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

Francisco Altamirano^{1,2}, César Oyarce^{1,2}, Patricio Silva^{1,2}, Marcela Toyos^{1,2}, Carlos Wilson^{1,2}, Sergio Lavandero^{1,2,3}, Per Uhlén⁴ and Manuel Estrada^{1,2}

¹Facultad de Medicina, Instituto de Ciencias Biomédicas, ²Centro FONDAP Estudios Moleculares de la Célula and ³Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Avenida Independencia 1027, Santiago 8380453, Chile

⁴Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm SE-17177, Sweden

(Correspondence should be addressed to M Estrada at Facultad de Medicina, Instituto de Ciencias Biomédicas, Universidad de Chile; Email: iestrada@med.uchile.cl)

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-acetoxy-methylester or by inhibitors of inositol 1,4,5-trisphosphate (IP₃) 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 IP₃/Ca²⁺ and MEK/ERK1/2 to induce cardiomyocyte hypertrophy.

Journal of Endocrinology (2009) **202**, 299–307

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 administered, 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 (IP₃)-mediated Ca²⁺ 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 ± S.E.M. In order to compare the difference between basal and post-stimulated conditions we carried out ANOVAs and the statistical 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 (Ser²⁴⁴⁸), S6K1 (Thr³⁸⁹), and 4E-BP1 (Ser⁶⁵) 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

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.

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 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 $\pm$ 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 $\mu\text{g}/\text{ml}$) or with the inhibitors of the IP_3 pathway: U-73122 (U73, 15 μM) or 2-APB (20 μM). Values are mean $\pm$ 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 $\mu\text{g}/\text{ml}$) or with the inhibitors of the IP_3 pathway: U-73122 (U73, 15 μM) or 2-APB (20 μM). Values are mean $\pm$ 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 $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$ compared with the basal value.

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

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

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 $\sim 1200 \mu\text{m}^2$. Testosterone-induced cellular growth showed a $\sim 26\%$ 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 $\pm$ 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 p110 α ; 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 (Papadopoulos *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 IP₃-mediated Ca²⁺ signals in cultured cardiomyocytes and skeletal muscle cells (Estrada *et al.* 2003, Vicencio *et al.* 2006). In skeletal muscle cells, this Ca²⁺ increase is required for ERK1/2 activation (Estrada *et al.* 2003). Cardiomyocytes exhibit spontaneous Ca²⁺ oscillations, which correspond to the excitation-contraction coupling mechanism (Frey & Olson 2003). Although some evidence indicates participation of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ influx and Ca²⁺ release (Ballou *et al.* 2003). However, there are no detailed studies showing which Ca²⁺ pools are involved in mTOR activation or any evidence describing mTORC1 activation through the Ca²⁺/MEK/ERK1/2 pathway. Our results expand the concept of privileged Ca²⁺ pools to cardiac cells. In cultured cardiomyocytes, testosterone-induced ERK1/2 phosphorylation was independent of extracellular Ca²⁺, whereas BAPTA-AM and inhibition of the IP₃ pathway blocked the ERK1/2 activation, suggesting the participation of an IP₃-mediated intracellular Ca²⁺ increase. The mechanisms of Ca²⁺-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 Ca²⁺ increase and the IP₃ 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 Ca²⁺ 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 IP₃/Ca²⁺ 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.

Declaration of interest

There is no conflict of interest.

Funding

This work was supported by Fondo Nacional de Ciencia y Tecnología (FONDECYT) Grant nos. 1060077 and 1090276 (to M E) and Fondo de Investigación Avanzada en Areas Prioritarias (FONDAP) Grant 1501006 (to M E and S L). F A is a recipient of a doctoral fellowship from Comisión Nacional de Ciencia y Tecnología (CONICYT).

Acknowledgements

We thank Dr Barbara Ehrlich, Yale University, for the preliminary results.

