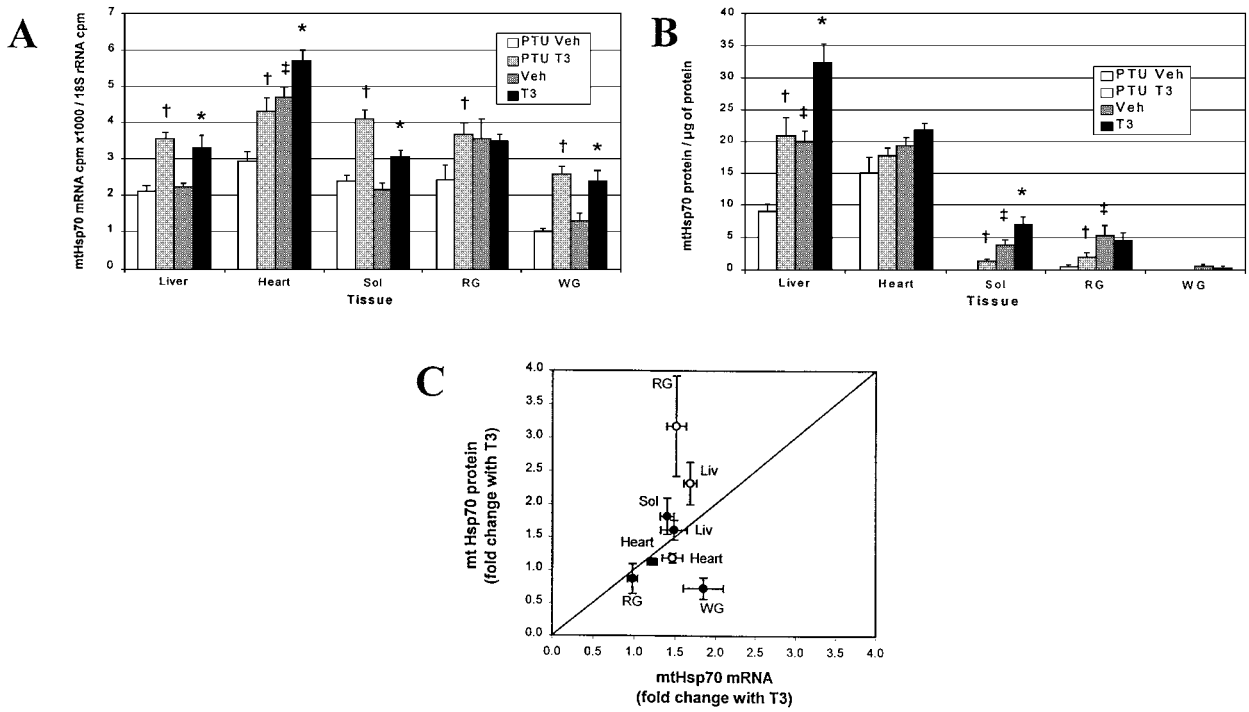


**Figure 1** Expression of mtHsp70 mRNA and protein in five tissues. (A) Slot blot analyses (12 µg of total RNA per slot) were used to quantify the relative levels of mtHsp70 mRNA between tissues and with thyroid hormone treatment. 18S rRNA was used to correct for differences in loading ($n$=4–8 animals/tissue). (B) Immunoblots were used to quantify the relative levels of mtHsp70 protein and the effect of $T_3$ ($n$=6 animals/tissue), (*$P$<0·05 between vehicle and $T_3$, †$P$<0·05 between PTU vehicle and PTU $T_3$, ‡$P$<0·05 between vehicle and PTU vehicle). (C) Comparison of the fold change in mRNA and protein in response to $T_3$ treatment. Values are re-expressed from the data shown in Figs 1A and B. The solid line indicates the line of identity, where the fold increase in mtHsp70 mRNA is equal to the fold increase in mtHsp70 protein. ● Series I animals, ○ Series II animals; Liv, liver; Sol, soleus muscle; RG, red gastrocnemius muscle; WG, white gastrocnemius muscle. In Series II, WG mtHsp70 protein levels were not detectable, while mRNA levels were induced 2·7-fold. Soleus values were 1·7-fold (mRNA) and 14·2-fold (protein). These data for soleus and WG are not illustrated. All data points are expressed as means ± S.E.M.

