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mTOR Complex 2 mediates Akt Phosphorylation that Requires PKC ϵ in Adult Cardiac Muscle Cells

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Abstract

Our earlier work showed that mammalian target of rapamycin (mTOR) is essential to the development of various hypertrophic responses, including cardiomyocyte survival. mTOR forms two independent complexes, mTORC1 and mTORC2, by associating with common and distinct cellular proteins. Both complexes are sensitive to a pharmacological inhibitor, torin1, although only mTORC1 is inhibited by rapamycin. Since mTORC2 is known to mediate the activation of a prosurvival kinase, Akt, we analyzed whether mTORC2 directly mediates Akt activation or whether it requires the participation of another prosurvival kinase, PKC ϵ (epsilon isoform of protein kinase-C). Our studies reveal that treatment of adult feline cardiomyocytes *in vitro* with insulin results in Akt phosphorylation at S473 for its activation which could be augmented with rapamycin but blocked by torin1. Silencing the expression of Rictor (rapamycin-insensitive companion of mTOR), an mTORC2 component, with a sh-RNA in cardiomyocytes lowers both insulin-stimulated Akt and PKC ϵ phosphorylation. Furthermore, phosphorylation of PKC ϵ and Akt at the critical S729 and S473 sites respectively was blocked by torin1 or Rictor knockdown but not by rapamycin, indicating that the phosphorylation at these specific sites occurs downstream of mTORC2. Additionally, expression of DN-PKC ϵ significantly lowered the insulin-stimulated Akt S473 phosphorylation, indicating an upstream role for PKC ϵ in the Akt activation. Biochemical analyses also revealed that PKC ϵ was part of Rictor but not Raptor (a binding partner and component of mTORC1). Together, these studies demonstrate that mTORC2 mediates prosurvival signaling in adult cardiomyocytes where PKC ϵ functions downstream of mTORC2 leading to Akt activation.

Keywords

Cardiomyocytes; PKC ϵ ; mTOR; Akt; S6K1; Rapamycin; Torin1

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Disclosures

None.

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1. Introduction

Subsequent to an increased hemodynamic overload in the form of either pressure or volume, the heart undergoes hypertrophic myocyte growth. Although this process is initially compensatory by normalizing the wall stress, the compensatory growth process can transition into a decompensatory mechanism, depending upon the nature and severity of the increased overload. For example, during an increased pathological load such as pressure overload (PO), cardiac hypertrophy initially proceeds as a compensatory mechanism, but when the PO is severe and sustained, the compensatory mechanism is lost leading to congestive heart failure (CHF). Therefore, characterizing key cardioprotective pathways triggered initially as part of the compensatory mechanism is important to efforts at sustaining these mechanisms and thus hopefully preventing the development of CHF.

mTOR is a serine/threonine kinase that links external stimuli to cell growth processes. A major characteristic of mTOR is its specific inhibition by rapamycin-FKBP12 complex. As a molecular sensor of cell energy and nutrition status, mTOR serves as a key signaling intermediate to regulate cell growth, cell size, protein synthesis, cytoskeleton dynamics, energy sensing and protein degradation. mTOR forms two independent signaling complexes (for reviews, [1–3], mTORC1 and mTORC2, consisting of Raptor and Rictor, respectively. mTORC1 primarily controls cell growth and size through p70 S6 kinase (S6K1), 4E-binding protein (4EBP) and protein phosphatase-2A (PP2A) [4], whereas mTORC2 is known to regulate Akt activation as part of cell survival [2, 5] and the actin cytoskeleton [6, 7]. Therefore, changes in the activation of one or both of these mTOR-mediated processes can contribute to the development of CHF.

Akt is a well-characterized effector molecule of mTORC2 [8] that functions as a progrowth and cell survival kinase [9]. Of the three isoforms (Akt1, Akt2 and Akt3) present in mammalian cells, Akt1 is widely expressed in several tissues including the heart [10, 11]. Akt undergoes activation subsequent to the generation of PIP3 by PI3K that recruits Akt to the plasma membrane [12]. At the membrane, Akt is phosphorylated at two critical sites, T308 and S473 predominantly by PDK1 and mTORC2 [2], although recent studies demonstrate that PAK1 [13] and ILK [14] also serve as upstream kinases for S473 phosphorylation. Several studies demonstrate that Akt is sufficient to block cell death induced by a variety of apoptotic stimuli [15, 16]. In the heart, transgenic mice overexpressing Akt exhibited increased cardiomyocyte size and contractility *in vivo* [17–19], and several studies link Akt activation in the heart to physiological hypertrophy [18, 20–22]. Furthermore, PO of Akt1-deficient mice via TAC results in exaggerated pathological hypertrophy and fibrosis [20, 23]. Other independent studies [24, 25] demonstrate that the nuclear localization of Akt antagonizes certain features of cardiac hypertrophy and improves systolic function in TAC-induced hypertrophy in mice. It is suggested that acute activation of Akt especially in the nucleus may be beneficial to the heart [26].

