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Stimulatory Effect of Insulin on Theca-Interstitial Cell Proliferation and Cell Cycle Regulatory Proteins through MTORC1 Dependent Pathway

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Abstract

The present study examined the effect of insulin-mediated activation of the mammalian target of rapamycin complex 1 (MTORC1) signaling network on the proliferation of primary culture of theca-interstitial (T-I) cells. Our results show that insulin treatment increased proliferation of the T-I cells through the MTORC1-dependent signaling pathway by increasing cell cycle regulatory proteins. Inhibition of ERK1/2 signaling caused partial reduction of insulin-induced phosphorylation of RPS6KB1 and RPS6 whereas inhibition of PI3-kinase signaling completely blocked the insulin response. Pharmacological inhibition of MTORC1 with rapamycin abrogated the insulin-induced phosphorylation of EIF4EBP1, RPS6KB1 and its downstream effector, RPS6. These results were further confirmed by demonstrating that knockdown of *Mtor* using siRNA reduced the insulin-stimulated MTORC1 signaling. Furthermore, insulin-stimulated T-I cell proliferation and the expression of cell cycle regulatory proteins CDK4, CCND3 and PCNA were also blocked by rapamycin. Taken together, the present studies show that insulin stimulates cell proliferation and cell cycle regulatory proteins in T-I cells via activation of the MTORC1 signaling pathway.

Keywords

Insulin; Theca-interstitial cells; MTORC1; RPS6KB1; Cell cycle regulatory proteins

1. INTRODUCTION

In the ovary, theca-interstitial (T-I) cells play a central role in androgen production. These androgens also provide substrates for estrogen synthesis in granulosa cells (Magoffin, 2002; Magoffin, 2005; Young and McNeilly, 2010). It is well documented that luteinizing hormone (LH) is a primary regulator of T-I cell function (Palaniappan and Menon, 2009; Wood and Strauss, 2002). Our recent studies show that LH-mediated activation of PI3-kinase/AKT/MTORC1 signaling enhances the expression of genes involved in T-I cell

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proliferation and androgen synthesis (Palaniappan and Menon, 2010; Palaniappan and Menon, 2012). Additionally, insulin, IGF1 and other growth factors are also key players in T-I cell growth, proliferation and function (Duleba et al., 1999a; Duleba et al., 1997; Duleba et al., 1999b; Kwintkiewicz et al., 2006; Spicer et al., 2008).

Insulin acts primarily through the insulin receptor, a receptor tyrosine-kinase, signaling through the PI3-kinase and mitogen activated protein kinase (MAPK) pathways (Diamanti-Kandarakis and Papavassiliou, 2006; Myers and White, 1993). PI3-kinase propagates intracellular signaling cascades regulating a wide range of cellular processes including cell growth and proliferation by activating downstream molecules such as AKT/PKB and MTORC1 (Fingar and Blenis, 2004; Ma and Blenis, 2009; Palaniappan and Menon, 2010; Zheng et al., 2012). A recent study suggests that RAS/MAPK signaling cascade also promotes MTORC1 signaling and cell growth (Carriere et al., 2011). MTOR exists as two different complexes within the cell, MTORC1 and MTORC2, but only MTORC1 is sensitive to inhibition by rapamycin (Guertin and Sabatini, 2007). MTORC1 is a master controller of protein synthesis, integrating signals from growth factors within the context of the energy and nutritional conditions of the cell. Activated MTORC1 regulates protein synthesis by directly phosphorylating EIF4EBP1 and RPS6KB1 (Fingar et al., 2004; Fingar et al., 2002; Manning and Cantley, 2003; Palaniappan and Menon, 2010). Since insulin has been known to regulate MTORC1 signaling in target cells (Li et al., 2010; Rapley et al., 2011) in the present study we examined whether the proliferative effect of insulin on T-I cells is mediated by activating this pathway. Our results show that insulin activates T-I cell proliferation and the expression of cell cycle regulatory components (CDK4, CCND3 and PCNA) by triggering the MTORC1-dependent pathway.

2. MATERIALS AND METHODS

2.1. Materials

Medium 199, McCoy's 5A medium, L-glutamine and HEPES buffer were purchased from Invitrogen/GIBCO (Carlsbad, CA). Penicillin–streptomycin was purchased from Roche Diagnostics (Indianapolis, IN). Collagenase (CLS I) and deoxyribonuclease I were obtained from Worthington Biochemical Corp. (Freehold, NJ). Bovine insulin, bovine serum albumin (BSA) and TUBB (β-tubulin) antibody were purchased from Sigma Chemical Co. (St. Louis, MO). MTORC1 inhibitor, rapamycin and antibodies against phosphorylated RPS6KB1 (Thr^{389}), phospho- RPS6KB1 (Thr^{421}/Ser^{424}), phospho-EIF4EBP1 ($Thr^{37/46}$), phosphorylated ribosomal protein S6 ($Ser^{235/236}$), total RPS6KB1, total RPS6, total EIF4EBP1, CCND3, CDK4 and the *Mtor* siRNA kit were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against Phospho ERK1/2, total ERK and PCNA were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was obtained from Chemicon (Temecula, CA). Anti-mouse, anti-rabbit IgG horseradish peroxidase conjugates, enhanced chemiluminescence kit, the Femto Supersignal Substrate System and Restore Western blot stripping buffer were purchased from Pierce (Rockford, IL). Reagents as well as the primers and probes for the cyclin D1 (*Ccnd1*) and cyclin D3 (*Ccnd1*) real-time PCR were from Applied Biosystems (Foster City, CA). All other reagents used were conventional commercial products.

2.2. Animals

Sprague-Dawley female rats (25 days old) were purchased from Charles River Laboratories (Wilmington, MA). All the experimental protocols used in this study were approved by the University Committee on the Use and Care of Animals. Animals were housed in a temperature-controlled room with proper dark-light cycles as per the guidelines provided by

the University Committee on the Use and Care of Animals. The animals were euthanized by CO_2 asphyxiation. The ovaries were removed under sterile conditions and were processed immediately for the isolation of T-I cells.

