Testosterone induces cardiomyocyte hypertrophy through mammalian target of rapamycin complex 1 pathway

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Abstract

Elevated testosterone concentrations induce cardiac hypertrophy but the molecular mechanisms are poorly understood. Anabolic properties of testosterone involve an increase in protein synthesis. The mammalian target of rapamycin complex 1 (mTORC1) pathway is a major regulator of cell growth, but the relationship between testosterone action and mTORC1 in cardiac cells remains unknown. Here, we investigated whether the hypertrophic effects of testosterone are mediated by mTORC1 signaling in cultured cardiomyocytes. Testosterone increases the phosphorylation of mTOR and its downstream targets 40S ribosomal protein S6 kinase 1 (S6K1; also known as RPS6KB1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The S6K1 phosphorylation induced by testosterone was blocked by rapamycin and small interfering RNA to mTOR. Moreover, the hormone increased both extracellular-regulated kinase (ERK1/2) and protein kinase B (Akt) phosphorylation. ERK1/2 inhibitor PD98059 blocked the testosterone-induced S6K1 phosphorylation, whereas Akt inhibition (Akt-inhibitor-X) had no effect. Testosterone-induced ERK1/2 and S6K1 phosphorylation increases were blocked by either 1,2-bis(2-aminophenoxy)ethane–N,N,N,N-tetraacetic acid-acetoxymethylester or by inhibitors of inositol 1,4,5-trisphosphate (IP$_3$) pathway: U-73122 and 2-aminoethyl diphenylborate. Finally, cardiomyocyte hypertrophy was evaluated by, the expression of β-myosin heavy chain, α-skeletal actin, cell size, and amino acid incorporation. Testosterone increased all four parameters and the increase being blocked by mTOR inhibition. Our findings suggest that testosterone activates the mTORC1/S6K1 axis through IP$_3$/Ca^{2+} and MEK/ERK1/2 to induce cardiomyocyte hypertrophy.

Introduction

The heart is a target tissue for anabolic/androgenic steroids, testosterone being the main physiological hormone (Muller et al. 2003). Testosterone and its synthetic cognates have been used both clinically and illicitly to increase muscle mass (Evans 2004). Exogenously administrated, elevated testosterone concentrations induce cardiac hypertrophy in vitro (Marsh et al. 1998) and in vivo (Cabral et al. 1988, Malhotra et al. 1990, Nahrendorf et al. 2003), but the molecular mechanisms are still poorly understood. Protein synthesis is essential for both normal and hypertrophic growth of cardiomyocytes (Hannan et al. 2003). In cardiac cells, protein synthesis is highly regulated by the mammalian target of rapamycin complex 1 (mTORC1), which stimulates protein translation and ribosome biosynthesis (Proud 2004). mTORC1 lies upstream of critical translation regulators such as the 40S ribosomal protein S6 kinase 1 (S6K1; also known as RPS6KB1) and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1;

Proud 2004). Activation of the mTORC1 pathway is a critical step to induce cardiac hypertrophy in vitro (Takano et al. 1996, Boluyt et al. 1997, Simm et al. 1998, Rolfe et al. 2005, Kenessey & Ojamaa 2006) and in vivo (Shioi et al. 2003, McMullen et al. 2004, Gao et al. 2006, Kuzman et al. 2007, Kemi et al. 2008). Pro-hypertrophic stimuli can regulate mTORC1 signaling, mainly activating either the phosphatidylinositol 3-kinase (PI3K)/Akt (Shioi et al. 2002, Kenessey & Ojamaa 2006) or the MEK/extracellular signal-regulated kinase 1/2 (ERK1/2) pathways (Wang & Proud 2002, Rolfe et al. 2005). Increased protein synthesis is a hallmark event for anabolic action of testosterone, but the relationship with mTORC1 is unknown. Here, we show that testosterone activates the mTORC1 pathway in cardiomyocytes through inositol 1,4,5-trisphosphate (IP$_3$)-mediated Ca^{2+} release and MEK/ERK1/2. Moreover, inhibition of mTORC1 abolished cardiomyocyte hypertrophy induced by testosterone, suggesting an important role of this pathway during the shift from normal to hypertrophied cardiomyocytes.

Materials and Methods

Reagents

Testosterone enanthate, cyproterone acetate, U-73122, 2-aminoethyl diphenylborate (2-APB), pancreatin, and 5-bromo-2-deoxyuridine were purchased from Sigma Chemical Company (St Louis, MO, USA). Rapamycin, Akt-inhibitor-X, and PD98059 were from Calbiochem (San Diego, CA, USA). Of 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid-acetoxymethylester (BAPTA-AM) and cell tracker green were from Molecular Probes, Invitrogen (Eugene, OR, USA). [³H]-leucine was from NEN Radiochemicals Perkin Elmer (Waltham, MA, USA). Collagenase type II was from Worthington Biochemical Corporation (Lakewood, CA, USA). All other reagents were of analytical grade.