Several studies including ours [27–31] have characterized the activation of the rapamycin-sensitive mTOR (mTORC1) pathway in promoting protein translation in hypertrophying cardiomyocytes. However, the possible protective role of mTORC2 through Akt activation, which is widely studied in cancer, has yet to be explored in hypertrophying heart. Our recent studies show that treatment of rapamycin either *in vivo* during PO or *in vitro* during agonist stimulation of isolated adult cardiomyocytes results in an augmented activation of Akt [32], indicating the possibility that blocking one arm of mTOR, namely mTORC1, could promote its other arm, namely mTORC2. Therefore, understanding how Akt activation occurs in cardiomyocytes during hypertrophic stimulation and how rapamycin augments such

activation are important to efforts at sustaining the cardioprotective effect during pathological load such as PO.

In addition to Akt, the epsilon isoform of protein kinase-C (PKC ϵ) has been shown to play a prosurvival role in multiple cell types [33] including cardiomyocytes [34]. The cardioprotective effect of PKC ϵ has been shown in the normal heart [35], hypertrophying heart [36], and preconditioned heart [34], and nonreceptor tyrosine kinases have been shown to play a role in these effects [37]. Although our earlier work shows the importance of PKC ϵ in promoting mTORC1-mediated S6K1 activation [38], its precise role in mTORC2-mediated Akt activation is unclear.

In the present study, we show that the agonist-stimulated activation of Akt and its further augmentation by rapamycin treatment are mediated by mTORC2 in adult cardiomyocytes. Although PKC ϵ has been shown to regulate Akt expression [33] it is not known if it contributes to Akt activation during agonist stimulation of cardiomyocytes. By performing gene silencing experiments, we show that mTORC2-mediate PKC ϵ and Akt activation where PKC ϵ functions upstream of Akt, suggesting mTORC2/PKC ϵ /Akt module plays a cardioprotective role by promoting prosurvival signaling in cardiomyocytes.

2. Materials and Methods

2.1. Reagents

The following chemicals were obtained—Insulin (Sigma, St. Louis, MO), rapamycin (LC laboratory, Woburn, MA), wortmannin (Calbiochem, San Diego, CA). Torin1 was obtained from Dr. N. S. Grey (currently available as torin1 at TOCRIS Bioscience, Bristol, UK). All other chemicals were obtained from Sigma, St. Louis, MO.

2.2. Antibodies

Antibodies were obtained from the following companies—anti- mTOR, pS2448-mTOR, pS2481-mTOR, Akt, pS473-Akt, S6K1, pT389-S6K1, pS1135-Rictor, IRS-1 and pS636/639-IRS-1 (Cell Signaling, Beverly, MA), PKC ϵ , pS729-PKC ϵ , (Santa Cruz Biotechnology, Santa Cruz, CA) Raptor and Rictor (Bethyl Labs, Montgomery, TX), mTOR (BD Biosciences, San Jose, CA), GAPDH (Fitzgerald, Concord, MA), and horseradish peroxidase-labeled secondary antibodies (Promega, Madison, WI). Primary antibodies were used at a 1:1000 dilution for immunoblotting.

2.3. Adenoviruses

Generation of β -gal and PKC ϵ adenoviruses have been described previously [39]. Adenovirus for the expression of Rictor-shRNA (Ad-eGFP-rictor-shRNA) was purchased from Vector Biolabs (Philadelphia, PA).

2.4. Adult cardiomyocyte culture model

Adult ventricular feline cardiomyocytes regularly isolated in our core facilities via a hanging heart preparation using enzymatic digestion [40]. Isolated cardiomyocytes were suspended in a 1.8 mM calcium containing mitogen-free M-199 medium at pH 7.4. Cardiomyocytes were plated on laminin-coated tissue culture plates at a density of (7.5×10^4 cells/ml media) and incubated at 37°C in humidified air with 5% CO₂. After allowing 4 h for attachment, media was changed with serum-free Medium 199 (M199, GIBCO-BRL, Inc., Grand Island, NY) containing 200 units/ml penicillin and 200 μ g/ml streptomycin (GIBCO-BRL). Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington, DC, 1996) and

were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

2.5. Stimulation of Akt in cultured cardiomyocytes

Freshly isolated adult feline cardiomyocytes were cultured overnight and stimulated with 100 nM insulin in the presence or absence of various pharmacologic inhibitors. Untreated cells served as controls. For treatment with pharmacologic inhibitors, cells were pre-incubated for 30 min or 1 h and then stimulated for various time periods as indicated in the figure legends. For adenoviral expression, freshly isolated cardiomyocytes were plated on laminin-coated trays and incubated for 4 h prior to infection. Cells were then incubated overnight in serum-free M-199 media containing the adenovirus at MOI (multiplicity of infection) levels described in the figure legends. Cells infected with an equal MOI of β -galactosidase (β gal) adenovirus served as control. The media was replaced after 24 h and allowed to incubate for an additional 24 h prior to agonist stimulation.

