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Rapamycin Prevents Thyroid Hormone-Induced Cardiac Hypertrophy

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Thyroid hormones (THs) have many effects on the cardiovascular system including cardiac hypertrophy. Although THs induce cardiac hypertrophy, the mechanism through which they exert this effect is unknown. We previously found that THs activate signaling related to increased protein synthesis [mammalian target of rapamycin (mTOR) and p70 S6 kinase] in the heart. It is unknown whether this activation contributes to TH-induced hypertrophy or whether it is merely incidental. In this study, we used rapamycin to inhibit mTOR function in mice and neonatal cardiomyocyte cultures treated with THs to test whether mTOR/S6 kinase signaling is involved in TH-mediated cardiac hypertrophy. C57 mice were treated with T_4 for 3 d, 1 wk, 2 wk, or 1 month with either placebo, T_4 (50 μg/100 g body weight·d), rapamycin (200 μg/100 g body weight·d) or T_4/rapamycin by sc slow-release pellets. At the end of the treatment period, hemodynamics and physical data were collected and hearts were frozen for Western blot analysis or myocytes were isolated. The effects of T_3 and rapamycin were also investigated using neonatal cardiomyocytes. THs activated specific components of the AKT signaling pathway *in vivo* and *in vitro*. THs induced cardiac hypertrophy, which was completely inhibited by rapamycin. Our results suggest that TH-induced hypertrophy is mediated by AKT/mTOR/S6 kinase signaling, which is important in the regulation of protein synthesis, a hallmark of cardiac hypertrophy. (*Endocrinology* 148: 3477–3484, 2007)

THYROID HORMONES (THs) (T_3 and T_4) have many effects on the heart including the induction of cardiac hypertrophy, although little is known about the mechanism. THs exert many of their effects through binding to nuclear receptors that activate or suppress gene transcription. Contrary to the classical genomic mechanism of THs, recent studies suggest that THs also act through a nongenomic mechanism by binding to a membrane receptor to activate signaling (1). Signal transduction pathways such as phosphatidylinositol 3-kinase/AKT and MAPK have been implicated in the effects of TH such as angiogenesis (2) and cardiac hypertrophy (3). Recently, we showed that T_3 activates AKT in neonatal cardiomyocytes (NCMs), which prevented serum starvation-induced cell death. Overall, it is quite clear that THs activate signal transduction pathways in NCMs that are responsible for carrying out some of the hormone effects. However, less is known about the role of signal transduction pathways in the effects of TH *in vivo*.

Interestingly, TH-induced hypertrophy is phenotypically similar to the physiological hypertrophy mediated by AKT signaling, suggesting overlapping mechanisms (4). In a previous study done in our lab, we found that T_4 activates signaling related to increased protein synthesis [mammalian target of rapamycin (mTOR) and p70 S6 kinase] (5). Because activation of signal transduction pathways is often a transient event, a comprehensive time course study is needed to more completely understand what pathways are activated at different time points. Also, if the AKT signaling pathway contributes to the effects of THs on the heart, blocking this pathway should attenuate some of the effects such as hypertrophy and increased contractility.

Although mTOR signaling has been implicated in TH-induced myocyte hypertrophy (3), the role of p70 S6 kinase and mTOR *in vivo* has not been investigated. It has been shown that mTOR is involved in cardiac hypertrophy due to pressure overload (6, 7). Because increased protein synthesis is a hallmark of hypertrophy, activation of mTOR might be an important pathway through which THs induce hypertrophy. Here, we present evidence that rapamycin inhibits cardiac myocyte hypertrophy, thereby implicating mTOR/S6 kinase signaling in TH-induced cardiac hypertrophy.

Materials and Methods

Experimental design

Two-month-old C57BL/6J female mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were randomly separated into different treatment groups. The different treatments included placebo, rapamycin (200 μg/100 g body weight·d; Wyeth Pharmaceuticals, Cambridge, MA), T_4 (50 μg/100 g body weight·d; Sigma Chemical Co., St. Louis, MO), or T_4 plus rapamycin. Each treatment was administered for 3, 7, 14, or 30 d by slow-release implantable pellets obtained from Innovative Research (Sarasota, FL). The hearts were removed, blotted, weighed, and either snap-frozen or used for isolation of myocytes via aortic perfusion with collagenase. All procedures in this study were approved by the University of South Dakota Animal Care and Use Committee and followed institutional guidelines for animals.

Western blotting

Frozen whole left ventricular tissue was powdered in liquid nitrogen and RIPA buffer with protease inhibitor cocktail (EMD Biosciences Inc., San Diego, CA), phosphatase cocktail inhibitor (Sigma), and 1 mM phenylmethylsulfonyl fluoride. Each sample was incubated at 4 C for 15 min and sonicated to completely homogenize the tissue. Cell lysates were

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Abbreviations: FKBP12.6, 12-kDa FK506-binding protein; NCM, neonatal cardiomyocyte; RyR, ryanodine receptor; TH, thyroid hormone.