Culture of neonatal rat cardiomyocytes

All procedures for animal use were in accordance with guidelines approved by the Bioethical Committee at the Facultad de Medicina, Universidad de Chile. Primary neonatal rat cardiomyocytes were prepared from hearts of 1–3 days old Sprague–Dawley rats as previously described (Vicencio et al. 2006). This protocol produces cultures of cardiomyocytes that are at least 95% pure (Vicencio et al. 2006). To prevent overgrowth of fibroblasts and smooth muscle cells, 10 μM bromodeoxyuridine was used in the cell culture medium. Cardiomyocytes were cultured in a medium containing DMEM:M-199 (4:1) supplemented with 10% FBS and 1% penicillin–streptomycin.

siRNA transfection

Small interference RNA to mTOR (siRNA–mTOR) was obtained from Cell Signaling Technology (catalog number #6381, Danvers, MA, USA). After 24 h of cultures, cells grown on 60 mm dishes were transfected with siRNA–mTOR (50 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The knockdown of mTOR protein expression was confirmed by western blot.

Western blot

Cardiomyocytes were grown on 60 mm dishes and serum starved for 24 h before being exposed to testosterone for various times. The experimental procedure for western blot and cell lysates has been previously described (Estrada et al. 2003). Briefly, equal amounts of proteins were separated in SDS-PAGE polyacrylamide gels, and then transferred to nitrocellulose membranes. The following primary antibodies and their dilutions were used: anti-p-mTOR (1:1000; Cell Signaling); anti-mTOR (1:1000; Cell Signaling); anti-p-S6K1 (1:1,000; Upstate); anti-S6K1 (1:1000; Upstate); anti-p-4E-BP1 (1:1000; Cell Signaling); anti-p-Akt (1:1000; BD Bioscience); anti-Akt (1:1000; Santa Cruz, Santa Cruz, CA, USA); anti-p-ERK1/2 (1:1000; Cell Signaling); and anti-ERK1/2 (1:1000; Santa Cruz); anti-β-myosin heavy chain (β-MHC) (1:10 000, Sigma); anti-skeletal actin (SKA) (1:2000, Sigma); and anti-β-actin (1:10 000, Sigma). The protein bands in the blots were visualized using an ECL detection kit (Pierce, Piscataway, NY, USA) and the intensity of the bands was determined by scanning densitometry.

Cell size measurements

Cardiomyocytes were cultured for 24 h. Thereafter, the culture medium was replaced with a medium supplemented with testosterone and/or rapamycin and cultured for additional 48 h. To quantify the cell size changes induced by the hormone, cardiomyocytes were incubated with the vital fluorescent dye cell tracker green (Molecular Probes) for 45 min. Acquired confocal fluorescence images (LSM Pascal 5, Zeiss, Oberkochen, Germany) were analyzed and compared using the Image J Software (Bethesda, MA, USA). At least eight different fields from three independent cultures to each condition (>100 cells) were determined.

Aminoacid incorporation

After 24 h of culture, cardiomyocytes were incubated with [³H]-leucine (2.5 μCi/ml; NEN) for additional 48 h in the presence or absence of testosterone. Next, the cells were washed four times with ice-cold PBS and treated with 10% trichloroacetic acid at 4 °C for 1 h. The samples were centrifuged for 20 min at 16 000 g, and the pellets were washed once with ice-cold absolute acetone and dissolved in 0·2 M NaOH (Kenessey & Ojamaa 2006). Aliquots of triplicate samples per group were counted in a liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA). The data correspond to the ratio of basal counts/min with respect to each experimental condition.
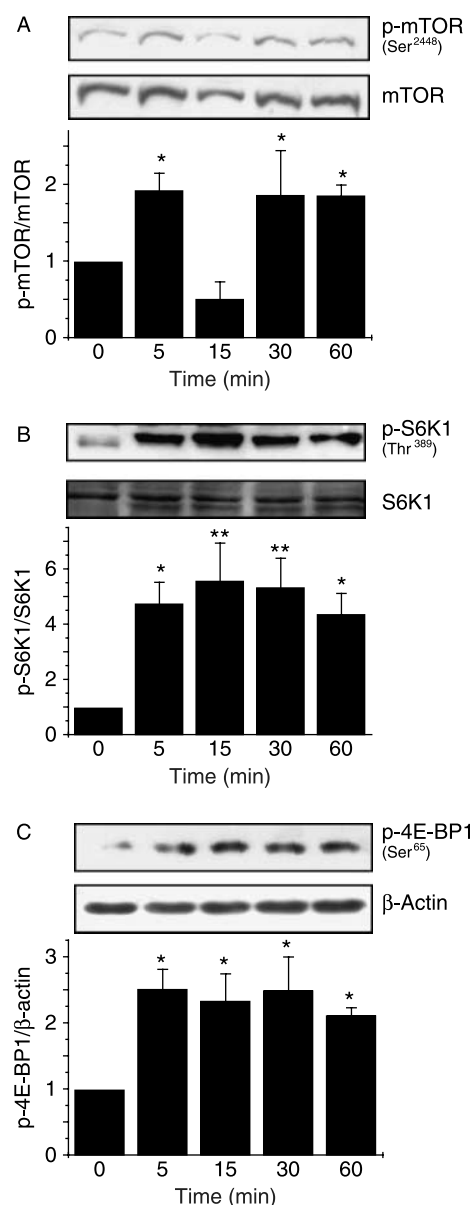
Statistical analysis