2.6. Western blotting

Triton X-100 soluble fraction of cardiomyocytes was prepared following extraction with Triton X-100 lysis buffer as previously established [41, 42]. Briefly, detergent soluble proteins were prepared by extracting cell samples with Triton X-100 buffer containing protease and phosphatase inhibitors (Sigma protease inhibitor cocktail and phosphatase inhibitor cocktails I and II) and centrifuging at $14,000 \times g$ for 15 min. An equal volume of SDS-sample buffer was then added to the supernatant and boiled for 5 min. The protein concentration was determined using BCA reagent (Pierce) and adjusted for comparison. Approximately 10 μ g of protein from each sample was resolved by SDS-PAGE and transferred electrophoretically to Immobilon-P membranes. After blocking the membranes for 1 h using 5 % milk and 2% BSA in TBST buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl and 0.1% Tween-20), primary antibodies diluted in TBST were added and incubated overnight at 4°C. Membranes were washed three times for 5 minutes each in TBST and incubated with the appropriate horseradish peroxidase-labeled secondary antibody in TBST buffer for 1 h at room temperature. After five final washes for five minutes each, proteins were detected using enhanced chemiluminescence, ECL (PerkinElmer, Wellesley, MA) and X-OMAT imaging film (Kodak). Phosphorylation and the total level of each signaling intermediate were determined by Western blot analysis followed by densitometry using NIH Image J program. The phosphorylation of each protein was normalized to their respective total protein level. The summary data for all the experiments are expressed in graphical form as means $\pm$ S.E.

2.7. Immunoprecipitation

Freshly isolated adult feline cardiomyocytes were incubated overnight in serum-free M199 media. Insulin stimulation and torin1 or rapamycin treatment of cells were performed at the concentrations and time periods indicated. Vehicle treated cells served as controls. About 7.5×10^5 cardiomyocytes were used for each immunoprecipitation, and for this cell lysates were prepared as described previously [7]. Briefly, cells were scraped in 500 μ l CHAPS lysis buffer (40 mM Hepes [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β -glycerolphosphate, 50 mM NaF, Sigma protease inhibitor cocktail (P 8340) and phosphatase inhibitor cocktails I and II (P 2850 and P 5726) with 0.3% CHAPS). The cells were lysed and then spun at $14,000 \times g$ for 15 min at 4 °C. Next, 50 μ l lysates were precleared by rotating at 4°C for 2 h with 30 μ l of prewashed Protein A/G agarose beads (Santa Cruz) and then spun at $1000 \times g$ for 2 min. The supernatant was separated from the beads and reserved for immunoprecipitation. The precleared lysates were then subjected to immunoprecipitation using 2 μ g of each primary antibody at 4°C overnight. Next, 30 μ l Protein A/G agarose beads (Santa Cruz) were added to the lysates the next day and rotated at

4 °C for 2 h. The immunoprecipitates were spun at $1000 \times g$ for 2 min, washed 4 times with CHAPS lysis buffer, and 30 μ l of 2x SDS-sample buffer was added to the beads. Samples were boiled for 5 min and spun at $14,000 \times g$ for 30 s to pellet the beads. Proteins were analyzed by Western blotting.

2.8. Statistics

Differences between the groups were compared by one-way ANOVA followed by a Tukey test for multiple comparisons. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. mTORC2-mediated Akt activation in adult cardiomyocytes

Our previous *in vitro* [38] studies identified that a PKC-mediated pathway contributes to the activation of mTOR and its downstream target S6K1 during agonist stimulation of adult cardiomyocytes, indicating the importance of PKC isoforms in mTORC1-mediated S6K1 activation. Our subsequent work showed that rapamycin treatment that blocked mTORC1/S6K1 simultaneously could enhance insulin- and endothelin-stimulated Akt activation [32]. Since mTORC2 has been shown to activate Akt in multiple cell types [43], we analyzed in the present study whether mTORC2 is primarily responsible for the agonist-stimulated Akt activation in adult cardiomyocytes and for the augmentation of this effect by rapamycin. Compared to control cells that showed a very low level of phosphorylation, insulin stimulated cardiomyocytes exhibited a very high level of phosphorylation at the S473 site of Akt and at the T389 site of S6K1, indicating activation of both these kinases (Fig. 1A). As we published previously, rapamycin pretreatment, while totally blocking T389 phosphorylation of S6K1, enhanced S473 phosphorylation of Akt [32], suggesting that Akt phosphorylation proceeds independent of mTORC1. To further demonstrate the role of mTOR, we used torin1 a recently characterized mTOR inhibitor that specifically blocks the catalytic activity of mTOR, and thus affects both mTORC1 and mTORC2. Pretreatment of adult cardiomyocytes with torin1 substantially blocked the insulin-stimulated Akt phosphorylation and its further potentiation by rapamycin as well as S6K1 phosphorylation (Fig. 1A). Together these data indicate that mTORC2 but not mTORC1 mediate the insulin-stimulated Akt activation and that blocking mTORC1 with rapamycin under these conditions could lead to enhanced activation of Akt via mTORC2.