2.3. Isolation and culture of theca-interstitial cells

The T-I cells were isolated, dispersed and cultured following a protocol previously published from our laboratory (Palaniappan and Menon, 2009; Palaniappan and Menon, 2010). Briefly, freshly collected ovaries were placed in Medium 199 containing 25mM Hepes (pH 7.4), 2 mM L-glutamine, 1 mg/ml BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin. The ovaries were then freed from adhering fat and actively punctured with a 27-gauge needle under a dissecting microscope to release the granulosa and blood cells. The remaining ovarian tissue was then washed three times with medium to release any remaining granulosa cells. The tissue was then minced and incubated for 30 min at 37 ° C in the same medium, supplemented with 0.65 mg/ml collagenase type 1 plus 10 μg/ml deoxyribonuclease. The dispersion was encouraged by mechanically pipetting the ovarian tissue suspension with a 10 ml pipette. The theca-interstitial cells released by this digestion were centrifuged at 250g for 5 min and washed in medium two times to eliminate remaining collagenase. The dispersed cells were then resuspended in McCoy's 5A medium containing 2 mM L-glutamine, 1 mg/ml BSA, 100 U/ml penicillin and 100 μg/ml streptomycin and subjected to unit gravity sedimentation for 5 min to eliminate small fragments of undispersed ovarian tissue. Cell viability was assessed by trypan blue exclusion and averaged above 90%. The dispersed cells were seeded in 60 mm plates (3×10^6 viable cells). The plated cells were maintained overnight in McCoy's 5A medium containing 2 mM L-glutamine, 1 mg/ml BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air–5% CO_2 at 37°C. After allowing cells to attach, they were treated with insulin for different time intervals, and inhibitor was used as indicated in the figure legends. Insulin and inhibitor concentrations were selected on the basis on our previous studies (Palaniappan and Menon, 2009; Palaniappan and Menon, 2010; Rice et al., 2005; Will et al., 2012).

2.4. Cell viability, cell number and proliferation assay

T-I cells were seeded into 96-well plates and cultured overnight with McCoy's medium containing 0.1 % BSA. After attachment, cells were pretreated with rapamycin for 1h followed by insulin (1μg/ml) for 24 h. After the treatment periods, cell viability was determined by MTT assay as previously described (Mosmann, 1983). To test whether insulin increases T-I cell number, cells were seeded into 24 well plates and cultured overnight with McCoy's medium containing 0.1 % BSA. After allowing to attach, the cells were treated with insulin (1μg/ml) for 24h and 48h and cell number was determined by using Countless Automated Cell Counter (Invitrogen). For BrdU cell proliferation assay, cells were labeled with BrdU followed by insulin treatment for 24h. Cell proliferation was assayed by measuring the incorporation of BrdU using BrdU immunoassay kits (Calbiochem, La Jolla, CA) as previously described (Palaniappan and Menon, 2010).

2.5. Real-Time PCR

The role of MTORC1 in insulin -mediated *Ccnd1* and *Ccnd3* mRNA expression were examined by pretreating the cells with or without rapamycin (20 nM) for 1 h, followed by insulin for 4 h. At the end of incubation, the cells were harvested, and total RNA extracted using TRIzol reagent following the manufacturer's instructions. (Life Technologies). *Ccnd1* and *Ccnd3* mRNA expression were analyzed by real-time PCR as previously described (Palaniappan and Menon, 2010). The changes in *Ccnd1* and *Ccnd3* expression were calculated using the Ct method (Livak and Schmittgen, 2001) with 18S rRNA as the internal control.

2.6. Western blot analysis

After various treatments as described in the respective figure legends, cell monolayers were washed with PBS, and then solubilized using radioimmunoprecipitation assay (RIPA) buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). Cell lysates were then sonicated and centrifuged for 10 min at 13,000 X g. The protein content of the supernatants was determined using BCA reagent (Pierce). Proteins (30–50μg/lane) were separated by electrophoresis using 10% or 4–20% gradient SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad) before immunoblot analysis as previously described (Palaniappan and Menon, 2010). Protein loading was monitored by reprobing the same blots with appropriate antibodies as indicated in the figure legends.

2.7. siRNA-mediated silencing of mTOR

The protocol for siRNA-mediated knockdown of *Mtor* in T-I cells was previously published from our laboratory (Palaniappan and Menon, 2010). The control and *Mtor* siRNA sequences are as follows: control sense siRNA 5′CGUACGCGGAAUACUUCGA 3′; antisense 5′UCGAAGUAUUCCGCGUACG 3′ and *Mtor* sense siRNA 5′ UGAACCCUGCCUUUGUCAUGC 3′; antisense 5′GCAUGACAAAGGCAGGGUUCA 3′. Briefly, T-I cells were transfected with control siRNA (non-targeted) or *Mtor* siRNA (targeted) using a Nucleofector transfection reagent (Amaxa), as per the manufacturer's instructions. After transfection, cells were resuspended in 5% FBS/McCoy's medium and plated. Forty-eight hours later media was replaced with serum free medium for overnight culture and then treated without or with insulin for an additional 30 min. MTOR, phospho-specific RPS6KB1 Thr^{389}, RPS6KB1 Thr^{421}/Ser^{424}, RPS6 $Ser^{235/236}$ and EIF4EBP1 $Thr^{37/46}$ were examined by Western blot analysis using specific antibodies.

2.8. Statistical analysis

Statistical analysis was carried out using one-way ANOVA followed by the Tukey multiple comparison test using Prism software (GraphPad Prism, version 3.0; GraphPad Inc., San Diego, CA). Values were considered statistically significant at $P < 0.05$. Each experiment was repeated at least three times, with similar results. Blots are representative of one experiment, and graphs represent the mean ± SE of three replicates.

