THYROID HORMONE STIMULATES PROTEIN SYNTHESIS IN THE CARDIOMYOCYTE BY ACTIVATING THE Akt-mTOR and p70S6K PATHWAYS

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Running Title: T3-Induced Cardiomyocyte Hypertrophy via PI3K-Akt-p70S6K

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Thyroid hormones affect cardiac growth and phenotype; however, the mechanisms by which the hormones induce cardiomyocyte hypertrophy remain uncharacterized. Tri-iodo-L-thyronine (T3) treatment of cultured cardiomyocytes for 24 hrs resulted in a 41±5% (p<0.001) increase in [3H]-leucine incorporation into total cellular protein. This response was abrogated by the phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin. Co-immunoprecipitation studies showed a direct interaction of cytosol-localized thyroid hormone receptor, TRα1, and the p85α subunit of PI3K. T3 treatment rapidly increased PI3K activity by 52±3% (p<0.005), which resulted in increased phosphorylation of downstream kinases, Akt and mammalian target of rapamycin (mTOR). This effect was abrogated by pretreatment with wortmannin or LY294002 (LY). Phosphorylation of p70S6K, a known target of mTOR, occurred rapidly following T3 treatment and was inhibited by rapamycin and wortmannin. In contrast, phosphorylation of the p85 variant of S6K in response to T3 was not blocked by LY, wortmannin or rapamycin, thus supporting a T3-activated pathway independent of PI3K and mTOR. 40S ribosomal protein S6, a target of p70S6K, and 4E-BP1, a target of mTOR, were both phosphorylated within 15—25 min. of T3 treatment and could be inhibited by wortmannin and rapamycin. Thus, rapid T3-mediated activation of PI3K by cytosolic TRα1, and subsequent activation of the Akt-mTOR-S6K signaling pathway may underlie one of the mechanisms by which thyroid hormone regulates physiological cardiac growth.

The observation that thyroid hormone treatment of patients and experimental animal models of heart failure can improve cardiac function has been attributed in part to its regulation of cardiac genes (1-5). It has also been well documented that thyroid hormones stimulate physiologic cardiac hypertrophy (6); however, the intracellular mechanisms underlying this response remain poorly defined. Patients with chronic hyperthyroidism experience a marked reduction in systemic vascular resistance with increased cardiac contractility and cardiac output, often associated with ventricular hypertrophy (7,8). However, the role of thyroid hormones on gene expression fails to satisfactorily explain the effects observed on cardiomyocyte growth. Recent published studies supporting the cytosolic localization and non-transcriptional activities of thyroid hormone receptors may underlie thyroid hormone-induced physiological growth (9-11). Evidence of protein-protein interactions between cytosolic thyroid hormone receptors and the p85α regulatory subunit of phosphatidylinositol 3-kinase (PI3K), and activation of PI3K activity by T3 treatment have been reported (12,13). Activation of the PI3Kα-Akt-mTOR pathway by IGF-1 and transgenic animal models expressing molecular components of this pathway have implicated the PI3K-Akt signaling pathway in determining heart size and physiologic cardiac growth (14-18). Recently, Kuzman et al (19) reported that the Akt-mTOR signaling pathway was activated in the hypertrophied hearts of hyperthyroid animals. Furthermore, activation of PI3K and Akt appears to be a common feature of the cardioprotective mechanisms of numerous peptide hormones and growth factors including IGF-1, insulin, adrenomedullin and estrogen (20-23). It remains to be ascertained whether activation of this pathway by thyroid hormones...
explains some of its cardioprotective effects and unresolved non-nuclear mechanisms of action (24-27).

The objective of the current study was to determine whether T3-induced physiologic cardiac growth is mediated via the PI3K-Akt-mTOR signaling pathway by the activation of PI3K through a direct interaction with TRα1. Thus, cytosolic localization of TRα1 in cardiomyocytes may serve a role in regulating protein synthesis, maintaining growth and cell survival.