Results are expressed as mean $\pm$ S.E.M. In order to compare the difference between basal and post-stimulated conditions we carried out ANOVAs and the statistical a differences were determined by Tukey's post-test. A value of $P<0·05$ was considered statistically significant.

Results

Testosterone activates mTORC1 pathway in cultured cardiomyocytes

Since mTORC1 is a major regulator of cell growth through its downstream targets S6K1 and 4E-BP1, we evaluated the phosphorylation changes of mTOR (Ser[2448]), S6K1 (Thr[389]), and 4E-BP1 (Ser[65]) induced by testosterone in cardiomyocytes
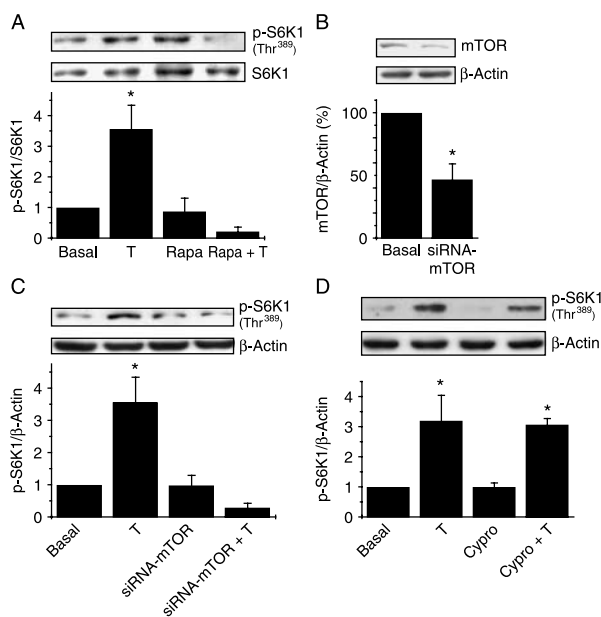
(Fig. 1). These phosphorylation sites are well recognized for their ability to reflect the actual mTORC1 activity (Wang & Proud 2007). Testosterone (100 nM) increased the mTOR phosphorylation in a biphasic manner with an initial increase

at 5 min which was approximately twofold the basal value ($P<0.05$, $n=3$) followed by a similar phosphorylation increase after 30 min, which was sustained for the studied times (Fig. 1A). Furthermore, phosphorylation of both S6K1 (Fig. 1B, 15 min, $P<0.01$, $n=3$) and 4E-BP1 (Fig. 1C, 5 min, $P<0.05$, $n=3$) were also increased. These data indicate that testosterone induces full activation of the mTORC1 pathway in cardiomyocytes.

S6K1 was evaluated next because phosphorylation changes in this protein reflect mTORC1 activation (Wang & Proud 2007). The activity of this complex can be blocked by the immune-suppressants macrolide and rapamycin (Chen et al. 1995). Figure 2 shows that testosterone-induced S6K1 phosphorylation increase was blocked by 20 nM rapamycin (Fig. 2A, $P<0.01$, $n=4$). To further determine the direct action of mTORC1 on S6K1, we used small interference RNA to mTOR (siRNA–mTOR). In cardiomyocytes transfected with siRNA–mTOR the expression of mTOR

Figure 1 Testosterone activates mTORC1, S6K1, and 4E-BP1 in cultured cardiomyocytes. Cells were stimulated with testosterone (100 nM) for 5, 15, 30, or 60 min. Phosphorylated mTOR (Panel A), S6K1 (Panel B), and 4E-BP1 (Panel C) levels were determined by western blot from whole cell lysates, using specific antibodies against the active phosphorylated proteins. mTOR and S6K1 phosphorylation were re-probed and normalized with their respective total protein, whereas 4E-BP1 was normalized with β-actin. Densitometric analyses show the ratio of phosphorylated and total proteins. Values are mean±s.e.m. ($n=3$ to each condition). *$P<0.05$, **$P<0.01$ compared with the basal value.