To further explore if Akt activation during insulin stimulation proceeds predominantly via mTORC2-mediated mechanism in adult cardiomyocytes, we performed Rictor knockdown experiments using adenoviral expression of shRNA along with $\pm$ torin1 pretreatment (Fig. 1B). Compared to β -gal infected control cells, Rictor shRNA expression at 200 MOI substantially reduced the level of Rictor in adult cardiomyocytes. Furthermore, this reduction by shRNA was found to be stronger in torin1 treated cells. The loss of Rictor was partly accompanied with reduced level of Akt S473 phosphorylation (Fig. 1B, lane 3 in the lighter exposure) but not the T389 phosphorylation of S6K1. As expected, pretreatment of cells with torin1 substantially reduced both Akt phosphorylation at the S473 site and S6K1 phosphorylation at T389 site. Any residual phosphorylation at the S473 site in torin1 treated cells was found to be further abolished in cells with Rictor knockdown (comparing lanes 5 and 6 with lane 4 in the darker exposure). These experiments with shRNA knockdown and torin1 pretreatment reveal that mTORC2 is primarily responsible for Akt S473 in adult cardiomyocytes while mTORC1 is responsible for T389 phosphorylation of S6K1.

As shown earlier (Fig. 1A), treatment of adult cardiomyocytes with insulin results in the T389 phosphorylation of S6K1, a critical site of phosphorylation required for S6K1 activation [44], and under these conditions, several signaling intermediates that could serve as S6K1 substrates can be expected to undergo phosphorylation. Therefore, our next series

of studies were based on recent work that showed at least two negative regulatory mechanisms on mTORC2-mediated Akt activation via S6K1-mediated phosphorylation: one is phosphorylation of insulin receptor substrate-1 (IRS1) at the S636/639 sites that affects IRS1-mediated PI3K activation and downstream signaling [45], and the second is Rictor phosphorylation at the T1135 site that is known to affect mTORC2 downstream signalling [46]. Our data show that pretreatment of cells with either rapamycin or torin1 that blocked S6K1 phosphorylation (Fig. 1A) inhibited phosphorylation of both these substrates (Fig. 1C). Therefore, augmentation of insulin-stimulated Akt activation by rapamycin was accompanied with the loss of phosphorylation of IRS-1 and Rictor on their negative regulatory sites, which might result in enhanced activation of mTORC2/Akt. In addition, we measured the level of mTOR phosphorylation both at the S2448 site, which was previously characterized to be mediated by S6K1 [47], and at the S2481 site via autophosphorylation [48]. Rapamycin blocked phosphorylation at these sites, although torin1 showed stronger inhibition on T2481 autophosphorylation as previously suggested [48].

3.2. PKC ϵ -mediated Akt activation in adult cardiomyocytes

Next, we explored whether PKC ϵ , a well known prosurvival kinase is involved in mTORC2-mediated S6K1 activation. For this, we used dominant negative PKC ϵ (DN-PKC ϵ) adenovirus, which we have previously employed to study the role of PKC ϵ during mTORC1-mediated S6K1 activation [38]. Infecting cardiomyocytes with PKC ϵ adenovirus at 200 and 400 MOI resulted in the expression of substantial level of DN-PKC ϵ when compared to β -gal infected cells (Fig. 2A). Analysis of insulin-stimulated Akt activation in these cells showed a significant decrease in Akt phosphorylation, and this drop was observed more at the 400 MOI. However, DN- PKC ϵ expression did not affect the insulin-stimulated mTOR phosphorylation at S2481 or S2448. These data indicate that PKC ϵ is involved in insulin-stimulated Akt activation, although it did not affect mTOR phosphorylation at the S2481 and S2448 sites.