In general, a close parallel (Fig. 1C) existed between the $T_3$-induced increases in protein and mRNA levels of Series I tissues. The sole exception was WG. In this tissue, and in Series II tissues (soleus and RG), the change in mRNA concentration was not accompanied by an equivalent change at the protein level, suggesting some post-transcriptional regulation of mtHsp70 expression under these conditions.

The increase in mtHsp70 protein evident in heart tissue was localized to the mitochondria. $T_3$ induced a 1·8-fold ($n$=16, $P$<0·05) increase in mtHsp70 in isolated cardiac mitochondria (Fig. 2).

### *Import of mtHsp70 into isolated cardiac mitochondria*

To evaluate the effect of $T_3$ on mtHsp70 import, *in vitro* translated mtHsp70 was incubated with mitochondria isolated from hearts obtained from animals in Series I and II (Fig. 3). A typical import time course autoradiogram is shown in Fig. 3A. The human mtHsp70 protein used in this study was rapidly imported and processed by rat heart mitochondria. This was expected based on evidence that the activity of the protein import machinery is not species-specific (Craig *et al.* 1998, Takahashi *et al.* 1998). Quantification of import reactions derived from several experiments (Fig. 3B) indicates that the import of mtHsp70 proceeded linearly over time. Import was significantly lower in animals administered PTU+vehicle ($P$<0·05). Compared with this PTU-vehicle group, import was 60% higher in PTU+$T_3$-treated animals, 60% higher in vehicle-treated animals, and 90% higher in $T_3$-treated animals when all incubation time points were considered. In addition, the rate of increase in mtHsp70

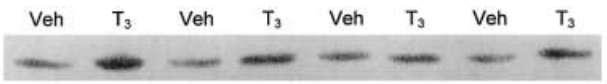


**Figure 2** A typical immunoblot illustrating mtHsp70 levels in isolated cardiac mitochondria from four vehicle- (Veh) and four thyroid hormone- ($T_3$) treated rats (25 µg of mitochondrial protein applied per lane).