**MATERIALS AND METHODS**

**Isolation, culture and viral transduction of neonatal rat ventricular myocytes**

Animals were treated in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHHS Publication No.85-23), and study protocols were approved by the Institutional Animal Care and Use Committee. Ventricular myocytes were isolated from hearts of 2-day old rats by collagenase digestion as we have previously described (9). Myocytes were plated at approximately 1.5 x 10⁴/cm² on collagen-coated 6-well plates or 60 mm dishes and cultured for the first 20 hours in DMEM/F12 medium containing 10% FBS, L-glutamine, cytosine β-D-arabinofuranoside (Ara-C, 10 uM) and antibiotics. After the first 20 hours, the NRVM cultures were washed with HBSS and transduced with adenovirus expressing TRα1 (2 moi) (Ad-TRα1) as we have previously published (9). Select cultures were co-transduced with adenovirus expressing a mutant p85α subunit of PI3K that lacks the p110-binding site (Ad-Δp85PI3K, kindly provided by Dr. M. Kasuga, Kobe University, Japan). In most experiments, cultures were transduced with Ad-TRα1 unless indicated otherwise. Cells were washed and maintained in serum-free medium (containing transferrin (5 mg/L), selenium (5 ug/L) for 48 hrs prior to experimentation as indicated in Results. Reagents used in cell culture were the highest quality available from Calbiochem (San Diego, CA) or ICI Biomedicals Inc. (Aurora, OH). Final concentrations were: T3 (10⁻⁸ M), LY294002, (5x10⁻⁵ M), wortmannin (2x10⁻⁶ M), rapamycin (2.5x10⁻⁸ M), IGF-1 (10⁻⁸ M).

**Measurement of protein synthesis**

After 24 hrs in serum-free medium, the cardiomyocytes were treated with T3 (10⁻⁸ M) for 24 hrs or left untreated. In some cultures, wortmannin was added either together with T3 for 24 hrs or for the final 6 hrs of the experiment. Six hrs before harvest, L-[3,4,5³H]leucine (5 uCi/ml; 117 Ci/mmol) was added to the culture medium to measure incorporation into newly synthesized protein. Total cellular proteins were precipitated in ice cold 10% trichloroacetic acid and collected by centrifugation (14,000xg, 10 min., 4ºC). The protein pellets were washed twice by resuspension in cold 10% TCA and collected by centrifugation. The final pellets were dissolved in 0.2N NaOH by incubation at 60ºC for 30 min. Protein concentrations were determined by the bicinchoninic acid protein assay (Micro BCA assay; Pierce Biotechnology, Rockford, IL) and the radioactivity measured by liquid scintillation counting.

**Measurements of protein and DNA**

Cardiomyocytes were quantitatively scraped from culture dishes into 0.2N perchloric acid and collected by centrifugation (10,000xg for 10 min.). The resulting precipitate was dissolved in 0.3N KOH by incubation at 60ºC for 20 min. Aliquots were used for analysis of total protein by the BCA method, and for DNA determination by fluorescence spectrophotometry using 33258 Hoechst dye and calf thymus DNA as standard (DNA assay kit, Sigma).

**RNA analysis**

Total cellular RNA was prepared from ~10⁶ cardiomyocytes using a commercially available kit (RNeasy Mini Kit; Qiagen, Valencia, CA). Northern blotting methods were used for mRNA analysis of cardiac α- and β-myosin heavy chains (MyHC) and sarcoplasmic reticulum calcium-activated ATPase (SERCA2) as previously published (10).
Cell fractionation and immunoblot analysis

Cells were homogenized in buffer containing 20 mM Hepes pH 7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol, 0.2% NP-40 plus protease and phosphatase inhibitors, and fractionated by centrifugation at 12,000xg for 1 min at 4º C. The resulting supernatant was used as the cytosolic fraction. Protein concentrations were determined by Micro BCA assay, and equal amounts of protein were analyzed by Western blotting as previously published (9,10). Antibodies used were: anti-TRα1 (PA1-P211A; Affinity BioReagent, Golden, CO), anti-P13Kp85 (Upstate Cell Signaling Solutions, Lake Placid, NY), and anti-Akt, p-Akt(Ser473), p-Akt(Thr308), mTOR, p-mTOR(Ser2448), p70S6K, p-p70S6K(Thr389), 4E-BP1, p-4E-BP1(Ser65), S6 ribosomal protein, p-S6(Ser235/236) from Cell Signaling Technology (Beverly, MA). Secondary antibodies used were either goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase. Protein bands were detected using chemiluminescence reagent and visualized by exposure to x-ray film. Protein band intensity was quantified by laser scanning densitometry (GS-800 Calibrated Densitometer; BioRad; Hercules, CA).