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TABLE 1. Effects of T_4 and rapamycin on hemodynamics

Treatment	HR (bpm)	LVPs (mm Hg)	LVPd (mm Hg)	dP/dT (mm Hg/sec)	−dP/dT (mm Hg/sec)
3 d					
Placebo (n = 9)	578 ± 54	116 ± 3.5	5.3 ± 1.0	10,834 ± 788	8,586 ± 837
T_4 (n = 10)	631 ± 26[a]	111.8 ± 4.4[a]	7 ± 0.9[a]	10,181 ± 434[a]	8,718 ± 497
7 d					
Placebo (n = 7)	561 ± 33	136 ± 21	7.5 ± 1.9	11,609 ± 1,407	9,129 ± 1,283
T_4 (n = 7)	642 ± 39[a]	125 ± 5.2	6.3 ± 0.7	11,867 ± 850	10,071 ± 843
14 d					
Placebo (n = 7)	558 ± 71	110 ± 7.3	6.0 ± 2.4	9,599 ± 1,824	7,945 ± 1,421
Rapamycin (n = 7)	583 ± 14	119 ± 3.4[a]	4.8 ± 1.3	11,121 ± 712	8,532 ± 685
T_4 (n = 8)	594 ± 47	125 ± 4.3[a]	7.1 ± 2.2	10,855 ± 1,034	9,821 ± 707[a]
T_4/rapamycin (n = 7)	608 ± 27	125 ± 8.1[a]	5.7 ± 1.4	11,509 ± 1,102[a]	9,635 ± 947[a]
30 d					
Placebo (n = 6)	501 ± 32	119 ± 2.7	5.1 ± 1.4	9,056 ± 2,430	6,914 ± 1,542
Rapamycin (n = 3)	532 ± 82	139 ± 8.9	4.6 ± 0.9	10,012 ± 2,772	8,195 ± 3,303
T_4 (n = 7)	562 ± 35	121 ± 8.0	4.6 ± 0.9	9,923 ± 1,423	8,554 ± 1,419
T_4/rapamycin (n = 8)	517 ± 53	125 ± 13	6.6 ± 3.7	9,913 ± 1,616	7,548 ± 1,702

HR, Heart rate; LVPd, left ventricular diastolic pressure; LVPs, left ventricular systolic pressure; dP/dT, maximum rate of pressure increase; −dP/dT, maximum rate of pressure decrease.

[a] $P < 0.05$ compared with placebo.

centrifuged at 12,000 × *g* for 15 min. The supernatant was collected and stored in aliquots at −80 C. Protein concentrations of the cell lysates were determined by a bicinchoninic acid protein assay. Samples were then mixed with Laemmli buffer containing 5% β-mercaptoethanol and loaded onto SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes, and blots were probed with antibodies specific to phospho-p70 S6 kinase (Ser 421/424), phospho-p70 S6 kinase (Thr 389), total p70 S6 kinase, phospho-S6 ribosomal protein (Ser 235/236), total S6 ribosomal protein, phospho-ERK (Thr 202/Tyr 204), total ERK, phospho-AKT (Ser 473), phospho-AKT (Thr 308), and total AKT (Cell Signaling Technology, Beverly, MA). Bands were visualized using chemiluminescence (Pierce, Rockford, IL), and relevant band intensities