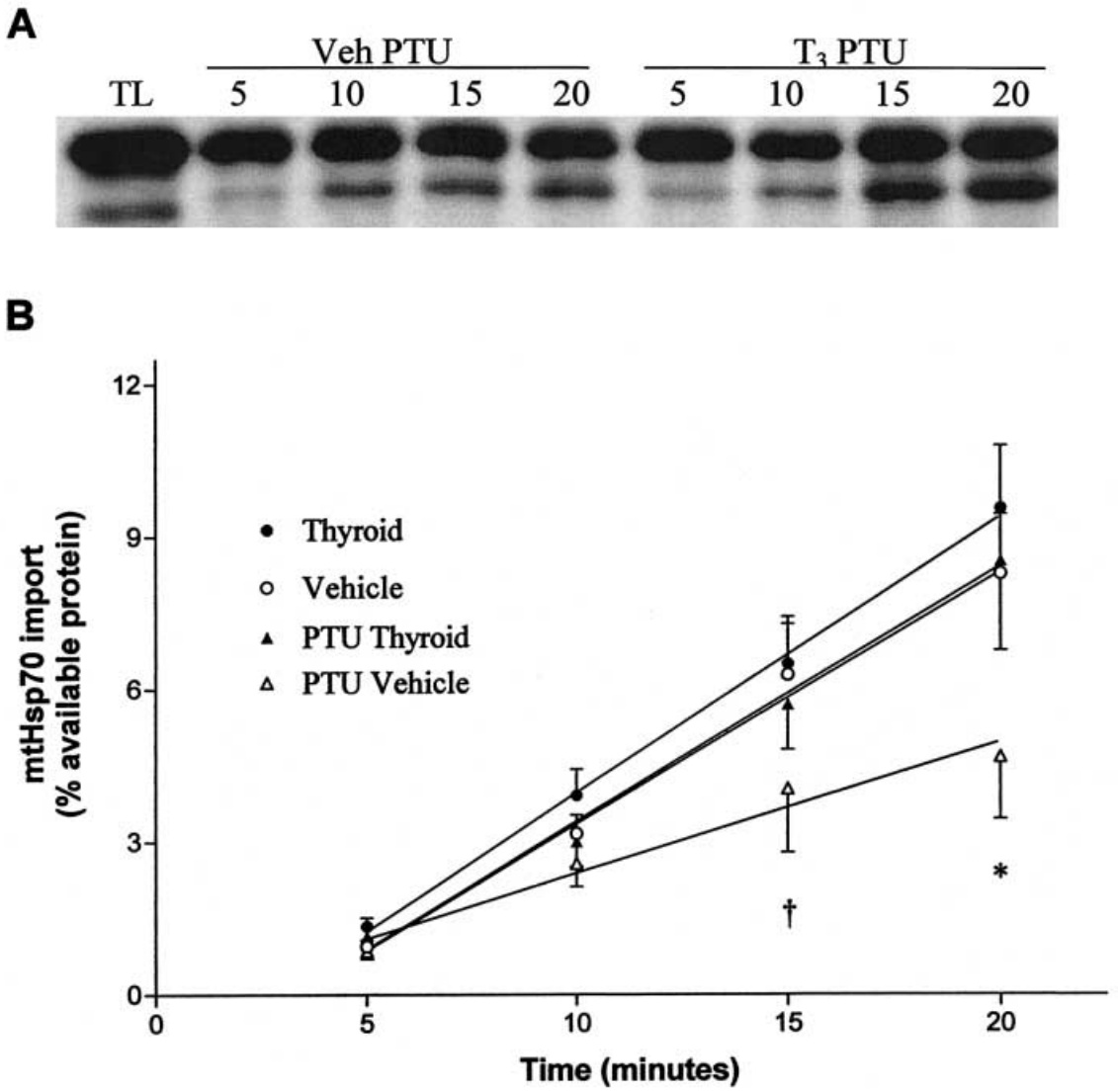


**Figure 3** Import of mtHsp70 into isolated cardiac mitochondria. (A) Autoradiograph of a representative reaction comparing import of mtHsp70 in mitochondria obtained from vehicle-treated PTU rats (Veh PTU) and from thyroid-treated PTU rats ($T_3$ PTU) at 5, 10, 15, and 20 min of import (TL, translation lane, where 4 µl of translation product was loaded). The lower band represents the imported mtHsp70. (B) Quantification of the autoradiograph from multiple experiments ($n=15$ animals for vehicle and thyroid values, $n=9$ animals for vehicle PTU and thyroid PTU values). A two-way ANOVA revealed a significant effect of treatment between the vehicle PTU and thyroid PTU group ($P<0{\cdot}05$). *Post hoc* comparisons demonstrated that the vehicle PTU group was different from all groups after 20 min (*) and different from thyroid and vehicle groups after 15 min (†). All data points are expressed as means ± S.E.M.

import was significantly lower in PTU-vehicle animals than in any of the other groups ($P<0{\cdot}05$). Compared with the PTU-vehicle group, the rate of import was 126% higher in PTU+$T_3$ animals, 130% higher in vehicle-treated animals, and 150% higher in $T_3$-treated animals. Thus, the administration of $T_3$ to PTU-treated animals normalized the rate of import to that found in non-PTU vehicle animals.