Measurement of PI3 kinase activity

Cardiomyocytes were treated with T3 (10^-8 M) or IGF-1 (10^-8 M) for 10 min. or left untreated. Cells were lysed in ice cold buffer containing 137 mM NaCl, 20 mM Tris.HCl, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, 1 % NP-40, protease and phosphatase inhibitors, and the soluble fraction obtained by centrifugation at 10,000xg was used for immunoprecipitation. Cytosolic protein (500 ug) in 1 ml final volume was incubated overnight with 8 ug anti-P13Kp85 antibody at 4ºC. The immune complex was precipitated using TrueBlot anti-rabbit Ig IP beads (eBioscience), and then incubated with either anti-P13K p85 or anti-TRα1 antibody for 1 hr, 4ºC. The antibody complex was precipitated with anti-rabbit Ig IP beads and collected by centrifugation (2500xg, 5 min.). Precipitates were extensively washed prior to resolution by electrophoresis. Non-specific immune precipitation was determined using anti-rabbit IgG. Immunoblot analysis with TrueBlot HRP anti-rabbit IgG (used according to manufacturer’s instructions, eBioscience) was required to eliminate interference with the detection of TRα1 protein.

Statistical analysis

All data are presented as mean ± SE derived from a minimum of two separate cell preparations. One-way ANOVA was used for statistical analysis of mean values between experimental groups, and Student-Newman-Keuls was used for pairwise multiple comparisons. Differences between means were considered significant at p <0.05. Data were analyzed using SigmaStat 3.1 (Systat Software, Inc., Richmond, CA).

RESULTS

T3-stimulated protein synthesis is mediated by PI3K activation

We determined that T3 treatment of cultured cardiomyocytes for 24 hrs resulted in significant cell growth as measured by the 15-20% increase (p<0.02) in protein to DNA ratios (Fig.1A). Similar effects were seen in cardiomyocytes that
were transduced with Ad-TRα1 and cultures that were uninfected (UI). We evaluated whether this growth effect of T3 treatment resulted from an increase in rate of protein synthesis by measuring the incorporation of [3H]leucine into total cell protein over a 6h time period. Using Ad-TRα1 transduced cultures, we showed that T3 significantly increased protein synthesis by 41 ± 5%, and that this increase could be prevented by inhibition of PI3K activity by simultaneously treatment of the cells with wortmannin (W) for either 6 or 24 hrs (Fig.1C). These data support a role for the PI3K pathway in T3 stimulation of protein synthesis and cardiomyocyte growth. Furthermore, Northern blot analysis ascertained that T3 treatment of the Ad-TRα1-transduced cardiomyocytes resulted in a physiological hypertrophic phenotype with stimulation of α-myosin heavy chain (αMyHC) and SERCA2 mRNA expression and repression of βMyHC (Fig.1B). These T3-induced phenotypic changes were similar to those observed in non-transduced cardiomyocytes (data not shown), and in cardiomyocytes transduced with control Ad-neβgal (nuclear localized β-galactosidase) as we have previously published (10).

TRα1 complexes with p85α subunit of PI3K and T3 stimulates PI3K activity

Since our previous studies had shown that the TRα1 isoform localized to both nuclear and cytoplasmic compartments of the cardiomyocyte, we investigated whether the receptor interacted directly with the p85α subunit of PI3K as had been previously reported in human skin fibroblasts (12). To address this possibility, cardiomyocytes were transduced with adeno virus expressing TRα1 and cultured for 48 hrs prior to harvest. Cytosolic fractions were subjected to immunoprecipitation with either anti-TRα1 or anti-p85α antibodies and probed for the presence of both proteins in the immune complex by Western blot analysis. As shown in Fig.2A, immune complexes containing the two proteins were observed both in the presence and absence of T3, suggesting that complex formation was ligand independent. Furthermore, we were able to show immune complex formation of endogenous TRα1 and p85α proteins when immunoprecipitated with the p85 antibody (UI) (Fig.2A). However, the low affinity of commercially available anti-TRα1 antibodies for effective use in immunoprecipitation of the low amounts of endogenous TRα1, precluded successful immunoprecipitation of the TRα1-p85 complex using anti-TRα1 antibodies.