Figure 2 Effect of mTORC1 inhibition on testosterone-induced S6K1 phosphorylation in cultured cardiomyocytes. Cells were pre-incubated with rapamycin (rapa, 20 nM) for 30 min and then stimulated with testosterone (T, 100 nM) for 15 min (time for the S6K1 phosphorylation peak). (Panel A) Representative western blot and densitometric analysis showing the inhibitory effects of rapamycin on S6K1 phosphorylation ($n=4$). (Panel B) Cells transiently transfected with siRNA–mTOR exhibit ∼53% reduced protein expression with respect to non-transfected cells ($n=3$). (Panel C) The increase in the S6K1 phosphorylation induced by testosterone was inhibited by mTOR knockdown ($n=3$). (Panel D) Cells were pre-incubated with cyproterone (Cypro, 1 μM, $n=4$) for 30 min followed by stimulation with testosterone (T, 100 nM) for 15 min. The inhibition of androgen receptor did not modify the S6K1 phosphorylation increase induced by testosterone. Values are mean±s.e.m. ($n=3$ to each condition). *$P<0.05$, compared with basal value.

protein was reduced by 53% (Fig. 2B, $P<0.05$, $n=3$). In addition, knockdown of mTOR inhibited the testosterone-induced S6K1 phosphorylation increase (Fig. 2C, $P<0.05$, $n=3$). Taken together, these data suggest a direct effect of mTORC1 on S6K1 phosphorylation in testosterone-stimulated cardiomyocytes.

Previously, we have shown that testosterone induces rapid effects independent of its intracellular androgen receptor (AR) in skeletal muscle cells (Estrada et al. 2003), neuroblastoma cells (Estrada et al. 2006), and cardiomyocytes (Vicencio et al. 2006). To study the participation of AR on mTORC1 activation, we performed experiments using cyproterone, an inhibitor of the intracellular AR. Cyproterone (1 μM) did not modify the S6K1 phosphorylation increase induced by testosterone (Fig. 2D, $P<0.05$, $n=4$). These results suggest that mTORC1 activation by testosterone in cardiomyocytes is both specific and independent of the canonical AR pathway.
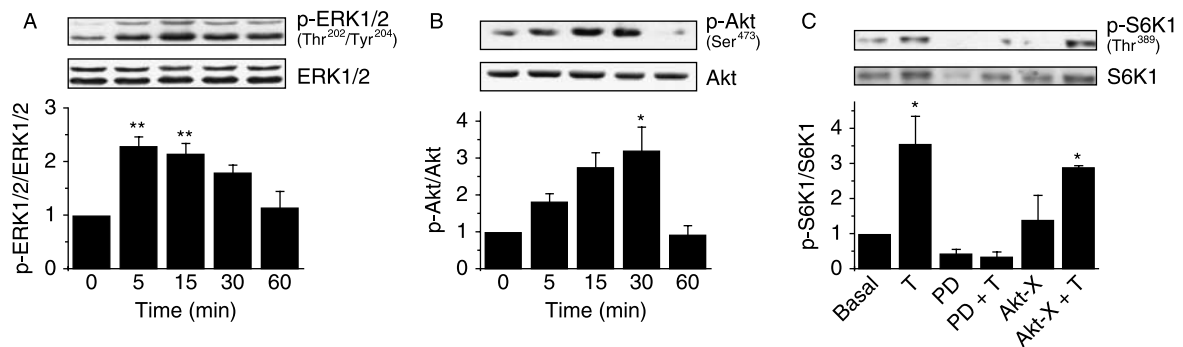
Testosterone activates ERK1/2 and Akt

mTORC1 is a multifunctional protein complex involved in survival, proliferation, differentiation, and growth (Richardson et al. 2004). mTORC1 is activated mainly by the upstream kinases ERK1/2 or Akt (Proud 2004). To explore the mechanism by which testosterone activates mTORC1, we first evaluated whether testosterone can activate these kinases. Testosterone induced an increase in phosphorylation of both ERK1/2 and Akt (Fig. 3). Time-course analysis for ERK1/2 activation shows a fast and transient peak at 5–15 min (Fig. 3A, $P<0.01$, $n=3$), whereas Akt phosphorylation displays a delayed increase, which peaked at 30 min after testosterone stimulation, returning to basal values after an hour (Fig. 3B, $P<0.05$, $n=3$). Phosphorylation kinetics for both ERK1/2 and Akt are in agreement with mTORC1 activation.

### ERK1/2, but not Akt, is involved in mTORC1 activation induced by testosterone

In order to determine the upstream signaling pathway involved in mTORC1 activation, we used specific inhibitors for MEK1/2 and Akt. Cardiomyocytes were pre-incubated with 50 μM PD98059, a MEK1/2 inhibitor, or with 10 μM Akt-inhibitor-X, a specific inhibitor that blocks Akt kinase activity but does not modify PI3K activity (Thimmaiah et al. 2005). Akt inhibition had no effect, whereas ERK1/2 inhibition blocked the testosterone-induced S6K1 phosphorylation (Fig. 3C, $n=4$). Together, these results indicate that MEK/ERK1/2 pathway, but not PI3K/Akt, is required for mTORC1 activation and subsequent S6K1 phosphorylation induced by testosterone in cardiomyocytes.