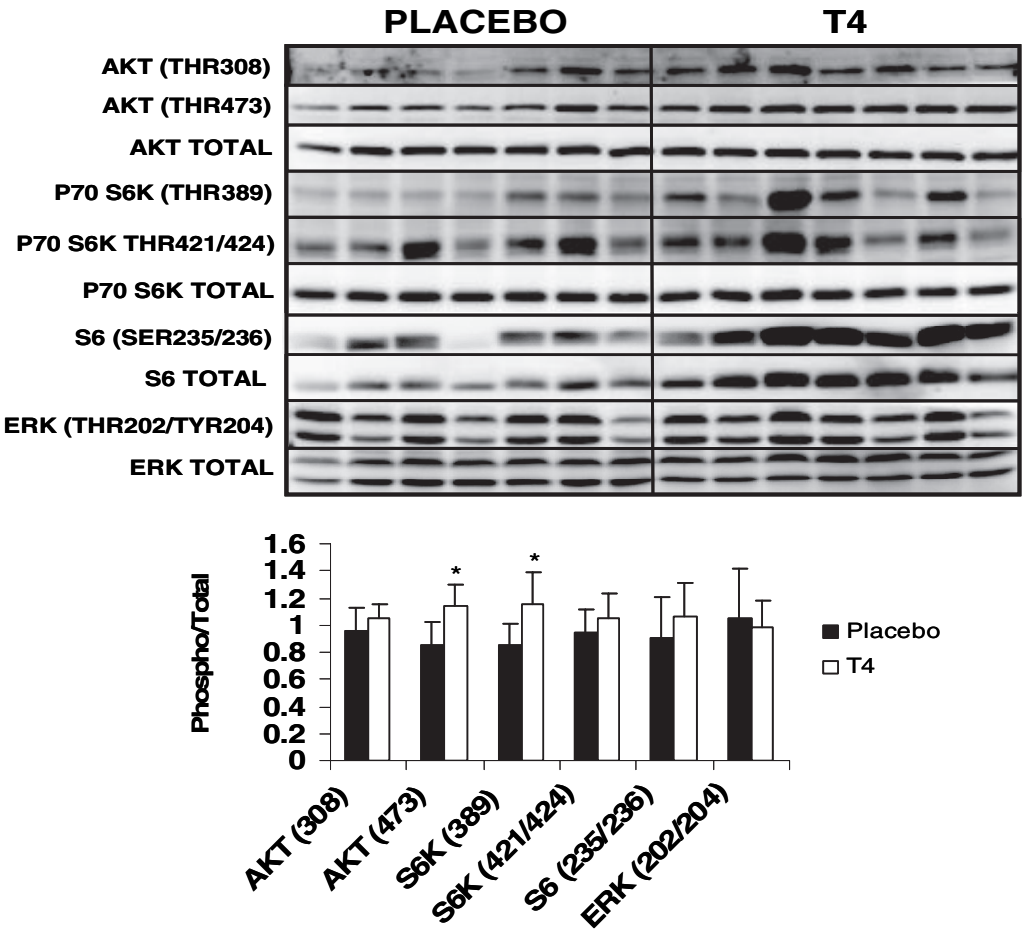

FIG. 1. Western blots from mice treated with T_4 for 3 d. Densitometry is expressed as a ratio of phosphorylated to total protein. *, $P < 0.05$ compared with placebo.

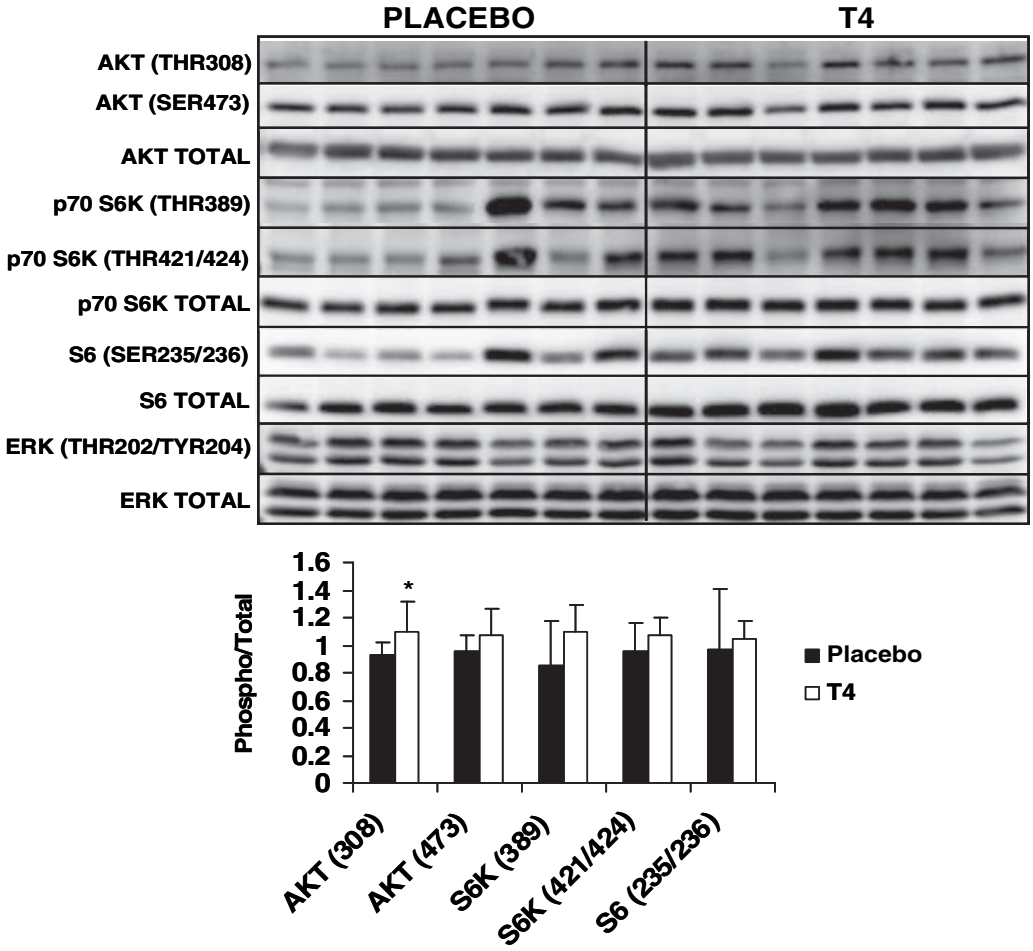

FIG. 2. Western blots from mice treated with T_4 for 7 d. Densitometry is expressed as a ratio of phosphorylated to total protein. *, $P < 0.05$ compared with placebo.

were quantified using a Versadoc Imaging System model 3000 (Bio-Rad Laboratories, Inc., Hercules, CA).