To determine whether T3 stimulated PI3K enzymatic activity, cardiomyocytes transduced with Ad-TRα1 were treated with T3 (10^-8 M) or vehicle for 10 min. prior to harvest. Cytosolic fractions were incubated overnight with anti-PI3K-p85α antibodies, and then the precipitated immune complexes were used for enzymatic analysis. Results in Fig.2B show that T3 rapidly increased PI3K activity by 52 ± 3% (p<0.005) compared with untreated myocytes. Shown for comparison is the stimulatory effect of IGF-1 on PI3K activity showing ~2-fold increase. As stated above, we were unsuccessful in using the anti-TRα1 antibodies to immunoprecipitate sufficient TRα1-PI3K complex for enzymatic analysis.

T3 rapidly induces phosphorylation of Akt and p70S6K that is sustained for 24 hrs

We examined whether the observed T3-stimulated PI3K activity could result in activation of the Akt/S6K signaling pathway as has been shown in IGF-1-mediated physiological cardiac hypertrophy and cardioprotection (15,16). As shown in Fig. 3, Ser473 phosphorylation of Akt was detected at 15 min after addition of T3, and phosphorylation was maintained up to 24 hrs, whereas total Akt protein levels were unaltered. Similarly, rapid and sustained phosphorylation of p70S6K occurred after T3 treatment, thus supporting a potential role of these signaling proteins in mediating a non-genomic cytosolic T3 response. Downstream targets of activated Akt and p70S6K pathways include several proteins directly involved in protein translation, such as 4E-BP1 and 40S ribosomal protein S6. Rapid and persistent phosphorylation of these two proteins were observed following treatment with T3 (Fig.3), providing evidence that rapid non-genomic responses to T3, potentially acting through cytosol-localized TRα1, may stimulate
protein synthesis resulting in cardiomyocyte growth.

**T3 activates mTOR through phospho-Akt**

We further studied the rapid responses to T3 using cardiomyocytes that were transduced with Ad-TRα1. As with cardiomyocytes not subject to Ad-TRα1 transduction (Fig.3), phosphorylation of Akt at Ser473 and Thr308 occurred rapidly within 7 min of T3 treatment (Fig. 4A). Cell fractionation studies showed that only very low amounts of phosphorylated Akt were present in the nucleus in unstimulated conditions. However, as phosphorylation of cytosolic Akt occurred in response to T3, increasing amounts pAkt appeared in the nucleus while pAkt content in the cytoplasm decreased, suggesting that T3 induced nuclear translocation of pAkt. Inhibition of PI3K activity by LY294002 (LY) attenuated the T3-mediated phosphorylation and nuclear translocation of Akt (Fig. 4A). Wortmannin similarly blocked the T3 response. Also shown is the well-known robust effect of IGF-1 on Akt phosphorylation (Fig.4A).

Akt activation has been implicated in numerous cellular functions including activation of protein translation through its role in phosphorylation and activation of the mammalian target of rapamycin (mTOR) (reviewed in 28). Fig.4B shows that mTOR phosphorylation (Ser2448) in response to T3 treatment occurred more slowly than Akt phosphorylation as would be expected if mTOR were downstream of Akt in the signaling pathway. Furthermore, mTOR phosphorylation was sustained and significantly higher (p<0.01) after 60 min of T3 treatment. Quantitation of the Western blot analyses illustrates the sequential phosphorylation and activation of these proteins within the signaling pathway (Fig. 4D). As with Akt, mTOR phosphorylation in response to T3 was abrogated by wortmannin, thus supporting a role of PI3K in this pathway (Fig. 4B).

**T3-activated mTOR phosphorylates 4E-BP1 and p70**

In response to growth factor stimulation, mTOR has been shown to increase protein translation by phosphorylating 4E-BP1 and thus prevent its association with the translation initiation factor eIF4E, and by activating p70S6K protein kinase (reviewed in 29). As shown in Fig. 4C, 4E-BP1 phosphorylation was evident within 25 min. of T3 treatment, clearly after the phosphorylation and activation of mTOR, supporting its location downstream of mTOR. Furthermore, this effect of T3 was completely inhibited by pre-treatment with wortmannin.