Role of Ca^{2+} signaling in testosterone-induced ERK1/2 phosphorylation

Rise in intracellular Ca^{2+} concentration has been suggested as a critical step for ERK1/2 activation (Agell et al. 2002). Previously, we demonstrated that testosterone increases intracellular Ca^{2+} levels in cardiomyocytes (Vicencio et al. 2006) and skeletal muscle cells (Estrada et al. 2003), through a pertussis toxin (PTX)-sensitive G-protein coupled receptor that activates phospholipase C (PLC) to generate IP_3, which evokes Ca^{2+} signaling. To determine whether intracellular Ca^{2+} is involved in the testosterone-induced ERK1/2 phosphorylation, cardiomyocytes were incubated in Ca^{2+}-free medium (1 mM EGTA) or pre-incubated with BAPTAAM (a chelator of intracellular Ca^{2+}). As is shown in Fig. 4, ERK1/2 phosphorylation was only blocked by BAPTA-AM (Fig. 4A, $n=3$). These observations suggest that Ca^{2+} release from intracellular stores is necessary for ERK1/2 activation. Next, to investigate the role of IP_3 signaling on ERK1/2 phosphorylation induced by testosterone, cardiomyocytes were pre-incubated with PTX (a G-protein

Figure 3 Activation of ERK1/2, but not Akt, is required for mTORC1 activation by testosterone in cultured cardiomyocytes. Time-course of ERK1/2 and Akt phosphorylation induced by testosterone (T, 100 nM) in cardiomyocytes. (Panel A) ERK1/2 phosphorylation shows a fast increase at 5 min, returning to basal values after 1 h ($n=3$). (Panel B) A delayed increase in the Akt phosphorylation is observed for up to 30 min ($n=3$), which returns to basal values after 1 h. (Panel C) Cells were pre-incubated with Akt-inhibitor-X (Akt-X) or PD98059 (PD). Inhibition of ERK1/2 blocked the increase in the S6K1 phosphorylation ($n=4$). Values are mean±s.e.m. *$P<0.05$, **$P<0.01$ compared with basal value.
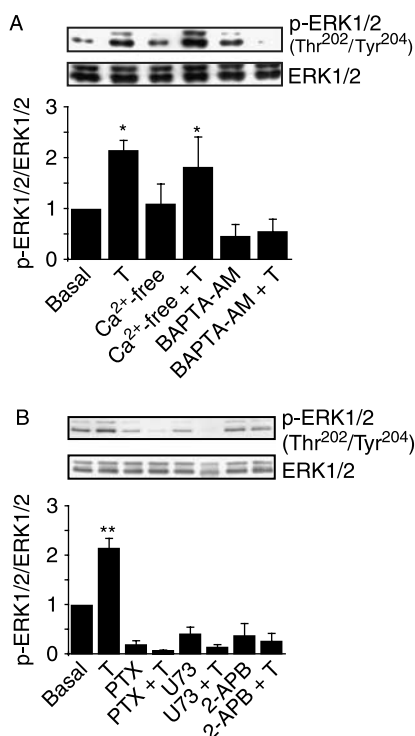
Figure 4 Role of Ca^{2+} in the testosterone-induced ERK1/2 phosphorylation in cultured cardiomyocytes. (Panel A) Cells were stimulated with testosterone (T, 100 nM) in Ca^{2+}-free medium (1 mM EGTA) or in the presence of BAPTA-AM (100 μM). Densitometric analysis shows ERK1/2 phosphorylation. (Panel B) Testosterone-induced ERK1/2 phosphorylation was studied in cells pre-incubated with PTX (1 μg/ml) or with the inhibitors of the IP_3 pathway: U-73122 (U73, 15 μM) or 2-APB (20 μM). Values are mean±S.E.M. ($n=3$ to each condition). *$P<0.05$, **$P<0.01$ compared with the basal value.

inhibitor) or with the IP_3 inhibitors: U-73122 (a PLC inhibitor) or 2-APB (an IP_3R blocker). All three inhibitors blocked the testosterone-induced ERK1/2 phosphorylation increase (Fig. 4B; $n=3$). These results suggest a sequence of events involving activation of a PTX-sensitive G-protein, IP_3-mediated Ca^{2+} release, and ERK1/2 phosphorylation.
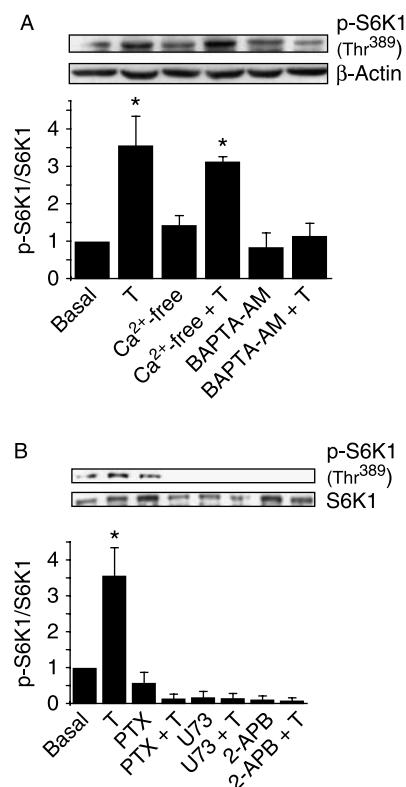
Testosterone-induced mTORC1 activation is dependent on intracellular Ca^{2+} levels