Myocyte isolation and cell volume measurements

Adult mouse myocytes were isolated as described by O'Connell *et al.* (8). Myocytes were fixed in 2% glutaraldehyde, and volume was measured using a Coulter Channelyzer as described in detail before (9).

Neonatal myocyte cultures

Neonatal myocytes were isolated from 0- to 2-d-old rat pups using a kit from Worthington Biochemical Corp. (Lakewood, NJ). The cells were then seeded in 60-mm (1.5×10^6 cells) dishes for Western blot analysis. Cells were cultured in 10% serum for 24 h after isolation. Serum-containing media was then removed, and cells were cultured in serum-free media for 24 h. T_3 (100 nM), rapamycin (1 nM), or a combination was then added to the media, and the cells were cultured for the respective times listed in the results. Cells were harvested in RIPA buffer or trypsinized for cell volume measurements using a Coulter Channelyzer.

Data analysis

All data are expressed as means ± SD. One-way ANOVA or *t* tests were used to detect significant differences between groups. The Student-Newman-Keuls test was used as a *post hoc* test. A *P* value ≤ 0.05 was considered significant.

Results

Effects of T_4 and rapamycin on hemodynamics

T_4 treatment predictably increased heart rate at 3 and 7 d (Table 1). However, there was not a significant difference in heart rate at the 14- or 30-d time points (Table 1). At 2 wk, +dP/dT (maximum rate of pressure increase) was significantly increased in the T_4/rapamycin group compared with placebo, and −dP/dT (maximum rate of pressure decrease) was significantly elevated in T_4 and T_4/rapamycin compared with control at 2 wk. There were no significant changes in hemodynamic measurements after 1 month of treatment.

Activation of signal transduction pathways at different time points

Three days. We measured the expression and phosphorylation of members of the AKT signaling pathways at each time point. At 3 d, phosphorylation of AKT (Ser473) and S6 kinase (Thr389) were significantly increased (Fig. 1). T_4 also increased expression of S6 ribosomal protein, which is downstream of S6 kinase. Because the total and phosphorylated forms were increased in proportion, there was not a significant difference in the phospho/total ratio of S6 ribosomal

protein. There were no changes in the phosphorylation of ERK.

Seven days. After 7 d of treatment, phosphorylation of AKT (Ser473) returned to relatively normal levels (Fig. 2). However, phosphorylation of AKT (Thr308) was significantly increased. Molecules downstream of AKT were not significantly changed, although there was a trend for increased phosphorylation of S6K (Thr389). Also, the expression of total S6 ribosomal protein remained increased at this time point. Again, there was no change in the phosphorylation of ERK.

Fourteen days. After 14 d of treatment, there was a significant increase in the phosphorylation of AKT 308 with rapamycin treatment alone (Fig. 3). Again, there was a trend for phosphorylation of S6K, but the changes were not significant. Although T_4 treatment did not increase the phosphorylation of S6, there was a reduction in the phosphorylated levels with rapamycin. Also, expression of total S6 ribosomal protein was increased with T_4 treatment. This increase in S6 expression was prevented by rapamycin.

Thirty days. T_4 increased the phosphorylation of S6 kinase and S6 ribosomal protein (Fig. 4). This effect was completely blocked in mice treated with both T_4 and rapamycin. There was no difference in phosphorylated AKT with T_4 treatment. However, there was a significant increase in phosphorylated ERK with T_4 treatment that was unaffected by rapamycin.

Rapamycin prevented T_4-induced myocyte hypertrophy

At 2 wk, heart weight was 32% greater in the T_4 group compared with placebo (Table 2). Heart weight in the T_4/rapamycin group was significantly lower than the T_4 group, suggesting rapamycin attenuated T_4-induced hypertrophy. Although the heart weight in the T_4/rapamycin group was significantly elevated compared with the placebo group, there was also a significant increase in body weight with T_4 treatment. Comparison of heart weight/