T3-mediated phosphorylation of the p70 and p85 variants of S6 kinase (S6K) are shown in Fig.5A&B. Phosphorylation of both S6K isoforms occurred rapidly and peaked at approximately 25-40 min. of T3 treatment. Pre-treatment of the cardiomyocytes with inhibitors of PI3K (LY294002, LY; wortmannin, W) or inhibitors of mTOR (rapamycin, R) prevented T3-induced phosphorylation of the p70 but not the p85 S6K variant, supporting distinct T3-mediated pathways activating these two S6K proteins (Fig.5A,B). Thus, the T3-mediated phosphorylation of p85S6K appears independent of the PI3K-mTOR signaling pathway. In contrast, phosphorylation of both p70 and p85 S6K variants by IGF-1 was completely prevented by pre-treatment with wortmannin (Fig. 5A).

**T3-activated p70S6K results in phosphorylation of 40S ribosomal protein S6**

p70S6K is recognized as the kinase that phosphorylates 40S ribosomal subunit protein, S6, that enables polyribosomal association with mRNAs containing 5' terminal oligopyrimidine (TOP) tracks (reviewed in 28). As shown in Fig.5C, phosphorylation of S6 protein was first observed at 15 min and sustained to one hour of T3 treatment. This effect was blocked by pre-treatment with wortmannin or rapamycin, suggesting that T3-mediated activation of p70S6K by the PI3K/mTOR signaling pathway was potentially responsible for ribosomal S6 protein phosphorylation and stimulation of protein translation.

**T3 effects are blocked by overexpression of a mutant p85α PI3K**

To further corroborate the results showing T3-mediated activation of the PI3K-Akt pathway, we overexpressed a mutant form of the p85α subunit of PI3K (Ad-Δp85PI3K). As shown in Fig. 5D, overexpression of the mutant...
by adenoviral mediated transfer into cardiomyocytes at two different multiplicities of infection (10 and 100 moi), prevented T3-induced phosphorylation of Akt(S473) and p70S6K. The effects of T3 on the phosphorylation of the p85α variant appeared unaffected by the mutant p85PI3K, further supporting distinct mechanisms of activation of these two proteins.

DISCUSSION

The present studies have provided insight into the molecular mechanisms by which thyroid hormones induce physiologic cardiac hypertrophy, and potentially function to maintain normal cell growth. Although previous studies have documented increased rates of protein synthesis and decreased protein degradation rates in cardiac tissue in response to thyroid hormone, the mechanisms underlying these responses have not been elucidated (6). Heretofore, biological effects of thyroid hormone have been largely attributed to nuclear transcriptional mechanisms of action that promote a normal cardiac phenotype, but fail to fully explain its growth promoting effects (30,31). However, recent reports have documented cytosolic and membrane-initiated effects of thyroid hormones that do not involve transcriptional activity (reviewed in 32,33). Kinugawa et al. (11) published that the stress-activated p38MAPK pathway was involved in overexpressed TRα1-induced pathologic, but not physiologic, cardiomyocyte hypertrophy. Kuzman et al. (19) showed increased phosphorylation of Akt, p70S6K and mTOR in hypertrophied hearts of thyroxine treated animals. These latter observations are consistent with recent reports supporting a role for the PI3K-Akt-mTOR pathway in regulating mammalian cell size, and in promoting physiologic cardiac hypertrophy in response to insulin and IGF-1 (34). Thyroid hormones have been reported to stimulate PI3K activity in skin fibroblasts and endothelial cells by non-nuclear mechanisms (12,13). These observations together with our recently published data showing cytoplasmic localization of TRα1 (9,10), provided the rationale to propose that thyroid hormone-induced physiologic cardiac growth is mediated by activating the PI3K-Akt signaling pathway through cytosol-localized TRα1.

In support of this hypothesis the present study shows co-immunoprecipitation of TRα1 and the p85α subunit of PI3K. Using anti-p85 antibodies, sufficient endogenous TRα1 protein could be co-immunoprecipitated. However, no commercially available anti-TRα1 antibodies proved satisfactory in the immunoprecipitation of endogenous TRα1, so that we transduced the cardiomyocytes with replication-deficient adenovirus expressing TRα1 at very low multiplicity of infection (2 moi). As the results show, this approach provided substantive data to support a direct protein-protein interaction between TRα1 and p85α. This protein interaction appeared to be independent of ligand binding; however, treatment with T3 rapidly activated PI3K activity to approximately 140% of the activity in untreated cardiomyocytes. Concurrent with this effect, was the ability of inhibitors of PI3K (wortmannin) to inhibit the T3-induced increase in protein synthesis lending support for a role of PI3K in this response. A downstream target of PI3K and dependent on its enzymatic product, PIP3, is the phosphoinositide-dependent kinase, PDK1, and its target, Akt (29,35). We were able to show that T3 rapidly phosphorylated Akt, and that pAkt was translocated to the nucleus. Both phosphorylation and nuclear translocation of pAkt in response to T3 could be blocked by inhibition of PI3K. Recent studies have shown that nuclear-targeted Akt has potent anti-apoptotic effects with significant protective effects on reducing myocardial infarct size (18). We showed that phosphorylation and activation of Akt was sustained even after 24 hrs of T3 treatment, further supporting its role in T3-mediated maintenance of cell growth and potentially cell survival.