References

- Agell N, Bachs O, Rocamora N & Villalonga P 2002 Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and calmodulin. *Cellular Signalling* **14** 649–654.
- Ballou LM, Jiang YP, Du G, Frohman MA & Lin RZ 2003 Ca(2+)- and phospholipase D-dependent and -independent pathways activate mTOR signaling. *FEBS Letters* **550** 51–56.
- Boluyt MO, Zheng JS, Younes A, Long X, O'Neill L, Silverman H, Lakatta EG & Crow MT 1997 Rapamycin inhibits alpha 1-adrenergic receptor-stimulated cardiac myocyte hypertrophy but not activation of hypertrophy-associated genes. Evidence for involvement of p70 S6 kinase. *Circulation Research* **81** 176–186.
- Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF *et al.* 2000 The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *EMBO Journal* **19** 6341–6350.
- Cabral AM, Vazquez EC, Moyses MR & Antonio A 1988 Sex hormone modulation of ventricular hypertrophy in sinoaortic denervated rats. *Hypertension* **11** 193–197.
- Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA, Gu Y, Dalton N, Chung C *et al.* 2002 Akt induces enhanced myocardial contractility and cell size *in vivo* in transgenic mice. *PNAS* **99** 12333–12338.
- Chen J, Zheng XF, Brown EJ & Schreiber SL 1995 Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *PNAS* **92** 4947–4951.
- Chiloeches A, Paterson HF, Marais R, Clerk A, Marshall CJ & Sugden PH 1999 Regulation of Ras. GTP loading and Ras–Raf association in neonatal rat ventricular myocytes by G protein-coupled receptor agonists and phorbol ester. Activation of the extracellular signal-regulated kinase cascade by phorbol ester is mediated by Ras. *Journal of Biological Chemistry* **274** 19762–19770.
- DeBosch B, Treskov I, Lupu TS, Weinheimer C, Kovacs A, Courtois M & Muslin AJ 2006 Akt1 is required for physiological cardiac growth. *Circulation* **113** 2097–2104.
- Estrada M, Espinosa A, Muller M & Jaimovich E 2003 Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. *Endocrinology* **144** 3586–3597.
- Estrada M, Uhlen P & Ehrlich BE 2006 Ca²⁺ oscillations induced by testosterone enhance neurite outgrowth. *Journal of Cell Science* **119** 733–743.
- Evans NA 2004 Current concepts in anabolic–androgenic steroids. *American Journal of Sports Medicine* **32** 534–542.
- Frey N & Olson EN 2003 Cardiac hypertrophy: the good, the bad, and the ugly. *Annual Review of Physiology* **65** 45–79.
- Gao XM, Wong G, Wang B, Kiriazis H, Moore XL, Su YD, Dart A & Du XJ 2006 Inhibition of mTOR reduces chronic pressure-overload cardiac hypertrophy and fibrosis. *Journal of Hypertension* **24** 1663–1670.
- Graves LM, He Y, Lambert J, Hunter D, Li X & Earp HS 1997 An intracellular calcium signal activates p70 but not p90 ribosomal S6 kinase in liver epithelial cells. *Journal of Biological Chemistry* **272** 1920–1928.
- Hannan RD, Jenkins A, Jenkins AK & Brandenburger Y 2003 Cardiac hypertrophy: a matter of translation. *Clinical and Experimental Pharmacology and Physiology* **30** 517–527.
- Izumo S, Lompre AM, Matsuoka R, Koren G, Schwartz K, Nadal-Ginard B & Mahdavi V 1987 Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. Interaction between hemodynamic and thyroid hormone-induced signals. *Journal of Clinical Investigation* **79** 970–977.
- Izumo S, Nadal-Ginard B & Mahdavi V 1988 Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *PNAS* **85** 339–343.
- Kang HY, Cho CL, Huang KL, Wang JC, Hu YC, Lin HK, Chang C & Huang KE 2004 Nongenomic androgen activation of phosphatidylinositol 3-kinase/Akt signaling pathway in MC3T3-E1 osteoblasts. *Journal of Bone and Mineral Research* **19** 1181–1190.
- Kemi OJ, Ceci M, Wisloff U, Grimaldi S, Gallo P, Smith GL, Condorelli G & Ellingsen O 2008 Activation or inactivation of cardiac Akt/mTOR signaling diverges physiological from pathological hypertrophy. *Journal of Cellular Physiology* **214** 316–321.
- Kenessey A & Ojamaa K 2006 Thyroid hormone stimulates protein synthesis in the cardiomyocyte by activating the Akt-mTOR and p70S6K pathways. *Journal of Biological Chemistry* **281** 20666–20672.