3. RESULTS

3.1. Insulin-induced T-I cell proliferation is rapamycin sensitive

The initial experiments examined toxicity of rapamycin treatment in cultured T-I cells using cell viability assay. To test this, cultured T-I cells were preincubated with or without rapamycin (20 nM) for 1h followed by treatment with insulin for 24 h. Cell viability was analyzed by MTT assay. The results presented in Figure 1A show that repamycin treatment at 20 nM concentration did not reduce cell viability compared with control or insulin treatment group. To determine, whether inhibition of MTORC1 signaling reduces insulin-induced T-I cell proliferation, cells were treated with rapamycin for 1 h followed by insulin for 24 h in the presence of BrdU. The results indicate that insulin treatment augmented BrdU incorporation, and this stimulatory effect was abolished by the addition of the MTORC1 inhibitor, rapamycin (Fig. 1B). To provide further evidence to show that insulin increases T-I cell number, cells were treated with insulin for 24 h and 48 h and cell number was assessed. As shown in Figure 1C, insulin treatment significantly increased T-I cell number at 48 h. Collectively, these results suggest that insulin-stimulated T-I cell proliferation occurs through the MTORC1-dependent signaling pathway.

3.2. Insulin stimulates activation of RPS6KB1, RPS6 and EIF4EBP1

MTORC1 is a master regulator of cell growth and proliferation that is deregulated in pathological conditions. Because insulin is known to increase T- I cell proliferation, we explored whether insulin would alter RPS6KB1 and EIF4EBP1 phosphorylation in cultured T-I cells, since RPS6KB1 and EIF4EBP1 are downstream targets of MTORC1 signaling. To test this, T-I cells were cultured with insulin for different time intervals, the phosphorylation of RPS6KB1, and its target RPS6 and EIF4EBP1 were examined by Western blot analysis using phospho-specific antibodies. The results (Fig. 2A) show that within five minutes of insulin treatment, RPS6KB1 was phosphorylated at Thr^{389} reaching maximal phosphorylation at 15 min (Fig. 2B) whereas RPS6 ($Ser^{235/236}$) and EIF4EBP1($Thr^{37/46}$) phosphorylation increased by 15 min and was further augmented at 30 min. At 60 min, the extent of RPS6KB1 and EIF4EBP1 phosphorylation was lower than that seen at 30 min (Fig. 2C and D). These results show that MTORC1 signaling is responsive to insulin in T-I cells.

3.3. Insulin stimulated RPS6KB1, RPS6 and EIF4EBP1 phosphorylation is sensitive to rapamycin

We then tested the effect of MTORC1 inhibitor, rapamycin, to further confirm that phosphorylation of RPS6KB1, RPS6 and EIF4EBP1 occurs downstream of MTORC1 activation in response to insulin. T-I cells were pretreated with rapamycin for 1 h followed by insulin treatment for 30 min. Immunoblot analysis was performed using phospho-specific RPS6KB1 Thr^{389}, RPS6KB1 Thr^{421}/Ser^{424}, RPS6 $Ser^{235/236}$ and EIF4EBP1 $Thr^{37/46}$ antibodies. The results (Fig. 3A) clearly show that insulin-induced phosphorylation of RPS6KB1 (Fig. 3B and C), RPS6 (Fig. 3D) and EIF4EBP1 (Fig. 3E) was prevented by treatment with rapamycin.

3.4. siRNA-mediated silencing of *Mtor* and its effect on RPS6KB1, RPS6 and EIF4EBP1 phosphorylation in response to insulin

To provide further evidence of MTORC1-mediated phosphorylation of RPS6KB1, RPS6 and EIF4EBP1, T-I cells were transfected with *Mtor* siRNA to knockdown *Mtor* expression using the protocol that we recently reported (Palaniappan and Menon, 2010). Cells were transfected with control siRNA or *Mtor* siRNA for 48 h followed by insulin treatment for 30 min. Cell lysates were analyzed for MTOR and phospho form of RPS6KB1 Thr^{389}, RPS6KB1 Thr^{421}/Ser^{424}, RPS6 $Ser^{235/236}$ and EIF4EBP1 $Thr^{37/46}$ by Western blot analysis. The results (Fig. 4A) show that as expected, MTOR protein expression was blocked by the siRNA targeting *Mtor*, compared to non-targeting siRNA (Fig. 4B). Furthermore, the knockdown of *Mtor* by *Mtor* siRNA reduced insulin- induced phosphorylation of RPS6KB1 Thr^{389}, RPS6KB1 Thr^{421}/Ser^{424}, RPS6 $Ser^{235/236}$ and EIF4EBP1 $Thr^{37/46}$ (Fig. 4C–F). These results conclusively show that insulin-mediated activation of MTORC1 is required for RPS6KB1 Thr^{389}, RPS6KB1 Thr^{421}/Ser^{424}, RPS6 $Ser^{235/236}$ and EIF4EBP1 $Thr^{37/46}$ phosphorylation.

3.5. Insulin stimulates RPS6KB1 and RPS6 phosphorylation through PI3-kinase dependent pathway

Initial experiments examined whether insulin activates ERK1/2 phosphorylation in T-I cells. To test this, cells were cultured with insulin for different time intervals, and the phosphorylation of ERK1/2 was examined by immunoblot analysis. The results show that within 5 min of insulin addition ERK1/2 is robustly phosphorylated (Fig 5A and B). We have previously shown that insulin increases AKT phosphorylation at Ser 473. Furthermore, pretreatment with PI3-kinase inhibitor, Wortmannin completely blocked insulin-induced phosphorylation of AKT (Palaniappan and Menon, 2009).

Because insulin is known to regulate both PI3-kinase/AKT pathway and ERK1/2 pathway, inhibitors were employed to determine the signaling pathway involved in insulin-induced phosphorylation of RPS6KB1 and its downstream effectors, RPS6. To test this, T-I cells were preincubated with PI3-kinase inhibitor, Wortmannin or MEK inhibitor for 30 min and 1 h, respectively followed by stimulation with insulin for 30 min. The cell lysates were then assayed for the phosphorylation of RPS6KB1 (Thr 389) and RPS6 (Ser 253/236) by immunoblot analysis. As expected, insulin treatment increased phosphorylation of RPS6KB1 and RPS6, whereas pretreatment with MEK inhibitor caused partial inhibition of RPS6KB1 and RPS6 phosphorylation. By contrast, pretreatment with Wortmannin produced complete inhibition of RPS6KB1 and RPS6 phosphorylation in response to insulin (Fig. 5C–E). Taken together, these results suggest that insulin-mediated MTORC1 activation is primarily regulated by PI3-kinase dependent signaling pathways in T-I cells.