Next, we explored whether Akt S473 phosphorylation by various agonists and the augmented effect by rapamycin require PKC ϵ . For this, both β -gal and DN-PKC ϵ expressing cardiomyocytes were pretreated with $\pm$ rapamycin and then stimulated with phenylephrine (PE), insulin or TPA (12-O-tetradecanoylphorbol-13-acetate). Cell extracts were analyzed for changes in the S473 phosphorylation status of PKC ϵ (Fig. 2B). In β -gal expressing control cells, both PE and insulin showed lower and higher levels of Akt S473 phosphorylation respectively while TPA showed no such activation. Both PE and insulin-stimulated Akt phosphorylation was further augmented in rapamycin treated cells. These findings performed in adult cardiomyocytes were similar to our earlier observation [32]. However, a similar experiment in DN-PKC ϵ cells showed loss of both the agonist-stimulated Akt phosphorylation and its further augmentation by rapamycin. These data clearly demonstrate the importance of PKC ϵ in mediating Akt S473 phosphorylation. Furthermore, since DN-PKC ϵ did not affect agonist-stimulated mTOR phosphorylation (Fig. 2A), it suggests that PKC ϵ functions downstream of mTORC2 in mediating Akt S473 phosphorylation.

3.3. mTORC-mediated PKC ϵ phosphorylation in adult cardiomyocytes

To explore whether mTOR plays an upstream role in PKC ϵ activation, we first measured the level of S729 phosphorylation of PKC ϵ in insulin stimulated adult cardiomyocytes pretreated with $\pm$ rapamycin or torin1. Although our data showed that PKC ϵ is required for Akt activation (Fig. 2), only a low level detection of PKC ϵ phosphorylation at S729 was observed in adult feline cardiomyocytes with $\pm$ insulin treatment (data not shown). Since it is possible that the antibody against phospho-S729 PKC ϵ reacts poorly with feline samples, DN-PKC ϵ was exogenously expressed in adult cardiomyocytes in order to raise the baseline

level of PKC ϵ and then analyzed if mTOR plays an upstream role in PKC ϵ phosphorylation (Fig. 3A) by treatment with $\pm$ torin1. Compared to uninfected cells or β -gal expressing cells, S729 phosphorylated PKC ϵ could be readily detected in DN-PKC ϵ expressing adult feline cardiomyocytes. Importantly, treatment with torin1 blocked the phosphorylation, indicating that mTORC1 and/or mTORC2 is playing an upstream role in S729 phosphorylation of PKC ϵ . To demonstrate that mTORC2 contributes to PKC ϵ phosphorylation, we once again raised the baseline levels of PKC ϵ in cells via DN-PKC ϵ expression and then the cells were reinfected with either β -gal or sh-Rictor. Cardiomyocytes that were not infected for the second time served as an untreated additional control (Fig. 3B). Expression of sh-Rictor efficiently knocked down the Rictor level in cardiomyocytes as observed in previous experiments (data not shown). Western blot analysis revealed both baseline and insulin-stimulated phosphorylation of PKC ϵ at S729 was undetectable in sh-Rictor expressing cells (Fig. 3B). Finally, to demonstrate that mTORC2 and not mTORC1 is responsible for the PKC ϵ S729 phosphorylation, we used DN-PKC ϵ expressing cardiomyocytes that were stimulated with insulin and studied the effect of rapamycin and torin1. Our earlier results (Fig. 1A) showed that rapamycin blocked mTORC1 but not mTORC2 while torin1 blocked both the mTOR complexes. Results shown in Fig. 3C revealed that only torin1 but not rapamycin could block the PKC ϵ phosphorylation, indicating that mTORC2 but not mTORC1 plays an upstream role for the S729 phosphorylation of PKC ϵ .

3.4. Assembly of PKC ϵ and Akt with mTORC2 in adult cardiomyocytes

Next, we performed a series of immunoprecipitation experiments to demonstrate PKC ϵ and Akt association with mTORC2. For this, adult cardiomyocytes were stimulated with insulin in the presence or absence of wortmannin, a PI3K inhibitor. Unstimulated cells served as control. Cell lysates were prepared in a buffer system previously described for studying mTOR complexes [7] and used to immunoprecipitate Raptor, Rictor, phospho-S473 Akt and Akt antibodies (Fig. 4). Western blot analyses of the Raptor and Rictor immune complexes show that the specific antibodies immunoprecipitated their respective proteins along with mTOR (Fig. 4A). Western blots for PKC ϵ and phosphorylated Akt show their corresponding bands predominantly in the Rictor immunoprecipitates of insulin treated cells, where their presence was found to be lost with wortmannin treatment. Furthermore, reciprocal immunoprecipitation with phospho-S473 Akt antibody showed the presence of Akt and PKC ϵ in the immune complexes (Fig. 4B). We also tried to pull down the Rictor/PKC ϵ /Akt complex using regular Akt antibody (Fig. 4C). Regular Akt antibody, unlike the phospho-S473 Akt antibody, was able to pull down only Akt from control or insulin-treated cell extracts, but not the complex consisting of PKC ϵ or Rictor. It is possible that the epitope for this antibody is masked in the protein complex. In this case, the results with this antibody serve just as an internal control. However, Rictor antibody, similar to the experiment shown in Fig. 4A, was able to immunoprecipitate the complex consisting of Akt, PKC ϵ and Rictor from insulin treated cells (Fig. 4C). These experiments reveal that PKC ϵ and Akt associate with mTORC2 during insulin stimulation.