Because the testosterone-triggered ERK1/2 phosphorylation requires intracellular Ca^{2+}, inhibition of IP_3-mediated Ca^{2+} increase should block mTORC1 activation. In order to test this hypothesis, a similar strategy to determine the Ca^{2+} dependence of ERK1/2 was performed. In Ca^{2+}-free medium, testosterone increased S6K1 phosphorylation, whereas in cardiomyocytes pre-incubated with BAPTA-AM, this effect was completely blocked (Fig. 5A, $n=3$). To further determine the Ca^{2+} pool required for mTORC1 activation, cardiomyocytes were incubated with PTX, U-73122 or 2-APB. As expected, inhibition of IP_3-signaling

blocked the S6K1 phosphorylation increase (Fig. 5B, $n=3$). Together, these results indicate that testosterone-induced mTORC1 signaling involve both ERK1/2 and IP_3-mediated intracellular Ca^{2+} increase.

Testosterone-induced cardiomyocyte hypertrophy involves mTORC1

Elevated testosterone concentrations are associated with cardiac hypertrophy *in vitro* and *in vivo* (Cabral *et al.* 1988, Malhotra *et al.* 1990, Marsh *et al.* 1998, Nahrendorf *et al.* 2003). In order to assess if testosterone triggers cardiomyocyte hypertrophy via mTORC1 signaling, we examined different hypertrophy parameters. First, we evaluated testosterone effects on protein expression of β-MHC and α-SKA, which are considered cardiac hypertrophy markers (Frey & Olson 2003). Stimulation of cardiomyocytes with testosterone for 24 h increased protein expression by approximately twofold for β-MHC (Fig. 6A, $P<0.01$, $n=4$) and approximately

Figure 5 Role of Ca^{2+} on mTORC1 activation induced by testosterone in cultured cardiomyocytes. (Panel A) Cells were stimulated with testosterone (T, 100 nM) in Ca^{2+}-free medium (1 mM EGTA) or in the presence of BAPTA-AM (100 μM). Densitometric analysis shows S6K1 phosphorylation. (Panel B) Testosterone-induced S6K1 phosphorylation was investigated in cells pre-incubated with PTX (1 μg/ml) or with the inhibitors of the IP_3 pathway: U-73122 (U73, 15 μM) or 2-APB (20 μM). Values are mean±S.E.M. ($n=3$ to each condition). *$P<0.05$ compared with the basal value.
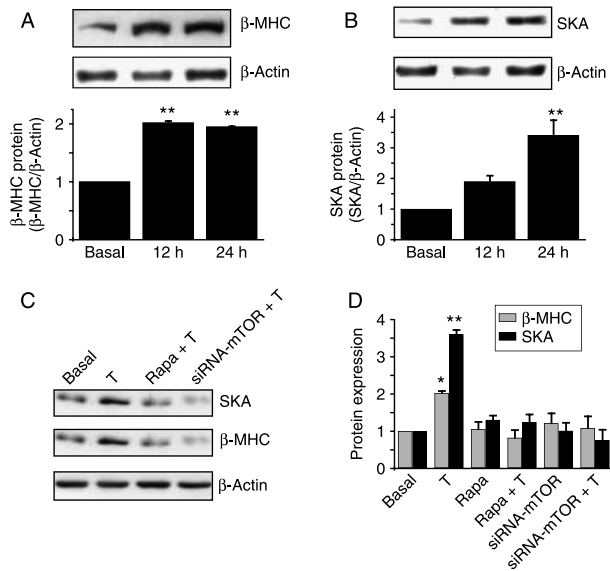
Figure 6 *In vitro* effect of testosterone and mTORC1 inhibition in cardiomyocyte hypertrophy. (Panels A and B) Cells were stimulated with testosterone (T, 100 nM) for 12 and 24 h. The expression levels of β-MHC (Panel A) and SKA (Panel B) were determined by western blot ($n=4$). (Panel C) mTORC1 inhibition with rapamycin (rapa, 20 nM, $n=4$) or siRNA–mTOR ($n=3$) abolished the testosterone-induced β-MHC and SKA protein expression increase. (Panel D) Densitometric analysis shows the protein expression for the indicated experimental condition. Values are mean±s.e.m. *$P<0.05$, **$P<0.01$ compared with the basal value.

been explained by activation of the intracellular AR (Marsh et al. 1998). Here, we show that in addition to the canonical genomic mechanism, testosterone activates mTORC1 to induce cardiomyocyte hypertrophy. Our data show that cyproterone, an inhibitor of the intracellular AR, did not affect mTORC1 activation induced by testosterone.