3.6. Insulin stimulates cell cycle regulatory proteins through the MTORC1-dependent pathway

Cell cycle progression from G1 to the S phase is regulated by D-type cyclins, which modulate the activities of the cyclin-dependent kinases, a process sensitive to MTORC1 inhibitor, rapamycin (Fingar et al., 2004; Fingar et al., 2002). To determine the effect of rapamycin on *Ccnd1*and *Ccnd3* mRNA expression, T-I cells were preincubated with rapamycin (20 nM) for 1 h followed by the addition of insulin for 4 h. Total RNA was extracted and the mRNA levels of *Ccnd1*and *Ccnd3* were determined by real-time PCR. The results indicate that insulin -stimulated *Ccnd1*and *Ccnd3* mRNA expression was blocked by MTORC1 inhibitor, rapamycin (Fig. 6A and B). The effect of rapamycin on insulin-induced cell cycle regulatory protein expression was then examined. Cells were preincubated with the MTORC1 inhibitor, rapamycin for 1 h, followed by insulin treatment for 24 h. The cell lysates were examined for the cell cycle regulatory proteins such as CDK4, CCND3, and PCNA by Western blot analysis. The results showed that while insulin treatment alone caused significant increases in the expression of the cell cycle regulatory proteins, the addition of the MTORC1 inhibitor, rapamycin, strongly prevented these increases, suggesting that insulin-induced MTORC1 signal is necessary for CDK4, CCND3 and PCNA protein expression (Fig. 6 C–F).

4. DISCUSSION

Present study provides new insights into how insulin-mediated activation of MTORC1 signal regulates T-I cell proliferation. We show that insulin activates the downstream targets of MTORC1, RPS6KB1 and EIF4EBP1. Furthermore, inhibition of MTORC1 activation by rapamycin or siRNA-mediated knockdown of *Mtor* abolished the insulin-induced phosphorylation of RPS6KB1 and its target RPS6, and EIF4EBP1 phosphorylation. In addition, insulin-stimulated T-I cell proliferation and expression of cell cycle regulatory proteins were prevented by the MTORC1 inhibitor, rapamycin.

The serine/threonine kinase MTORC1 plays a key role in regulating cellular processes including transcription, protein synthesis, cell growth and proliferation (Ma and Blenis, 2009; Palaniappan and Menon, 2010). It is well established that insulin regulates MTORC1 signaling through TSC1-TSC2 protein complex (Inoki et al., 2005). This protein complex normally acts as a negative regulator of MTORC1 signaling. In response to mitogenic signals, the TSC2 protein complex is inactivated by phosphorylation at Thr 1462 which leads to activation of the MTORC1 signaling cascade (Hou et al., 2010; Inoki et al., 2005). Recently, we and others have also demonstrated that gonadotropin inhibits TSC2, leading to activation of MTORC1 signaling and augmenting the phosphorylation of downstream targets, RPS6KB1 and EIF4EBP1 (Alam et al., 2004; Kayampilly and Menon, 2007; Palaniappan and Menon, 2010). In the present study, we show that treatment with insulin

induces phosphorylation of RPS6KB1 and EIF4EBP1. Furthermore, insulin-stimulated phosphorylation of RPS6KB1 and EIF4EBP1 is abrogated by MTORC1 inhibitor, rapamycin, or by siRNA-mediated knockdown of *Mtor*, suggesting that insulin-mediated activation of MTORC1 signal is essential for T-I cell proliferation. Studies using molecular approaches have identified RPS6KB1 as an important molecule for cell proliferation and growth (Chou and Blenis, 1995; Lane et al., 1993; Price et al., 1992; Reinhard et al., 1994; Shima et al., 1998). Furthermore, RPS6KB1 is a key regulator of mRNA translation and plays a central role in cell cycle progression through the G1 phase of proliferating cells (Bandi et al., 1993; Chou and Blenis, 1995; Ruvinsky and Meyuhas, 2006; Tee et al., 2005; Wullschleger et al., 2006). Since RPS6 is a substrate for RPS6KB1, its phosphorylation is also correlated with protein synthesis. In the present study, rapamycin treatment blocks insulin-induced phosphorylation of RPS6KB1 and RPS6 and cell cycle regulatory protein expression, suggesting that activation of MTORC1 is required for cell cycle regulatory protein expression. Therefore, based on these findings, we suggest that the increase in theca cell number seen under pathological conditions such as PCOS may be due to hyperactivation of mTORCI signaling pathway by insulin and LH. This is further supported by our recent finding that LH/hCG induces theca cell proliferation and cell cycle regulatory proteins by cAMP-mediated activation of PI3-kinase/AKT/MTORC1 pathways (Palaniappan and Menon, 2010).

Recently, it has been reported that prenatal exposure to excess androgen alters the intraovarian insulin signaling (Ortega et al., 2010). In addition to insulin, intraovarian growth factors such as IGF-1 have been known to regulate theca cell growth and proliferation (Duleba et al., 1997; Kwintkiewicz and Giudice, 2009). It is important to mention that a high level of insulin has been shown to activate IGF-1 receptors. Indeed, insulin and IGF-1 have common intracellular signal transduction pathways (PI3-kinase and MAPK) after ligand-induced receptor activation (Gallagher and LeRoith, 2010). Because of this reason, the insulin concentration used in the present study may also activate IGF-1 receptor. Recent studies have shown that IGF-1 also activates MTORC1 signaling in various cell types (Arvisais et al., 2010; Ning and Clemmons, 2010; Wahdan-Alaswad et al., 2010). MTORC1 activation promotes cellular anabolic processes such as protein and lipid synthesis, cell growth, and cell cycle progression, driving cell proliferation via its downstream targets RPS6KB1 and EIF4EBP1 (Dowling et al., 2010; Ekim et al., 2011; Fingar et al., 2004). In this study, we clearly showed the MTORC1-dependent phosphorylation of RPS6KB1 and EIF4EBP1 in response to insulin. Inactivation of MTORC1 by a pharmacological inhibitor or by siRNA-mediated knockdown of MTORC1 suppresses the insulin-induced phosphorylation of RPS6KB1 and EIF4EBP1, leading to inhibition of T-I cell proliferation and cell cycle regulatory protein expression. Therefore, it is suggested that activation of MTORC1 regulates T-I cell proliferation at least in part by up regulating the cell cycle machinery.