*MPP activity*

To assess the potential effect of $T_3$ on the processing of mtHsp70, the activity of the mitochondrial processing peptidase was measured. In establishing the assay conditions, preliminary experiments indicated that MPP activity was sensitive to the presence of metal cations (e.g. $Mn^{2+}$), and that it could be inhibited by pre-incubation

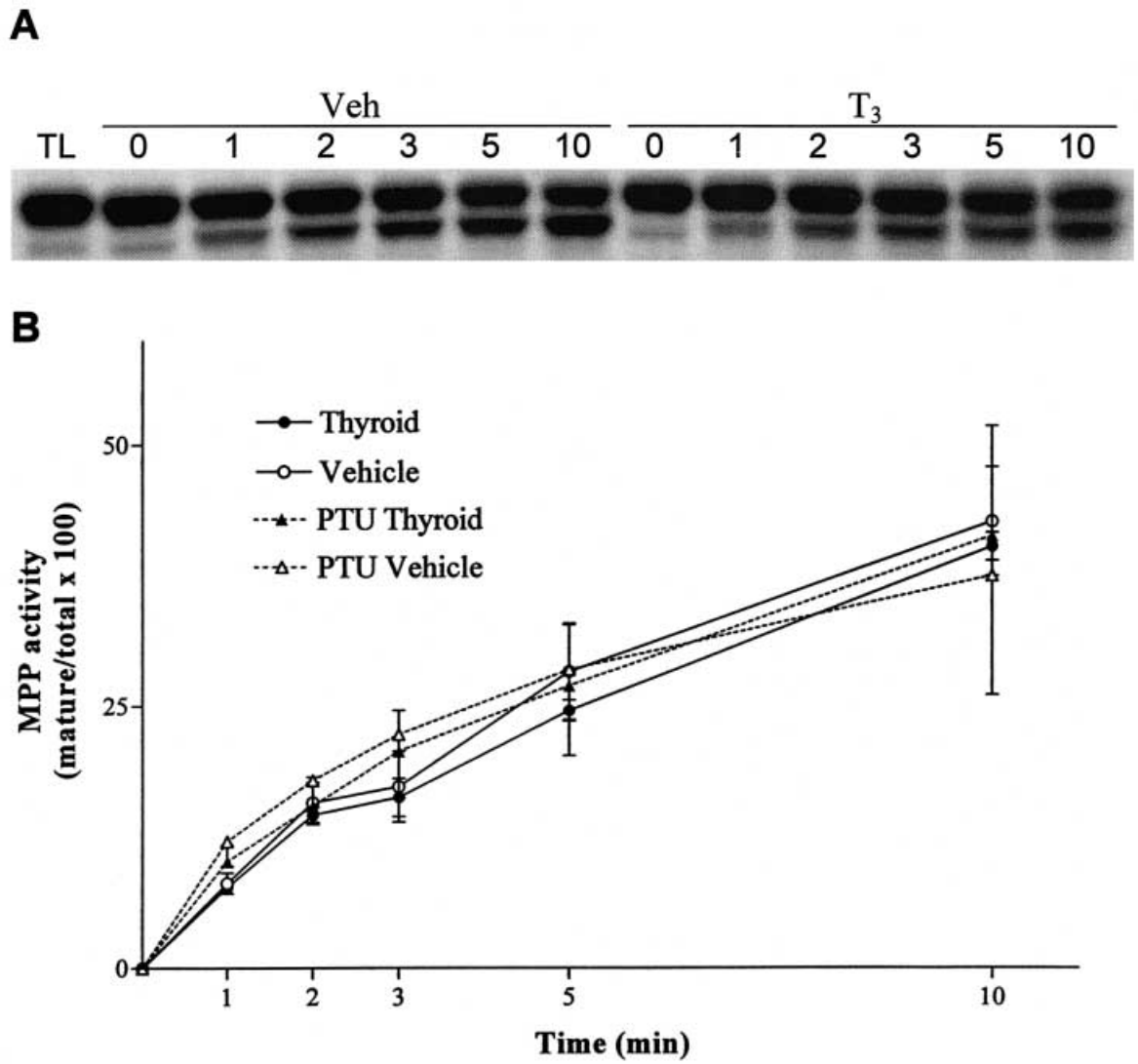


**Figure 4** *In vitro* assay of MPP activity. (A) Autoradiograph of typical MPP assay. Mitochondrial extracts were prepared from isolated cardiac mitochondria (Veh, vehicle treated rats; $T_3$, thyroid hormone treated rats; TL, translation lane) and incubated with *in vitro* radiolabeled mtHsp70 for the times (min) indicated. (B) Quantification of autoradiographs from multiple experiments. Vehicle and $T_3$ values are shown, as are vehicle PTU and $T_3$ PTU values. All data points are expressed as means ± S.E.M. ($n=4$ animals/treatment). There were no significant differences between groups.