Mammalian target of rapamycin (mTOR) has been shown to be important in maintaining cell size by regulating ribosomal biogenesis and protein translation (reviewed in 29, 36). Regulation of mTOR signaling is linked to PI3K/Akt by the tuberous sclerosis complex. As might be expected, activation of Akt by T3
resulted in the phosphorylation of mTOR in a
time frame consistent with its location
downstream of Akt in the signaling pathway.
This effect was inhibited by wortmannin, further
supporting the observed stimulatory effect of T3
on PI3K activity. Two downstream targets of
mTOR, 4E-BP1 and S6K1, are important
regulators of protein translation, and their
regulation by phosphorylation enables protein
synthesis to proceed commensurate with growth
factor and nutrient availability (28). Thus,
phosphorylation of 4E-BP1 can be used as a
measure of signaling through the mTOR
pathway. In response to T3, phosphorylation of
4E-BP1 was observed at a later time point than
mTOR phosphorylation (25 min vs. 15 min).
4E-BP1 plays a key regulatory role in the
initiation of protein translation by binding to and
inhibiting eIF4E initiation complex formation
when it is hypophosphorylated as would occur in
cells deprived of growth factors (reviewed in
28,29,36). Phosphorylation of 4E-BP1 relieves
its inhibitory effect, thus enabling translation
initiation to occur as would potentially be the
case in T3 treated cardiomyocytes.

The S6 kinases are also important regulators
of protein translation by virtue of their ability to
phosphorylate a 40S ribosomal subunit protein,
S6, which enables the translation of mRNAs
containing a 5’ terminal oligopyrimidine (TOP)
track (37). In the present studies, T3 stimulated
phosphorylation of p70^{S6K}, followed by
sustained phosphorylation of S6 protein. These
effects were blocked by both wortmannin and
rapamycin, suggesting that T3-mediated
phosphorylation and activation of PI3K and
mTOR were necessary for this response. These
data support a direct role of T3 in stimulating
translation initiation, and perhaps a more
important function of thyroid hormone in
maintaining a basal level of protein synthesis
under physiological euthyroid conditions.

The p85 variant of S6K1 was also
phosphorylated by T3 treatment, but unlike
p70^{S6K}, this response was not inhibited by PI3K
inhibitors (LY or wortmannin) or by inhibition
of mTOR by rapamycin. Furthermore,
overexpression of a PI3K mutant (Δp85PI3K)
blocked T3-induced phosphorylation of p70^{S6K}
but was without effect on the p85^{S6K} variant.
Therefore, these data suggest that p85^{S6K} may
not be involved in the phosphorylation of
ribosomal S6 protein, and that the T3-induced
phosphorylation of this S6K variant occurs via a
mechanism that is distinct from the PI3K-mTOR
signaling pathway. The function of this protein
is largely unknown, except that it differs from
p70^{S6K} by the presence of an amino-terminal
nuclear localization signal (38). Studies are
currently underway to determine the function of
this protein in response to T3.

Recent studies addressing the non-nuclear,
non-transcriptional actions of thyroid hormones,
whether through cytosolic receptors or through
membrane-initiated processes (39,40), may
provide answers to many previously unexplained
actions of these hormones (24-27). Similar to
other steroid hormones like estrogens (23), the
nuclear and cytosolic activities of thyroid
hormones are likely to be a part of a
complementary regulatory network involved in
the maintenance of normal cellular function.
Results from the present study support cytosolic
actions of thyroid hormone that can be rapid in
onset, but potentially have long-term effects in
the maintenance of normal cell growth.