In summary, the findings reported here support the notion that insulin-mediated activation of MTORC1 signal controls T-I cell proliferation by increasing the expression of cell cycle regulatory proteins. Furthermore, it is suggested that in hyperthecosis seen in pathological conditions, activation of MTORC1 signaling by insulin might be responsible for the proliferation of T-I cells.

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Abbreviations

BrdU	bromodeoxyuridine
CCND3	Cyclin D3
CDK	cyclin-dependent kinase
EIF4EBP1	eukaryotic initiation factor 4E binding protein 1
ERK	extracellular signal-regulated kinase
IGF-1	Insulin like growth factor 1
MAP kinase	mitogen-activated protein kinase
MEK	MAPK kinase
MTOR	mammalian target of rapamycin
PCNA	proliferating cell nuclear antigen
PI3 kinase	phosphatidylinositol -3-kinase
RPS6	ribosomal protein S6
RPS6KB1	ribosomal protein S6 kinase 1
siRNA	small interfering RNA

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Research Highlights

- Insulin activates the MTORC1 signaling cascade in T-I cells.
- Pharmacological inhibitor or siRNA-mediated knockdown of *Mtor* prevents insulin- induced RPS6KB1 and EIF4EBP1, phosphorylation.
- Rapamycin blocks insulin-stimulated T-I cell proliferation and the expression of cell cycle regulatory proteins.

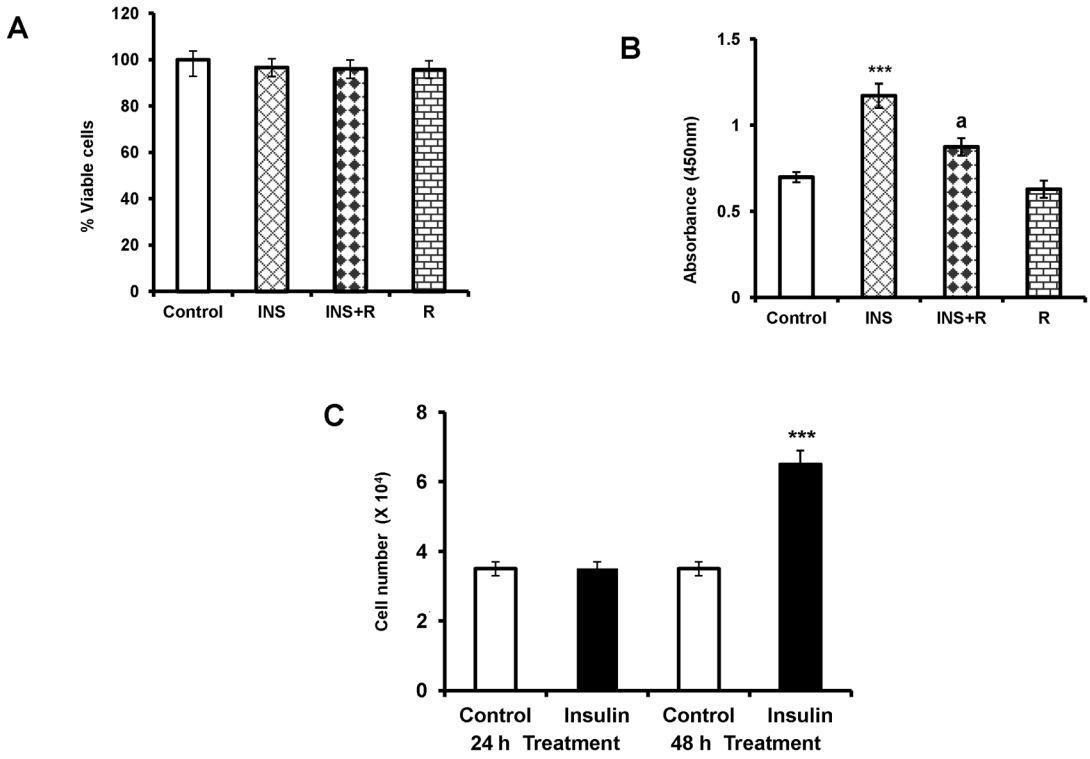

FIG. 1. Effect of MTORC1 inhibition on insulin-stimulated cell proliferation

(A), Ovaries were collected from 25 day old Sprague-Dawley rats. T-I cells were isolated by collagenase digestion and cells were plated with McCoy's medium. After 24 h attachment, cells were pretreated without or with rapamycin (20 nM) for 1 h prior to treatment for 24 h with insulin (1μg/ml). Control groups received vehicle (DMSO). The viability of the cells was assessed by MTT assay. Results are expressed as the percentage of viable cells compared with control. (B), Cells were incubated with MTORC1 inhibitor (rapamycin, 20 nM) for 1 h followed by insulin (1μg/ml) treatment for 24 h. Cells were labeled with BrdU and cell proliferation was assessed by BrdU incorporation as described in *Materials and Methods*. (C), Cells were treated with insulin (1μg/ml) for 24 h and 48 h and cell number was quantified by Cell Counter as described in *Materials and Methods*. All experiments were carried out three times with triplicates in each experiment. Error bars represent mean ± SE. ***, $P < 0.001$ *vs.* control. **a,** Significant differences ($P < 0.05$) compared with insulin treatment. INS= insulin; R= rapamycin