4. Discussion

Recent studies show that mTOR forms at least two independent complexes, mTORC1 and mTORC2, and that these two complexes primarily phosphorylate and activate S6K1 and Akt, respectively. Several early studies show that activation of both S6K1 and Akt promote ribosomal biogenesis and cell survival respectively in PO myocardium [49]. We [27, 38] and others [31, 50] have shown mTORC1-mediated S6K1 activation both in PO myocardium *in vivo* and in agonist-stimulated adult cardiomyocytes *in vitro* where specific PKC isoforms including PKC ϵ were found to play a critical role. However, other independent studies demonstrate that rapamycin administration during *in vivo* PO that affects S6K1 activation did not completely abolish the hypertrophic growth process and that drug treatment could

improve ventricular function [31]. In support of these observations, our recent work demonstrates that acute rapamycin treatment during both *in vivo* PO and *in vitro* stimulation of isolated adult cardiomyocytes with hypertrophic agonists results in enhanced Akt activation and decreased programmed cell death in hypertrophying myocardium [32]. In this context, previous studies indicate that the rapamycin effect on the enhancement of agonist-stimulated Akt activation could be observed in a few but not all cell types [43]. Whereas we showed augmented Akt activation by rapamycin in cardiomyocytes, these studies raised several important questions whether: (i) the agonist stimulated Akt activation and its further augmentation by rapamycin requires mTORC2, (ii) the previously reported negative regulation on mTORC2 via IRS-1 and/or Rictor phosphorylation is lost during rapamycin treatment, leading to enhanced mTORC2 activation, and (iii) the insulin-stimulated Akt activation in cardiomyocytes and its augmentation by rapamycin requires PKC ϵ , functioning downstream of mTORC2. Our present work supports all three possibilities.

In the present study, we used both rapamycin that specifically blocks mTORC1 and torin1 that inhibits mTOR catalytic activity, thus affecting both mTORC1 and mTORC2 downstream pathways. Previous studies show that during short term rapamycin treatment only mTORC1 is sensitive to inhibition but not mTORC2, and depending upon the cell type, rapamycin treatment could promote mTORC2-mediated Akt activation [43]. Our present data is in agreement with these observations. That is, treatment of adult cardiomyocytes with insulin results in the phosphorylation of Akt at the S473 and S6K1 at the T389 sites. As we published earlier [32], treatment of cells with rapamycin augments Akt activation while it completely abolishes S6K1 activation. However, treatment of cells with torin1 blocked both Akt and S6K1 phosphorylation. These studies with rapamycin clearly demonstrate that mTORC1 is responsible for S6K1 activation and that mTORC2 is responsible for Akt phosphorylation. Furthermore, the augmented effect by rapamycin on Akt phosphorylation in cardiomyocytes was also indeed mediated by mTORC2.

How does the acute rapamycin treatment with an accompanying loss of mTORC1-mediated S6K1 activation promote mTORC2-mediated Akt activation? Multiple mechanisms might contribute to such an augmented effect: (i) S6K1 has been shown to phosphorylate and negatively regulate IRS-1 signaling. That is, only with the unphosphorylated state of IRS-1 at the S636 and S639 sites [45], PI3K activation and downstream signaling could proceed, leading to mTORC2-mediated Akt activation. Therefore, loss of both S6K1 activation and the associated IRS-1 phosphorylation by rapamycin could result in an augmented Akt activation, (ii) S6K1 has been shown to phosphorylate Rictor at the negative regulatory T1135 site, causing loss of mTORC2 downstream signaling [46]. Therefore, when S6K1 activation is blocked due to acute rapamycin treatment, this negative feedback mechanism through Rictor might be unavailable, leading to enhanced Akt activation, (iii) Finally, mTOR itself has been shown to undergo phosphorylation at S2448 and S2481 sites where the latter was shown to occur through autophosphorylation [51]. Altered phosphorylation at these sites in mTOR by rapamycin treatment might affect the agonist stimulated Akt activation. Therefore, we analyzed these three possibilities in insulin treated cardiomyocytes, pretreated with $\pm$ rapamycin (Fig. 1C). Our data reveal that several sites in IRS-1, Rictor and mTOR undergo phosphorylation during insulin stimulation which could be brought back to their basal conditions with rapamycin or torin1 treatment. This indicates S6K1-mediated phosphorylation at these sites. Loss of specific phosphorylation in IRS-1 and Rictor might lead to a loss of negative regulation for mTORC2 signaling and Akt activation. In the case of mTOR, rapamycin and torin1 treatments brought the insulin stimulated S2448 phosphorylation back to the basal condition, indicating the loss of S6K1 mediated phosphorylation at this site. However, the loss of phosphorylation at the S2481 autophosphorylation site is more pronounced with torin1 than with rapamycin treatment. This indicates that mTOR kinase activity is not abolished by rapamycin unlike torin1,

although rapamycin did not enhance the autophosphorylation. These studies support our view that rapamycin treatment might lead to the loss of S6K1 -mediated negative regulation via phosphorylation of IRS-1 and/or Rictor at specific sites that results in enhanced mTORC2 activation and Akt phosphorylation.