REFERENCES

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**FIGURE LEGENDS**

**Figure 1.** *T3-stimulated protein synthesis is inhibited by wortmannin.*

(A) Neonatal rat cardiomyocytes were either transduced with replication deficient adenovirus expressing TRα1 (Ad-TRα1, 2 moi) or left untreated (UI) and then cultured in serum-free medium for 24 hrs prior to treatment with T3 (10 nM) or vehicle (0) for another 24 hrs. Cultures were harvested for total cell protein and DNA analysis and results expressed as protein/DNA ratios. Data are means ± SE, n=6 per treatment. **p<0.02 vs. 0 T3.

(B) Northern blot showing mRNA expression for β- and α-myosin heavy chain (βMyHC, αMyHC), sarcoplasmic reticulum calcium activated ATPase (SERCA2) and ribosomal 18S RNA after 24 hrs of T3 treatment (10 nM). Cardiomyocytes were transduced with Ad-TRα1 24 hrs prior to T3 treatment (+) or no treatment (-).

(C) Cardiomyocytes were transduced with Ad-TRα1 24 hrs prior to T3 treatment for 24 hrs. Control cultures (0) were left untreated. Wortmannin (2 μM) was added either simultaneously with T3 (+W 24h) or for the final 6 hrs (+W 6h) before harvest. Protein synthesis was measured by the incorporation of [3H] leucine into total cell protein by adding it to the medium for the last 6 hrs of the experiment. n=12 cultures per group from two different cell isolations. Bar graph is mean ± SE. Data were analyzed by analysis of variance, Student-Newman-Keuls test. *p<0.001 vs. 0, T3+W 6h and T3+W24h; t<0.01 vs. 0 and T3+W24h.

**Figure 2.** *TRα1 forms a complex with p85 subunit of PI3K and T3 activates PI3 kinase activity.*

(A) Cultured cardiomyocytes were either not transduced (UI) or transduced with Ad-TRα1 (2 moi) and cultured in serum-free medium for 48 hrs. Some cultures were treated with T3 (10 nM) (+) for 30 min. prior to harvest. Cell lysates from treated and untreated cultures were immunoprecipitated (IP) with either anti-TRα1, anti-p85α PI3K antibodies or control IgG, and then subjected to immunoblot (IB) analysis with antibodies as indicated. Cell lysates without IP (-) were used to show cellular content of p85α and TRα1. Blots are representative of 8 samples per treatment group from 3 separate cell isolations.

(B) Cardiomyocytes transduced with Ad-TRα1 were cultured as above and treated with T3 (10 nM) or IGF-1 (10 nM) for 10 min. prior to cell lysis as described in Methods. Immunoprecipitated PI3K from treated and untreated cultures were used to measure PI3 kinase activity by formation of radiolabeled L-α-phosphatidylinositol-4-phosphate (PIP) as detected by exposure of the TLC plate to x-ray film.
PIP product was quantified by laser densitometry and is shown graphically. p<0.005 T3 vs. untreated group (0). Bar graph represents mean ±SE, n=6-8 from two cell isolations.

**Figure 3. T3 rapidly activates Akt and S6 kinase**
Cardiomyocytes were cultured under serum-free conditions for 24 or 48 hrs prior to treatment with T3 over a 60 min period or for 24 hrs. Proteins from whole cell lysates were resolved by electrophoresis and cell signaling protein activation was determined by Western blot analysis of phosphorylated Akt, p70 S6K, ribosomal S6 protein and 4E-BP1, and then reanalyzed of the corresponding total protein (non-phosphorylated and phosphorylated). Blots are representative of 4 separate cell isolations, n=8-12 per time point.

**Figure 4. Time course of T3-induced phosphorylation of Akt, mTOR and 4E-BP1**
Cardiomyocytes transduced with Ad-TRα1 were cultured in serum-free medium for 48 hrs prior to treatment with T3 (10 nM) and harvested at the time points indicated. IGF-1 (10^{-8} M) treatment was for 15 min. (A) Nuclear (nuc) and cytoplasmic (cyto) fractions were isolated for protein analysis by Western blotting methods for detection of nuclear translocation of phosphorylated Akt in response to T3 treatment. Total cytosolic Akt shows equal sample loading at each time point. Cytosolic proteins were analyzed for phosphorylated and total mTOR (B) and 4E-BP1 (C) as described in Methods. Some cultures were pre-treated with wortmannin (W) or LY294002 (LY) for 30 min. prior to treatment with T3 for 15 min. (D) Densitometric quantitation of Western blots of cytosolic Akt and mTOR from four separate cell isolations were combined and are shown graphically; bar graph represents mean ±S.E. relative to untreated group (0 min. T3), n=8 per time point. Phospho-mTOR/mTOR at 15, 25, 40 and 60 min. T3 treatment are significantly different (p<0.01) vs. 0 min.; pAkt/Akt at 7, 15, 25 min. p<0.05 vs 0 min.T3.