Different hypertrophic stimuli can exploit divergent pathways to induce mTORC1-mediated cardiac hypertrophy. Thyroid hormone and physical exercise might activate mTORC1 through PI3K/Akt (Kenessey & Ojamaa 2006, Kemi et al. 2008), whereas phenylephrine will activate MEK/ERK1/2 (Rolfe et al. 2005). The importance of the MEK/ERK1/2 pathway on development of cardiac hypertrophy has been studied in transgenic mice with cardiac-restricted expression of an activated MEK1 (Bueno et al. 2000). These mice generate a concentric hypertrophy, which demonstrates the hypertrophic role of these signaling

threefold for SKA (Fig. 6B, $P<0.01$, $n=4$). Rapamycin blocked the increase in β-MHC and SKA expression triggered by testosterone (Fig. 6C and D, $n=4$). Moreover, siRNA–mTOR also blocked testosterone-induced increase of these hypertrophic markers, without changes in their basal expression (Fig. 6C and D, $n=3$).
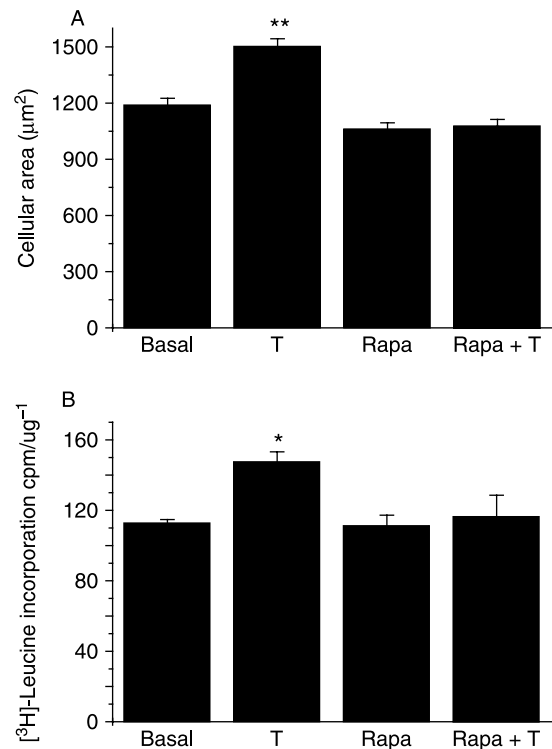
Figure 7A shows a 26% increase in cell size following testosterone treatment for 48 h compared with non-stimulated cells ($P<0.01$, ∼100 cells for each condition). Pre-incubation with rapamycin blocked the cell size increase induced by testosterone. mTORC1 is a critical regulator of protein synthesis. To further assess whether anabolic and hypertrophic effects of testosterone occur through the activation of this pathway, aminoacid incorporation was determined. As shown in Fig. 7B, testosterone increased aminoacid incorporation, which was inhibited by rapamycin ($P<0.05$; $n=4$). These results suggest that activation of mTORC1 signaling pathway plays a critical role in testosterone-induced cardiomyocyte hypertrophy.

Discussion

This study shows the first evidences that testosterone activates the mTORC1 signaling pathway in cultured neonatal cardiomyocytes. In cardiac cells, testosterone action has

Figure 7 Participation of mTORC1 in the testosterone-induced cardiomyocyte hypertrophy. (Panel A) Cell size was monitored after 48 h of treatment with testosterone (T, 100 nM). Cardiomyocytes were incubated with cell tracker green and visualized by confocal microscopy. Cell area in cardiomyocytes grown under control conditions was ∼1200 μm². Testosterone-induced cellular growth showed a ∼26% increase compared with control cells ($n=100$ cells to each condition). Cell size increase induced by testosterone was blocked in cells pre-incubated with rapamycin (rapa, 20 nM). (Panel B) Protein synthesis was determined by [³H]-leucine incorporation. Testosterone increased the amino acid incorporation, which was blocked by rapamycin ($n=4$). Values are mean ±s.e.m. *$P<0.05$, **$P<0.01$ compared with the basal value.

pathways. The PI3K/Akt pathway is also involved in the genesis and progression to a hypertrophic phenotype. Overexpression of either PI3K (subunit p110α; Shioi et al. 2000) or Akt (Condorelli et al. 2002, Shioi et al. 2002), in the heart, produces cardiac hypertrophy. Here, we determined that testosterone activates both ERK1/2 and Akt in cultured cardiomyocytes. However, ERK1/2, but not Akt, is necessary for the testosterone-induced mTORC1/S6K1 axis activation.