- Kuzman JA, O'Connell TD & Gerdes AM 2007 Rapamycin prevents thyroid hormone-induced cardiac hypertrophy. *Endocrinology* **148** 3477–3484.
- Malhotra A, Buttrick P & Scheuer J 1990 Effects of sex hormones on development of physiological and pathological cardiac hypertrophy in male and female rats. *American Journal of Physiology* **259** H866–H871.
- Marsh JD, Lehmann MH, Ritchie RH, Gwathmey JK, Green GE & Schiebinger RJ 1998 Androgen receptors mediate hypertrophy in cardiac myocytes. *Circulation* **98** 256–261.
- McMullen JR, Sherwood MC, Tarnavski O, Zhang L, Dorfman AL, Shioi T & Izumo S 2004 Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. *Circulation* **109** 3050–3055.
- Muller M, van der Schouw YT, Thijssen JH & Grobbee DE 2003 Endogenous sex hormones and cardiovascular disease in men. *Journal of Clinical Endocrinology and Metabolism* **88** 5076–5086.
- Nahrendorf M, Frantz S, Hu K, von zur Muhlen C, Tomaszewski M, Scheuermann H, Kaiser R, Jazbutyte V, Beer S, Bauer W *et al.* 2003 Effect of testosterone on post-myocardial infarction remodeling and function. *Cardiovascular Research* **57** 370–378.
- Papadopoulos N, Charalampopoulos I, Anagnostopoulou V, Konstantinidis G, Foller M, Gravanis A, Alevizopoulos K, Lang F & Stournaras C 2008 Membrane androgen receptor activation triggers down-regulation of PI-3K/Akt/NF-kappaB activity and induces apoptotic responses via Bad, FasL and caspase-3 in DU145 prostate cancer cells. *Molecular Cancer* **7** 88.
- Proud CG 2004 Ras, PI3-kinase and mTOR signaling in cardiac hypertrophy. *Cardiovascular Research* **63** 403–413.
- Richardson CJ, Schalm SS & Blenis J 2004 PI3-kinase and TOR: PIKTING cell growth. *Seminars in Cell and Developmental Biology* **15** 147–159.
- Rolfé M, McLeod LE, Pratt PF & Proud CG 2005 Activation of protein synthesis in cardiomyocytes by the hypertrophic agent phenylephrine requires the activation of ERK and involves phosphorylation of tuberous sclerosis complex 2 (TSC2). *Biochemical Journal* **388** 973–984.
- Shioi T, Kang PM, Douglas PS, Hampe J, Yballe CM, Lawitts J, Cantley LC & Izumo S 2000 The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO Journal* **19** 2537–2548.
- Shioi T, McMullen JR, Kang PM, Douglas PS, Obata T, Franke TF, Cantley LC & Izumo S 2002 Akt/protein kinase B promotes organ growth in transgenic mice. *Molecular and Cellular Biology* **22** 2799–2809.
- Shioi T, McMullen JR, Tarnavski O, Converso K, Sherwood MC, Manning WJ & Izumo S 2003 Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation* **107** 1664–1670.
- Simm A, Schluter K, Diez C, Piper HM & Hoppe J 1998 Activation of p70(S6) kinase by beta-adrenoceptor agonists on adult cardiomyocytes. *Journal of Molecular and Cellular Cardiology* **30** 2059–2067.
- Takano H, Komuro I, Zou Y, Kudoh S, Yamazaki T & Yazaki Y 1996 Activation of p70 S6 protein kinase is necessary for angiotensin II-induced hypertrophy in neonatal rat cardiac myocytes. *FEBS Letters* **379** 255–259.
- Thimmaiah KN, Easton JB, Germain GS, Morton CL, Kamath S, Buolamwini JK & Houghton PJ 2005 Identification of N10-substituted phenoxazines as potent and specific inhibitors of Akt signaling. *Journal of Biological Chemistry* **280** 31924–31935.

- Vicencio JM, Ibarra C, Estrada M, Chiong M, Soto D, Parra V, Diaz-Araya G, Jaimovich E & Lavandro S 2006 Testosterone induces an intracellular calcium increase by a nongenomic mechanism in cultured rat cardiac myocytes. *Endocrinology* **147** 1386–1395.
- Wang L & Proud CG 2002 Ras/Erk signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes. *Circulation Research* **91** 821–829.
- Wang X & Proud CG 2007 Methods for studying signal-dependent regulation of translation factor activity. *Methods in Enzymology* **431** 113–142.
- Willard FS, Berven LA & Crouch MF 2001 Lysophosphatidic acid activates the 70-kDa S6 kinase via the lipoxigenase pathway. *Biochemical and Biophysical Research Communications* **287** 607–613.

Received in final form 20 May 2009

Accepted 26 May 2009

Made available online as an Accepted Preprint

26 May 2009