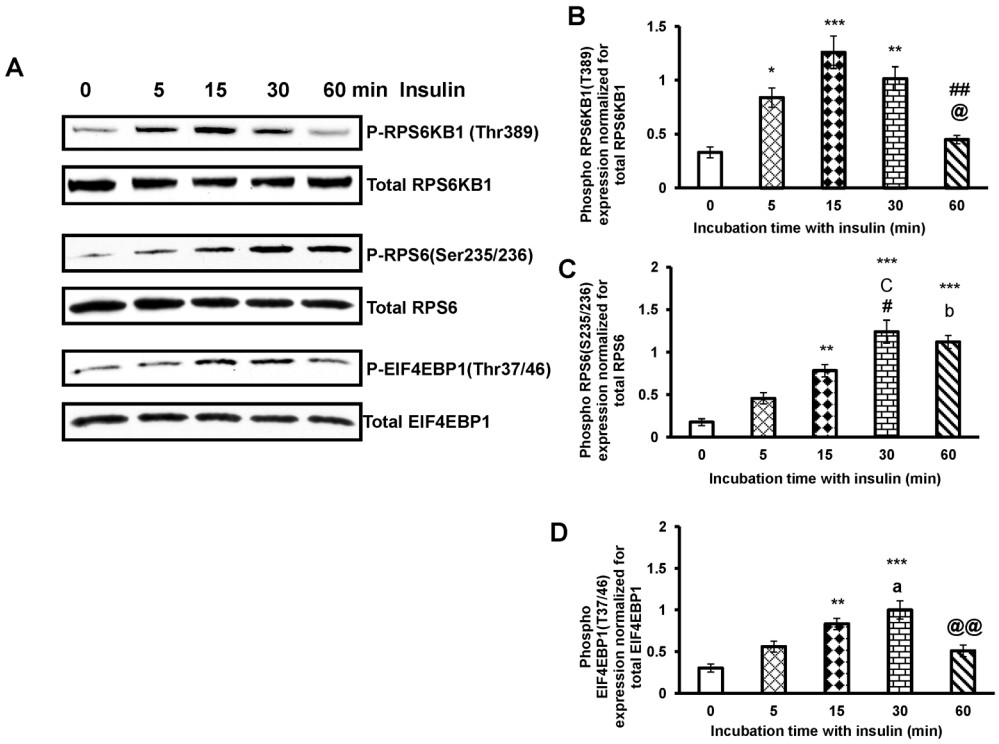

FIG. 2. Time course study of insulin effect on phosphorylation of RPS6KB1, RPS6 and EIF4EBP1

Cells were treated with insulin (1μg/ml) for different time periods. Cells were lysed using RIPA buffer and subjected to Western blot analysis using phospho-specific antibodies for RPS6KB1(Thr^{389}), RPS6($Ser^{235/236}$) and EIF4EBP1($Thr^{37/46}$). Protein loading was monitored by stripping and reprobing the same blot with RPS6KB1, RPS6 and EIF4EBP1 antibodies (A). The graphs (B, C and D) represent densitrometric scans of phospho RPS6KB1(Thr^{389}), phospho RPS6 ($Ser^{235/236}$) and phospho EIF4EBP1($Thr^{37/46}$) protein expression normalized for total RPS6KB1, RPS6 and EIF4EBP1, respectively. Blots are representative of one experiment, and the graphs represent the mean of three experiments. Error bars represent mean ± SE. *, $P < 0.05$; **, $P < 0.01$; and ***, $P< 0.001$ vs. 0 min insulin treatment. a, b and c represent significant differences compared with 5min insulin treatment (a, $P < 0.05$; b, $P < 0.01$ and c, $P < 0.001$, respectively). # and ## represent significant differences compared with 15min insulin treatment (#, $P <0.05$ and ##, $P < 0.01$, respectively). @ and @@ represent significant differences compared with 30 min insulin treatment (@, $P < 0.05$ and @@, $P < 0.01$, respectively).

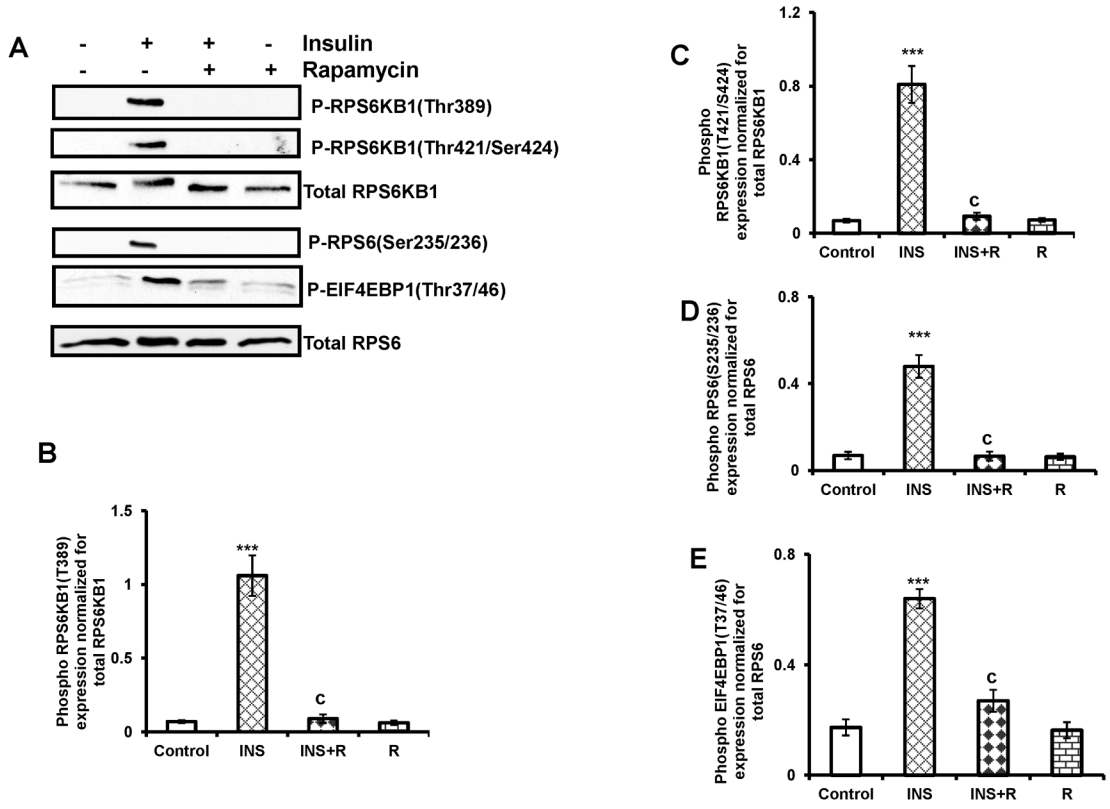

FIG. 3. Effect of rapamycin on insulin-stimulated phosphorylation of RPS6KB1, RPS6 and EIF4EBP1