**Figure 5. T3-stimulated phosphorylation of S6K and 40S ribosomal protein S6 is inhibited by wortmannin and rapamycin and by overexpression of a dominant negative mutant PI3K**
Ad-TRα1 transduced cardiomyocytes were cultured and treated with T3, IGF-1 and inhibitors as described in Fig.4. Pre-treatment with rapamycin (R) was for 60 min. prior to T3 treatment for 40 min. (A) (B) Immunoblot analyses of cytoplasmic fractions were probed with antibodies detecting phosphorylated and total S6K proteins. Phospho-S6K(T389) antibody recognizes both p70 and p85 variants of S6K, whereas anti-S6K antibody preferentially detects p70S6K. (C ) Same samples as above were used for immunoblot analysis of phosphorylated 40S ribosomal protein S6 in response to T3, showing complete inhibition by PI3K inhibitor wortmannin, and mTOR inhibitor rapamycin. Western blots are representative of samples from four separate cell isolations. (D) Cultured cardiomyocytes were transduced with Ad-TRα1 and adenoviruses expressing mutant p85PI3K (Ad-Δp85PI3K, at 10 and 100 multiplicity of infection, moi), and then cultured for 48 hrs in serum-free medium prior to T3 (10 nM) treatment for 15 min. Cytoplasmic fractions were analyzed for phospho-S6K(T389) and phospho-Akt(S473) content by immunoblot analysis. Increased expression of the p85α subunit of PI3K in Ad-Δp85PI3K transduced cells was verified by anti-PI3Kp85α antibody.
Figure 1

A.  

B. Northern Blot

C. 

\[ \text{[H]-Leucine incorporation (fold control)} \]

\[ 0 \quad T3 \quad T3 \quad T3 \quad +W \text{ 6h} \quad +W \text{ 24h} \]
Figure 2

A. 

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<td>T3</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

IB: p85α

IB: TRα1

B. 

0          T3

IGF-1

PI3K activity (% control)

-0.5  0.0  0.5  1.0  1.5  2.0  2.5

p<0.005

0  T3  IGF-1

PI3K activity (% control)
Figure 3

<table>
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<tr>
<th>T3</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60 min</th>
<th>0</th>
<th>24 hrs</th>
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</tbody>
</table>

- pAkt (S473)
- Akt
- p85(T389)
- p70(T389)
- p70S6K
- pS6(S235/236)
- S6
- p4E-BP1(S65)
- 4E-BP1
Figure 4

A. T3 (min) 0 0 7 15 25 40 60 +LY

\[ \text{nuc} ] \text{pAkt(S473)} \\
\text{cyto} \\
\text{cyto Akt} \\

B. T3 (min) 0 0 7 15 25 40 60 +W

\text{pAkt(T308)} \\
\text{Akt} \\
\text{p-mTOR(S2448)} \\
\text{mTOR} \\

C. T3 (min) 0 0 7 15 25 40 60 +W

\text{p4E-BP1(S65)} \\
\text{4E-BP1} \\

D. 

Graph showing phosphorylated/total protein levels over T3 treatment (min.)
Figure 5

A.

<table>
<thead>
<tr>
<th>T3 (min)</th>
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<th>0</th>
<th>7</th>
<th>15</th>
<th>25</th>
<th>40</th>
<th>60</th>
<th>+LY</th>
<th>IGF-1</th>
<th>+W</th>
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B.

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<th>+R</th>
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<th>7</th>
<th>15</th>
<th>25</th>
<th>40</th>
<th>60</th>
<th>+W</th>
<th>+R</th>
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D.

<table>
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<th>T3</th>
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<tr>
<td>pAkt(S473)</td>
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<td>PI3Kp85α</td>
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Thyroid hormone stimulates protein synthesis in the cardiomyocyte by activating the Akt-mTOR and p70 S6K pathways
Agnes Kenessey and Kaie Ojamaa

J. Biol. Chem. published online May 22, 2006

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