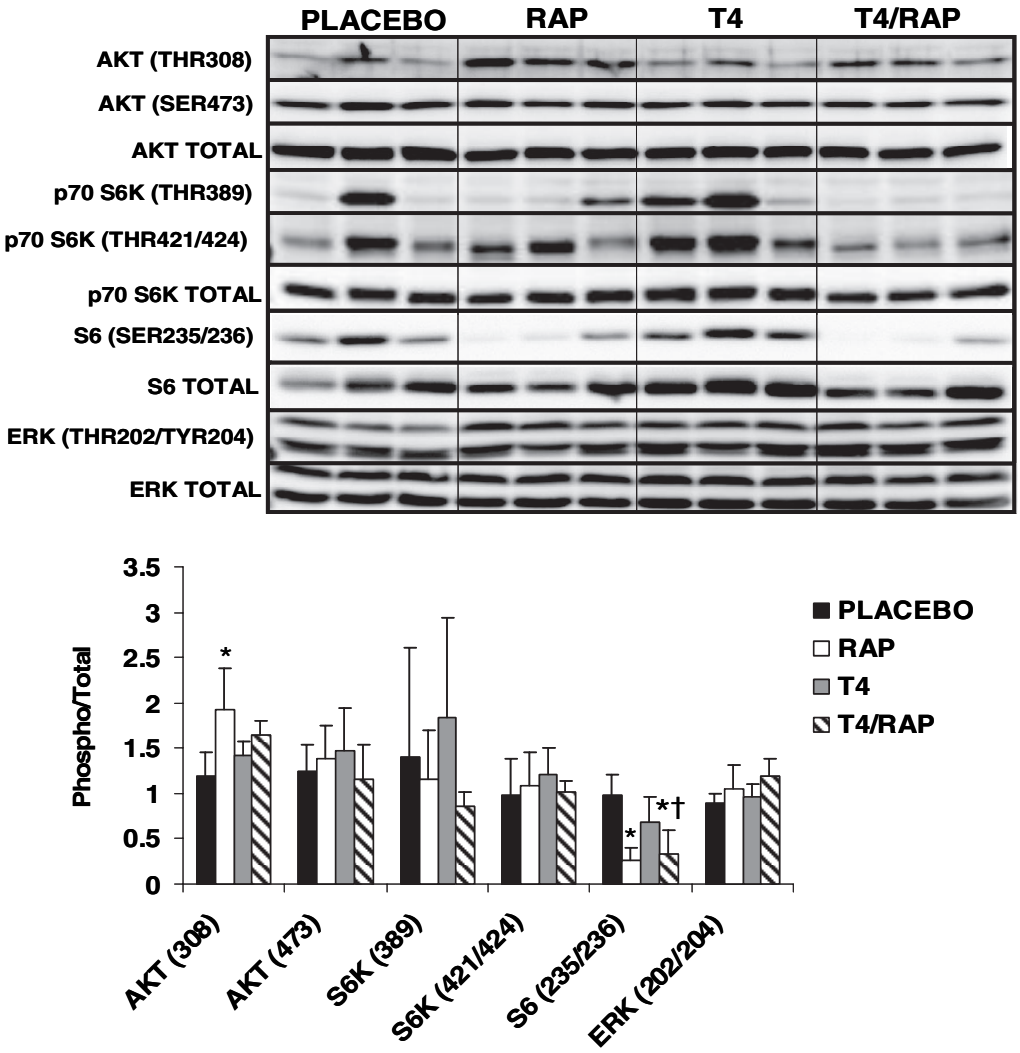

FIG. 3. Western blots from mice treated with rapamycin (RAP), T_4, or T_4/RAP for 14 d. Densitometry is expressed as a ratio of phosphorylated to total protein. *, $P < 0.05$ compared with placebo; †, $P < 0.05$ compared with T_4.