T-I cells were pretreated without or with rapamycin (20 nM) for 1 h prior to treatment for 30 min with insulin (1μg/ml). Control groups were treated with vehicle (DMSO). Cells were lysed and subjected to Western blot analysis using phosphorylation site-specific antibodies for RPS6KB1(Thr^{389}), RPS6KB1(Thr^{421}/Ser^{424}), RPS6 (Ser$^{235/236}$) and EIF4EBP1(Thr$^{37/46}$). The levels of RPS6KB1 or RPS6 protein were used as internal controls. Blots are representative of one experiment, and the graphs represent the mean of three experiments. Error bars represent mean ± SE. ***, P< 0.001 vs. control. c represent significant differences compared with insulin (c, P < 0.001). INS= insulin; R= rapamycin

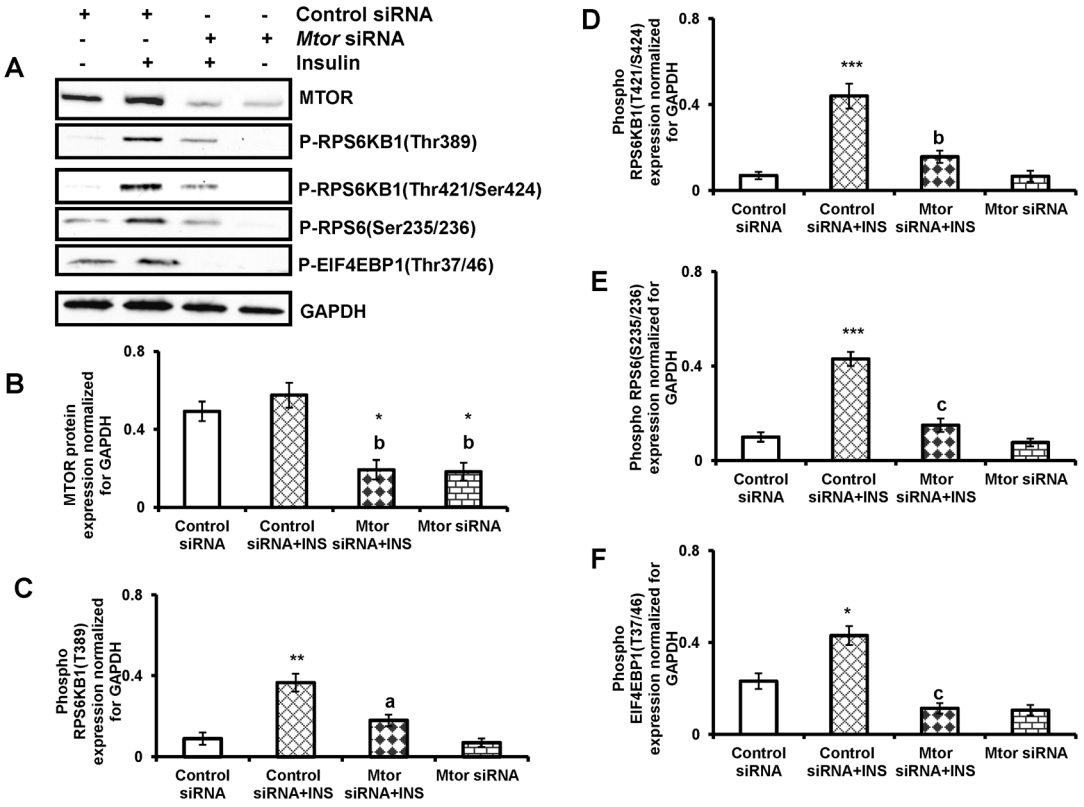

FIG. 4. *Mtor* siRNA inhibits insulin-induced phosphorylation of RPS6KB1, RPS6 and EIF4EBP1

T-I cells were transfected with 100 nM of control siRNA or *Mtor* siRNA for 48 h and then treated without or with insulin (1μg/ml) for 30 min. Proteins (30 μg) were separated by SDS-PAGE (4–20%), and then immunoblotted with MTOR, phospho-specific RPS6KB1 (Thr^{389}), RPS6KB1 (Thr^{421}/Ser^{424}), RPS6 ($Ser^{235/236}$) and EIF4EBP1($Thr^{37/46}$) antibodies. Protein loading was monitored by stripping and reprobing the same blot with antibody for GAPDH. The graphs (B, C, D and E) represent densitrometric scans of MTOR, phospho RPS6KB1(Thr^{389}), phospho RPS6 ($Ser^{235/236}$) and phospho EIF4EBP1($Thr^{37/46}$) protein expression normalized for GAPDH, respectively. Blots are representative of one experiment, and the graphs represent the mean of three experiments. Error bars represent mean ± SE. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ vs. control siRNA. a, b and c represent significant differences compared with control siRNA plus insulin treatment (a, $P < 0.05$; b, $P < 0.01$ and c, $P < 0.001$, respectively). INS= insulin