with o-phenanthroline, the metal ion chelator, as previously reported (Peralta *et al.* 1993). No effect of $T_3$ was evident on MPP activity (Fig. 4). MPP activity was much more rapid than import, since detectable processing of mtHsp70 to its mature form was evident as early as 1 min post-incubation. The amount of the signal represented by the mature form of mtHsp70 was approximately 10% by 1 min in MPP assays (average for all groups). In contrast, this level of conversion to mature mtHsp70 was only achieved after 20 min during protein import measurements.

*Degradation*

Since the $T_3$-induced increase in the levels of mtHsp70 in cardiac mitochondria could possibly be attributed to a reduction in mtHsp70 degradation, we assessed the intra-mitochondrial proteolysis of mtHsp70 in vehicle and $T_3$-treated animals. $T_3$ treatment had no effect on the degradation rate of mtHsp70 as measured over a 2-h period (Fig. 5), indicating the relative stability of the protein following import into mitochondria.


**Figure 5** Degradation of mtHsp70 in isolated cardiac mitochondria. Autoradiograph of a typical degradation assay comparing the level of mtHsp70 in mitochondria following import in vehicle (Veh) versus thyroid hormone ($T_3$) treated animals. Levels were measured at t=0 (termination of import), 0·5, 1, 1·5, and 2 h. PRE indicates mitochondria which were pre-incubated with valinomycin prior to import and degradation. Replication of multiple identical experiments ($n$=9 animals/treatment) did not reveal any effect of $T_3$ on the degradation of mtHsp70 (results not shown).

## Discussion

In response to whole body $T_3$ treatment, the heart hypertrophies. This is a result of marked alterations in the expression of contractile protein genes, accompanied by changes in the sarcoplasmic reticulum and mitochondria (Glass & Holloway 1990). It is well established that $T_3$ is a physiologically relevant inducer of mitochondrial biogenesis in cardiac muscle, eliciting changes in mitochondrial content and composition (Nishiki *et al.* 1978, Soboll 1993) which appear to parallel cell growth. Many of these effects are exerted at the level of transcription of nuclear-encoded mitochondrial genes (Nelson *et al.* 1995), but evidence for post-transcriptional regulation also exists (Hood *et al.* 1992). The results indicate that mtHsp70 protein concentration was lowest after PTU treatment alone, and highest in normal animals receiving $T_3$. This effect of $T_3$ on mtHsp70 was localized to mitochondria, and was due largely to an acceleration of precursor protein import kinetics. This acceleration of mtHsp70 import could not be associated with changes in processing, since MPP activity was much more rapid than import, and was not inducible by $T_3$.

We chose mtHsp70 for this study because it is a critical protein involved in the import (Kang *et al.* 1990), unfolding (Matouschek *et al.* 1997), refolding (Stuart *et al.* 1994), and degradation (Wagner *et al.* 1994) of precursor proteins. Although mtHsp70 has a role in both import and proteolysis, the $T_3$-induced increase in mtHsp70 within mitochondria was accompanied only by changes in import. This lack of effect of $T_3$ on intramitochondrial proteolysis was also found using a mutant form of cytochrome b2, which is misfolded and missorted to the matrix (Craig *et al.* 1998). These results were surprising in view of the role of mtHsp70 in facilitating the action of the matrix protease PIM1 (Wagner *et al.* 1994). Thus, it may be that mtHsp70 is found at sufficiently high levels within the mitochondria under normal conditions to support its role in the degradation of intramitochondrial proteins. Alternatively, mtHsp70 may only recognize proteins other than itself when directing proteins to the proteolytic pathway. In any event, the increase in mtHsp70 concentration within cardiac mitochondria as a result of $T_3$ treatment appears to be due to an increase in import, in the absence of changes in degradation.