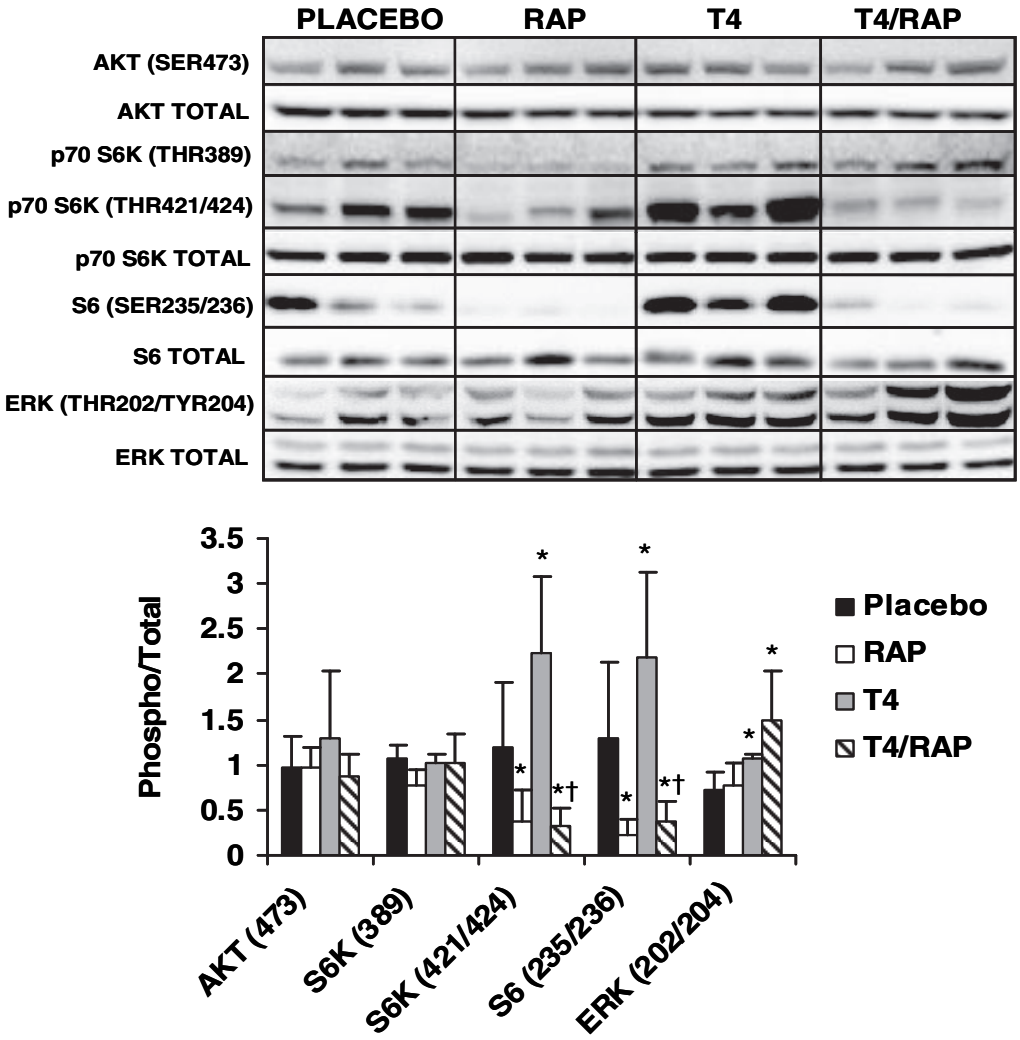

FIG. 4. Western blots from mice treated with rapamycin (RAP), T_4, and T_4/RAP for 30 d. Densitometry is expressed as a ratio of phosphorylated to total protein. *, $P < 0.05$ compared with placebo; †, $P < 0.05$ compared with T_4.

body weight between placebo and T_4/rapamycin suggests that rapamycin completely prevented the cardiac growth induced by T_4.

Similar results were obtained at the 1-month time point (Table 2). T_4 significantly increased heart weight compared with placebo (27%), which was completely prevented by rapamycin. To confirm that rapamycin prevented myocyte hypertrophy, we measured cell volume at the 1-month time point (Fig. 5). Percent changes in cell volume were virtually identical to changes in heart weight, confirming that rapamycin prevented T_4-induced myocyte hypertrophy.

Effects of TH in vitro

Treatment with T_3 for 15 min, 60 min, and 3 h did not have any effect on the levels of phosphorylated S6 ribosomal protein, which is downstream of both AKT and S6 kinase (Fig. 6). However, there was increased phosphorylation of S6 ribosomal protein at 24 h. At all time points, rapamycin reduced phosphorylation of S6 ribosomal protein, showing inhibition of this pathway.

When NCMs were treated with T_3 for 4 d, there was an increase in the phosphorylation of the AKT pathway (Fig. 7). Phosphorylation of AKT, S6 kinase, and S6 ribosomal protein was found to be increased in all. Rapamycin blocked T_3-induced phosphorylation of S6 kinase and S6 ribosomal protein. However, rapamycin did not decrease T_3-induced phosphorylation of AKT. In fact, phosphorylation of AKT was further increased by rapamycin treatment. ERK signaling was not affected by any of the treatments.

NCMs treated with T_3 for 4 d were also used to confirm that the effect of rapamycin on T_3-induced hypertrophy was direct rather than systemic. Volume of NCMs was measured with a Coulter Channelyzer after cell isolation by trypsinization. Cell volumes of T_3-treated myocytes were almost identical to cells cultured in media supplemented with 10% serum (Fig. 8). When T_3 treatment was combined with rapamycin, the increase in cell volume was attenuated. This confirms that T_3 stimulates myocyte hypertrophy directly rather than systemically, and this effect is blocked by rapamycin.

TABLE 2. Effects of T_4 and rapamycin on body and heart weight

Treatment	BW (g)	HW (mg)	HW/BW
3 d			
Placebo (n = 9)	17.2 ± 0.7	84 ± 2.3	4.9 ± 0.2
T_4 (n = 10)	17.9 ± 1.3	98 ± 5.4[a]	5.5 ± 0.2[a]
7 d			
Placebo (n = 9)	18.4 ± 0.8	86 ± 3.2	4.7 ± 0.2
T_4 (n = 10)	19.5 ± 0.8	105 ± 5.8[a]	5.4 ± 0.2[a]
14 d			
Placebo (n = 5)	18.0 ± 0.8	81.5 ± 7.9	4.6 ± 0.3
Rapamycin (n = 8)	18.6 ± 1.6	81.5 ± 3.9	4.5 ± 0.2
T_4 (n = 7)	20.2 ± 0.8[a]	107.2 ± 2.6[a]	5.3 ± 0.2[a]
T_4/rapamycin (n = 8)	20.4 ± 0.9[a]	95.6 ± 4.1[a,b]	4.7 ± 0.3[b]
30 d			
Placebo (n = 6)	20.8 ± 1.1	90.1 ± 7.3	4.3 ± 0.2
Rapamycin (n = 6)	20.1 ± 0.9	86.8 ± 2.1	4.3 ± 0.2
T_4 (n = 6)	21.5 ± 0.7	114.5 ± 12.4[a]	5.3 ± 0.2[a]
T_4/rapamycin (n = 6)	21.1 ± 0.9	92.8 ± 3.1[b]	4.4 ± 0.5[b]

BW, Body weight; HW, heart weight.
[a] $P < 0.05$ compared with placebo.
[b] $P < 0.05$ compared with T_4.