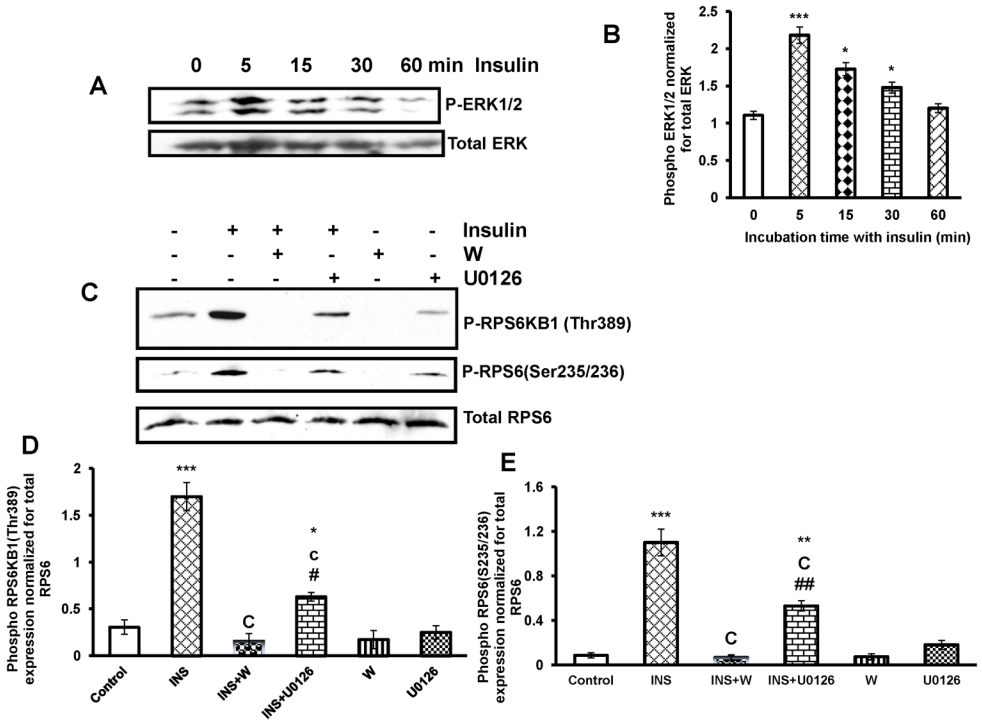

FIG. 5. Effect of PI3-kinase and MEK1 inhibition on insulin-stimulated phosphorylation of RPS6KB1 and RPS6

Cells were pretreated without or with the PI3-kinase inhibitor, Wortmannin (100 nM) for 30 min or the MEK inhibitor, U0126 (10μM) for 1 h, followed by insulin (1μg/ml) treatment for 30 min. Control groups were treated with vehicle (DMSO). The cell lysates were examined for phospho-specific RPS6KB1(Thr^{389}) and RPS6 (Ser235/236) by Western blot. Protein loading was monitored by stripping and reprobing the same blot with RPS6 antibody. The blot is representative of one experiment, and the graph represents the mean of three experiments. Error bars represent mean ± SE. *, $P < 0.05$; P **, $P < 0.01$; and ***, $P < 0.001$ vs. control. b and c represent significant differences compared with insulin (b, $P < 0.01$ and c, $P < 0.001$ respectively). # and ## represent significant differences compared with INS + W (#, $P < 0.05$ and ##, $P < 0.01$, respectively). INS= insulin; W= Wortmannin

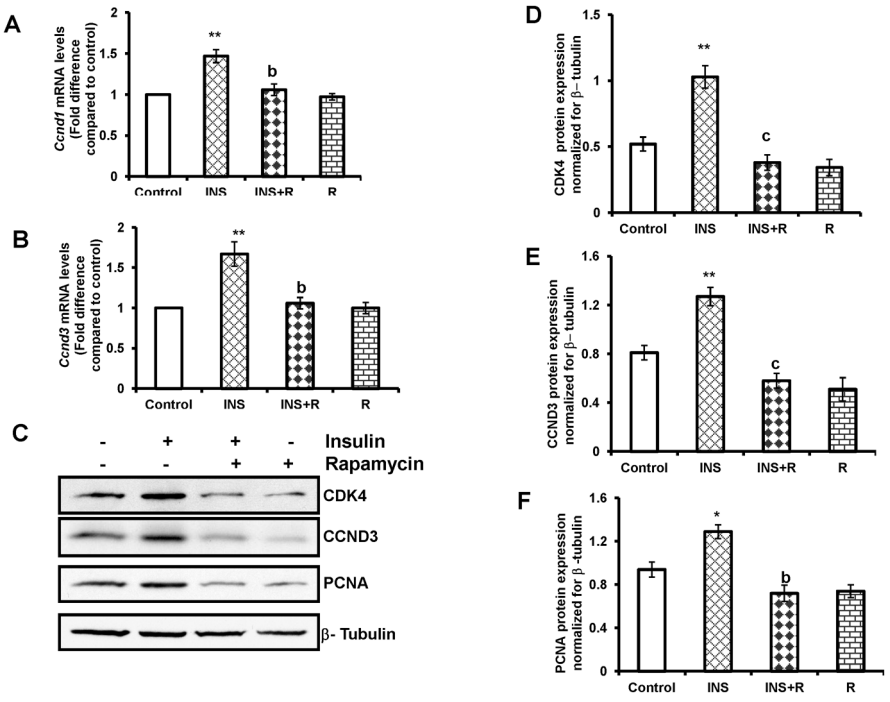

FIG. 6. Effect of rapamycin on insulin-stimulated cell cycle regulatory protein expression

Cells were pretreated without or with rapamycin (20 nM) for 1 h followed by insulin (1 μg/ml) treatment for 4 h (*Ccnd1*and *Ccnd3* mRNA expression) or 24 h (CDK4, CCND3 and PCNA protein expression). Control groups were treated with vehicle (DMSO). (A) and (B), Total RNA was reverse-transcribed and the resulting cDNA was subjected to real–time PCR using predesigned primers and probes for rat *Ccnd1*and *Ccnd3* as described in *Materials and Methods*. The graph (A) shows changes in *Ccnd1* mRNA expression normalized for 18S rRNA. The graph (B) shows changes in *Ccnd3* mRNA expression normalized for 18S rRNA. (C) The cell lysates were examined for CDK4, CCND3 and PCNA protein expression by Western blot analysis. The level of β-tubulin was used as loading control. The graphs (D, E and F) represent densitrometric scans of CDK4, CCND3 and PCNA protein expression, respectively, normalized for β-tubulin. Blots are representative of one experiment, and the graphs represent the mean of three experiments. Error bars represent mean + SE. *, $P < 0.05$ and **, $P < 0.01$ vs. control. b and c represent significant differences compared with insulin treatment (b, $P < 0.01$ and c, $P < 0.001$, respectively). INS= insulin; R= rapamycin