It is interesting to speculate that the effect of a $T_3$-induced enhancement of mtHsp70 import might serve to accelerate the import of other matrix-destined proteins, as well as itself, in a positive-feedback fashion. It has been suggested that mtHsp70 is an important regulatory protein within the import machinery (Dekker *et al.* 1997). However, evidence for mtHsp70's involvement as a rate-limiting step in the import process has yet to be determined, and will probably involve the selective over- or underexpression of mtHsp70 using gene-targeting techniques, along with coincident measures of protein import. We have recently shown that $T_3$ increases the level of the outer membrane import receptor Tom20 in cardiac muscle mitochondria (Craig *et al.* 1998), as well as in C2C12 muscle cells (JY Grey, MK Connor, JW Gordon & DA Hood, unpublished observations). Using coincident measures of protein import in intact cells, we found that changes in Tom20 levels, mediated either by $T_3$, forced overexpression via a Tom20 expression construct, or reduced expression using antisense oligonucleotides, led to parallel alterations in the import of proteins into the matrix space. This provides strong evidence that the mechanism of action of $T_3$ on the import process is, in part, exerted via changes in the expression of components of the outer membrane import receptor complex. It is also of interest to note that changes in protein import mediated by $T_3$, as observed using isolated mitochondria in the present study, are also reflected in studies using intact cells (JY Grey, MK Connor, JW Gordon & DA Hood, unpublished observations). However, it should be noted that in cells, import of radiolabeled precursor protein is expected to be a function of: (1) the concentration of the precursor, which may exist in the cytosol at or below the $K_m$ value for transport, and (2) the capacity of the protein import machinery for transport, determined by the concentration of Tom and Tim proteins in the membrane. Thus, a discordance between observations made *in vitro* and those using intact cells is possible, since import into isolated mitochondria using a precursor protein synthesized in a reticulocyte lysate system is designed to be independent of precursor protein concentration.

Finally, in light of evidence showing that mtHsp70 is inducible with $T_3$ treatment, it was of interest to study the regulatory events governing this increased expression. Using tissues possessing a wide range of mitochondrial contents (Hood 1990) and hence varying amounts of mtHsp70, it is evident from the data in Series I that the increase in mtHsp70 protein was largely accompanied by increases in mtHsp70 mRNA (Fig. 1C). These data provide an indirect indication that the predominant form of regulation is transcriptional in nature. Sequences with a close similarity (83%) to the concensus thyroid response element half-site consisting of AGGTCA are found

upstream of the murine mtHsp70 gene (Michikawa *et al.* 1993). Assuming that the currently unknown promoter sequence in the rat is similar, this lends support to the idea that the effect of $T_3$ on mtHsp70 expression is mediated, at least in part, via increases in transcription. However, an exception to this may be found in the WG, a tissue with a very low mitochondrial concentration as well as a low expression of $T_3$ receptors, typical of fast-twitch muscle types (Schuler & Pette 1998). In this case, the increase in mtHsp70 mRNA was not accompanied by an increase at the protein level, suggesting either a $T_3$-induced acceleration of mtHsp70 degradation, or a reduced translation of the transcript. Interestingly, a very different pattern of change in mtHsp70 mRNA and protein expression was observed in animals previously treated with PTU to reduce endogenous $T_3$ levels, followed by $T_3$ administration (Series II). In this situation, the effect of $T_3$ appeared to more dramatically alter the balance between transcriptional and post-transcriptional regulation in a tissue-specific fashion. This is illustrated most effectively by the comparison of soleus and RG muscles between Series I and Series II (Fig. 1C). Further studies incorporating the direct measurement of mtHsp70 transcription using nuclear run-on assays, combined with measures of protein turnover rates under the influence of $T_3$ will be necessary to identify the mechanism involved and improve our understanding of the regulation of mtHsp70 expression in these tissues.

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