Discussion

We present a novel mechanism through which THs induce cardiac hypertrophy *in vivo*. Our data suggest that mTOR activity is required for TH-induced hypertrophy. In this study, T_4 treatment affects AKT signaling in a temporal manner. The phosphorylation/activation of AKT/S6 kinase correlates with increased translation, which is a hallmark of cardiac hypertrophy (10). Interestingly, T_4 also seemed to increase the expression of S6 ribosomal protein at certain time points. The principal finding of this study was that rapamycin blocked the effects of THs on mTOR/AKT signaling, which prevented TH-induced hypertrophy.

In this study, the selected dose was intended to induce hyperthyroidism in the mice. However, significant increases in heart rate were observed only at the 3- and 7-d time points, although there was a trend for an increase at the other time points. Also, T_4 treatment did not have much of an effect on dP/dT except for an increase in the T_4/rapamycin group at 2 wk. The modest changes in heart rate and contractility from T_4 treatment likely reflect the fact that mice already have high sympathetic and low parasympathetic activity. Consequently, one would not expect these changes to be as pronounced as in larger species with lower native heart rates.

T_3 clearly increased AKT signaling *in vitro*. These data also showed that T_3 affected signaling only at later time points between 3 and 24 h. Activation of these pathways is further increased after 4 d of treatment. These data are very similar to data we showed in a previous study (11) and suggest that the effect of T_3 on signal transduction is delayed in action. This suggests that the mechanism of activating AKT signaling is mediated by a genomic mechanism rather than a nongenomic mechanism. However, the exact mechanism by which TH activates AKT signaling cannot be inferred and was not a major goal of this study.

The data presented in this study clearly show that T_4 activates AKT signaling, although activation of some molecules is only transient. T_4 increased AKT phosphorylation after 3 d of treatment, and phosphorylation levels were reduced at some point thereafter. Although the phosphorylation of AKT was not increased after 1 month of T_4 treatment, an AKT-mediated mechanism for the observed increases in mTOR/S6 kinase cannot be excluded. T_4 treatment also increased expression of S6 ribosomal protein. This increase in S6 expression was also reduced by rapamycin treatment. S6 ribosomal protein increases translation of the 5′-terminal oligopyrimidine mRNAs (12). This subset of mRNAs includes those for the cytoplasmic ribosomal proteins and other components of the translational machinery such as elongation factors. Therefore, increased expression and activity of S6 ribosomal protein could also contribute to

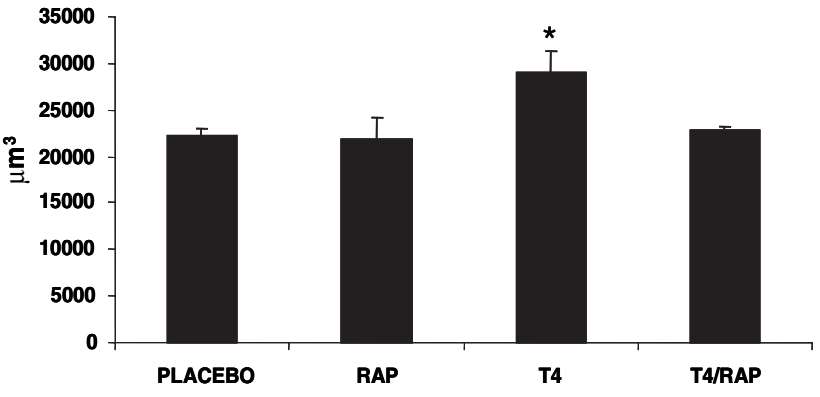

FIG. 5. Myocyte volume measurements from mice treated with T_4 for 30 d. *, $P < 0.05$ compared with placebo.

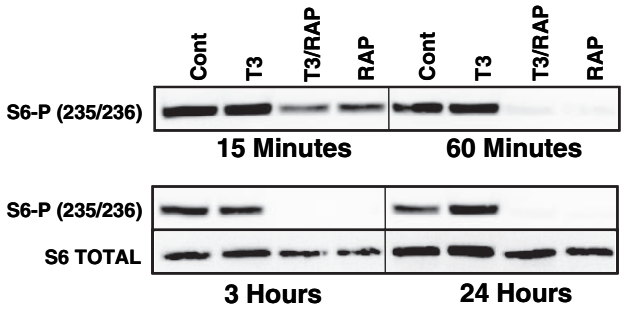

FIG. 6. Western blots measuring phosphorylation of S6 ribosomal protein from NCM extracts. NCMs were treated with T_3, rapamycin (RAP), or T_3/rapamycin.

growth-promoting effects of THs. It seems that both activation of signaling pathways as well as up-regulation of signaling proteins may contribute to the effect of THs on cardiac growth.