After showing that the agonist-stimulated Akt activation and its further augmentation are mediated via mTORC2 in adult cardiomyocytes, we next explored whether the activation of another pro-survival kinase, namely PKC ϵ , is required for the Akt-mediated prosurvival signaling. We have previously shown that PKC ϵ plays an upstream role in mTORC1-mediated S6K1 activation in adult cardiomyocytes [38]. Therefore, we explored if PKC ϵ plays either an upstream or downstream role in the mTORC2-mediated Akt activation. Our studies with dominant negative PKC ϵ adenovirus (Fig. 2A) clearly indicate that the insulin-stimulated Akt activation requires PKC ϵ . The following lines of evidence show that PKC ϵ functions downstream of mTORC2 and upstream of Akt S473 phosphorylation: First, similar to Akt S473 phosphorylation, PKC ϵ S729 phosphorylation by insulin is substantially reduced by torin1 but not by rapamycin. Second, the phosphorylation of exogenously expressed dominant negative PKC ϵ was also substantially blocked by torin 1 or absent in Rictor knockdown cells, suggesting that PKC ϵ is functioning downstream of mTOR. Third, expression of dominant negative PKC ϵ substantially blocks insulin-stimulated Akt S473 phosphorylation. Fourth, both PKC ϵ and phosphorylated Akt were found to be present more in the Rictor complex than in Raptor complex of insulin-stimulated cells. These data clearly show that S729 phosphorylation which is critical for the kinase function of PKC ϵ occurs downstream of mTORC2, although whether mTORC2 directly phosphorylates PKC ϵ at this site is not known from these studies. A recent study demonstrates that the mTORC2 subunit Sin1 is a direct binding partner of PKC ϵ and a critical component for PKC ϵ phosphorylation [52]. Previous studies *in vitro* have shown that PKC ϵ could phosphorylate Akt at the S473 site [53]. All these studies strongly suggest that agonist stimulated Akt activation in adult cardiomyocytes and the further augmentation of this effect by rapamycin proceed via mTORC2 where PKC ϵ serves as an integral kinase for the Akt S473 phosphorylation by functioning downstream of mTORC2.

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Highlights

In this study, we show that the insulin-stimulated Akt activation in isolated cardiomyocytes and its further augmentation by rapamycin treatment are mediated via mTORC2. Furthermore, by using pharmacological agents and performing gene silencing experiments, we show that mTORC2 indeed mediates critical phosphorylation at the S729 and S473 sites for the activation of PKC ϵ and Akt respectively and that PKC ϵ functions upstream of Akt S473 phosphorylation. Thus, these studies show for the first time that mTORC2 serves as the upstream activator of two major prosurvival kinases, PKC ϵ and Akt, suggesting a cardioprotective role by mTORC2/PKC ϵ /Akt axis in cardiomyocytes.

A **B**

Fig. 1.

mTORC2 mediates insulin stimulated Akt activation. (A) Freshly isolated adult feline cardiomyocytes were cultured on laminin-coated plates for 36 h and stimulated with 100 nM insulin for 60 min. For treatments with rapamycin (5 nM) and Torin1 (100 nM), cells were pretreated with the drugs 30 min prior to insulin treatment. Triton X-100 soluble proteins were prepared and used for Western blot analysis with anti-pAkt (S473), Akt, pS6K1 (T389) and S6K1 antibodies. Cell experiments were done in Triplicate. The summary data of quantification conducted using ImageJ for phosphorylated proteins against their respective total is represented in the graphs for both pAkt and pS6K1 as mean $\pm$ SEM. * $p < 0.05$ vs. control; ** $p < 0.05$ vs. insulin, # $p < 0.05$ vs. insulin, and ## $p < 0.05$ vs. insulin+rapamycin ($n = 4$). (B) Adult feline cardiomyocytes were infected with either β -gal or sh-Rictor adenoviruses at MOIs indicated in the figure. Cells were grown in insulin and serum free media for 36 h before being stimulated with insulin (100 nM) and part of the cultures were treated with 100 nM torin1. Cell extracts were prepared as suggested for Fig. 1A and Western blotted with anti-pAkt (S473), pS6K1 (T389), Rictor and GAPDH antibodies. Results were confirmed with two additional experiments. (C) Cell extracts prepared for Fig. 1A were used for Western blot analysis with anti-IRS-1 (S636/639), IRS-1, pRictor (T1135), Rictor, pmTOR (S2448), pmTOR (S2481), mTOR and GAPDH antibodies. Cell experiments were done in triplicate. The summary data of quantification conducted using ImageJ for phosphorylated proteins against their respective total is represented in the graphs as mean $\pm$ SEM. Statistical evaluation are shown for pIRS-1 and pRictor: * $p < 0.05$ vs. control; ** $p < 0.05$ vs. insulin, # $p < 0.05$ vs. insulin, and ## $p < 0.05$ vs. insulin+rapamycin ($n = 4$).