Akt overexpression induced cardiac hypertrophy at the molecular and histological levels (Condorelli et al. 2002). Furthermore, Akt null mice were defective in exercise-induced cardiac hypertrophy (DeBosch et al. 2006). These findings indicated that Akt signaling was indeed important for the physiological growth of the heart. Testosterone activates Akt signaling pathway in prostate cancer cells (Papadopoulou et al. 2008) and in osteoblasts (Kang et al. 2004). In this work, we demonstrate that testosterone rapidly increases Akt phosphorylation in cardiomyocytes. These pathways could participate in the hypertrophic response, survival or metabolism of the cardiomyocytes.

We previously determined that testosterone induces IP_3-mediated Ca^{2+} signals in cultured cardiomyocytes and skeletal muscle cells (Estrada et al. 2003, Vicencio et al. 2006). In skeletal muscle cells, this Ca^{2+} increase is required for ERK1/2 activation (Estrada et al. 2003). Cardiomyocytes exhibit spontaneous Ca^{2+} oscillations, which correspond to the excitation-contraction coupling mechanism (Frey & Olson 2003). Although some evidence indicates participation of Ca^{2+} signaling on the mTORC1 activation, there are no reports of this participation in cardiac cells. In rat liver epithelial cell lines, S6K1 phosphorylation induced by angiotensin II was prevented by BAPTA-AM (Graves et al. 1997). Lysophosphatidic acid stimulation of Swiss 3T3 fibroblasts promotes a fast S6K1 activation, which depends on PLC activity and intracellular Ca^{2+} increase (Willard et al. 2001). In Rat1 fibroblasts expressing the $α_{1A}$-adrenergic receptor, phenylephrine induces mTOR, S6K1, and 4E-BP1 activation dependent on both Ca^{2+} influx and Ca^{2+} release (Ballou et al. 2003). However, there are no detailed studies showing which Ca^{2+} pools are involved in mTOR activation or any evidence describing mTORC1 activation through the Ca^{2+}/MEK/ERK1/2 pathway. Our results expand the concept of privileged Ca^{2+} pools to cardiac cells. In cultured cardiomyocytes, testosterone-induced ERK1/2 phosphorylation was independent of extracellular Ca^{2+}, whereas BAPTA-AM and inhibition of the IP_3 pathway blocked the ERK1/2 activation, suggesting the participation of an IP_3-mediated intracellular Ca^{2+} increase. The mechanisms of Ca^{2+}-mediated ERK1/2 activation remain elusive; however, in cardiac cells a Ras-dependent activation of ERK1/2 by protein kinase C (Chiloeches et al. 1999) has been described. Accordingly, testosterone-induced S6K1 phosphorylation was completely blocked by inhibition of both intracellular Ca^{2+} increase and the IP_3 pathway. Moreover, testosterone-induced ERK1/2 phosphorylation in cardiomyocytes was

inhibited by pretreatment with PTX. These results are in agreement with the inhibition of ERK1/2/mTORC1/S6K1 pathway, by the G-protein inhibitor and with the Ca^{2+} requirement for ERK1/2 and S6K1 phosphorylation induced by testosterone.

Cardiac hypertrophy is produced in response to several pathophysiological conditions, such as mechanical stretching or neurohumoral deregulation. This process is characterized by increases in cardiomyocyte size and protein synthesis, as well as by the re-expression of various fetal genes (Izumo et al. 1987, 1988, Frey & Olson 2003). Testosterone induced a hypertrophic pattern in cardiomyocytes, evaluated by three parameters: 1) changes in the expression of β-MHC and SKA, which are well-known cardiac hypertrophy markers, 2) cardiomyocyte size, and 3) protein synthesis assessed by amino acid incorporation. Testosterone induced a significant increase in all three parameters that were blocked by the inhibition of mTORC1. Results suggest that the hypertrophic effects of testosterone on cardiomyocytes involve the mTORC1 pathway. In cardiomyocytes, testosterone action has been explicated only by activation of the intracellular AR (Marsh et al. 1998). We propose that cell growth produced by anabolic steroid hormones requires both androgen receptor (AR) activity and translation control through mTOR signaling pathway. Thus, both mTOR pathway and AR could control protein synthesis by a coordinated mechanism, where mTOR regulates translation and the AR regulates gene expression. However, further experiments are needed to determine the precise role of mTOR on AR signaling and vice versa.

In conclusion, we determined that testosterone activates mTORC1/S6K1 axis through MEK/ERK1/2 and IP_3/Ca^{2+} signaling. Moreover, testosterone-induced cardiomyocyte hypertrophy was prevented by mTORC1 inhibition. These data collectively support an anabolic mechanism for testosterone prior or parallel to the canonical AR pathway. Thus, the mTORC1/S6K1 pathway might be an important regulator of the metabolic signals for anabolic/androgenic steroid hormones in the heart.

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