Rapamycin has also been shown to promote other effects on cardiomyocytes. Rapamycin binds to 12-kDa FK506-binding protein (FKBP12.6), which interacts with the ryanodine receptor (RyR) to control calcium transients. The interaction of FKBP12.6 and RyR stabilizes the RyR and prevents aberrant release of calcium during the resting phase of the cardiac cycle. Dissociation of FKBP12.6 increases the probability of sarcoplasmic reticulum calcium channels being open and therefore can increase calcium availability and cardiac contractility. It has been shown that rapamycin increases calcium cycling in mouse myocytes (13). However, in a study done by Schoffstall *et al.* (14), they concluded that the antihypertrophic effect of rapamycin is not mediated through direct effects on myofilament contractility. In addition, we did not observe any significant differences in cardiac function with the treatment of rapamycin in this study. Although it is unlikely, we cannot rule out the possibility that the antihypertrophic effect of rapamycin is partly mediated by a mechanism other than control of protein translation signaling pathways.

Surprisingly, the mechanism of how TH induces cardiac hypertrophy is not well understood. There have been studies that show TH can induce hypertrophy directly in cultured cardiomyocytes and indirectly through alteration in loading conditions of the heart. It is well established that TH has

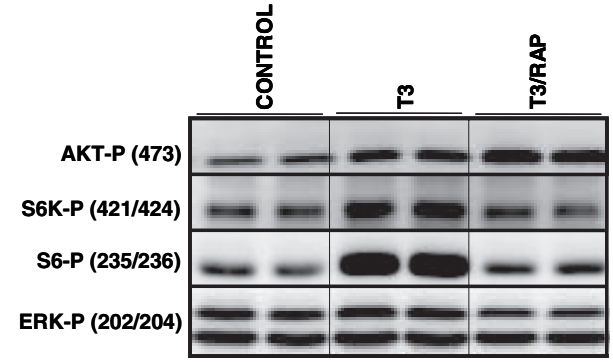

FIG. 7. Western blots for different components of the AKT signal transduction pathway in NCM extracts. NCMs were treated with T_3 or T_3/rapamycin.

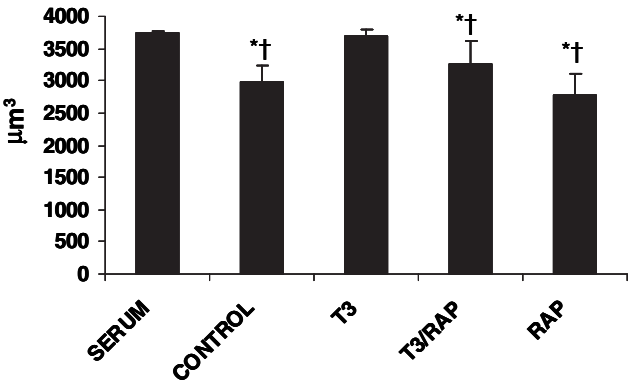

FIG. 8. NCM volume measurements from cells treated for 4 d. *, $P < 0.05$ compared with cells cultured with 10% serum; †, $P < 0.05$ compared with cells treated with T_3.

prominent effects on cardiac physiology that could result in compensatory chamber remodeling. It was previously shown that hearts heterotopically transplanted into rats that were treated with THs did not undergo hypertrophy (15, 16). These hearts were exposed to THs in the absence of loading conditions, suggesting that TH-induced hypertrophy is mediated through increased load. However, studies have also shown that T_3 can induce hypertrophy in cultured cardiomyocytes, suggesting a direct hypertrophic effect (17, 18). Cardiac hypertrophy is likely due to a combination of both direct and indirect mechanisms. In this study, we show that TH stimulates myocyte hypertrophy both *in vitro* and *in vivo*, and this effect is blocked by rapamycin. This confirms that rapamycin blocks the direct effect of THs on myocyte hypertrophy.

Another interesting finding in this study was increased phosphorylation of ERK at the 1-month treatment time point. ERK has been implicated in mediating some of the effects of THs such as myocyte hypertrophy (3, 19) and angiogenesis (1). Many studies have shown that THs can cause nongenomic activation of ERK, which contributes to the effects of TH. In this study, we did not see early activation of ERK. However, after 1 month of treatment, the phosphorylation of ERK is significantly increased. Overall, this study suggests that AKT is most likely important in many of the early effects of THs, whereas ERK may contribute to some of the late effects of THs. It is clear that THs can activate multiple signaling pathways; however, it will require more studies to further understand the timing and interaction of these pathways in promoting effects on the heart.

There are many possible mechanisms through which TH exerts the effects explained in this study. Bergh *et al.* (1) showed that T_4 can bind to $\alpha_v\beta_3$-integrin heterodimer and activate ERK. Another study recently done by Kenessey and Ojamaa (3) suggests that phosphatidylinositol 3-kinase is directly activated by the T_3/TH receptor-α complex. Alternatively, THs might stimulate the production and release of other growth factors that act in a paracrine manner to bind to membrane receptors and activate downstream signaling pathways. This is supported by our results showing that T_3 activated AKT at later time points only *in vitro*, suggesting the effect is mediated through a genomic rather than a nongenomic mechanism. Although the mechanisms by which

TH activates AKT signaling are not yet entirely clear, this study shows mTOR/AKT signaling is necessary for TH-induced cardiac hypertrophy.

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Disclosure Statement: The authors have nothing to disclose.

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