Fig. 2.

PKC ϵ plays an upstream role in Akt S473 phosphorylation. (A) Adult feline cardiomyocytes were infected with either β -gal or dominant negative PKC ϵ (DN-PKC ϵ) adenovirus at MOIs indicated in the figure. Cells were grown in insulin and serum free media for 36 h and part of the cultures were stimulated with 100 nM insulin for 1 h. Cell extracts were prepared as before (Fig. 1) and Western blotted with anti-pAkt (S473), Akt, pmTOR (S2448), pmTOR (S2481), mTOR, PKC ϵ and GAPDH antibodies. The summary data of quantification conducted using ImageJ for phosphorylated proteins against their respective total is represented in the graphs as mean $\pm$ SEM. * $p < 0.05$ vs. control (n = 4). (B) Plated cardiomyocytes were infected for 36–48 h with either β -gal (control) or DN-PKC ϵ adenoviruses (400 MOI) and then preheated with rapamycin (5 nM, 30 min) prior to 1 h stimulation with either phenylephrine (PE, 100 μ M), insulin (100 nM) or phorbol-12-myristate-13-acetate (TPA, 100 nM). Triton X-100 soluble proteins were prepared and used for Western blot analysis with anti-S473 Akt, Akt and GAPDH antibodies. Results were confirmed in one additional experiment.

Fig. 3. mTORC2 functions upstream for PKC_ε S729 phosphorylation. (A) Adult feline cardiomyocytes in culture were infected with 200 MOI of β-gal or DN-PKC_ε adenovirus and grown in insulin and serum free media for 36 h. Cells were then stimulated with 100 nM insulin for 1 h. For experiments with torin1, infected and uninfected (Cont) cells were pretreated with the 100 nM torin1 30 min prior to insulin treatment. Triton X-100 soluble proteins were prepared as before (Fig. 1) and Western blotted with anti-pPKC_ε (S729), PKC_ε and GAPDH antibodies. The results were confirmed in one additional experiment. (B) Adult feline cardiomyocytes in culture were infected first with 200 MOI of DN-PKC_ε adenovirus. 12 h later, media was changed and part of the culture was reinfected with 200 MOI of either β-gal or sh-Rictor adenovirus. Cells were incubated in insulin and serum free media for 36 h and then stimulated with ±100 nM insulin for 1 h. Cell extracts were prepared and Western blotted with anti-pPKC_ε (S729), PKC_ε and GAPDH antibodies. The results were confirmed in one additional experiment. (C) Adult feline cardiomyocytes were infected with 200 MOI of DN-PKC_ε adenovirus. Cells were grown in insulin and serum free media for 36 h and then stimulated with 100 nM insulin for 1 h. For treatments with 5 nM rapamycin and 100 nM torin1, cells were pretreated with the drugs 30 min prior to insulin treatment. Cell extracts were prepared and Western blotted with anti-pAkt (S473), pPKC_ε (S729), PKC_ε and GAPDH antibodies. The results were confirmed in one additional experiment.

Fig. 4. PKC ϵ and Akt associate with mTORC2 in insulin-stimulated adult cardiomyocytes. (A) Freshly isolated adult feline cardiomyocytes were cultured on laminin-coated plates. Part of the cultures was pre-incubated for 1 h with 100 nM wortmannin. Cells were then stimulated with $\pm$ 100 nM insulin for 1 h and lysates were prepared and used for immunoprecipitation with anti-Raptor and Rictor antibodies. The immune complexes were resolved on SDS-PAGE and Western blotted with anti-mTOR, Raptor, Rictor, PKC ϵ and pAkt (S473). Western blot analyses using these antibodies were also performed for the total lysate prepared from the insulin-stimulated cells and for the non-specific binding of proteins in the extract to ProteinA/G-agarose beads. (B) Cell lysates were prepared from adult cardiomyocytes stimulated with $\pm$ 100 nM insulin and used for immunoprecipitation with anti-pAkt (S473) antibody. To analyze for the nonspecific proteins, pAkt antibody in the absence of cell lysate (Ab+buffer) or insulin-stimulated cell lysate without the addition of antibody (Lys+Protein A/G beads alone) were also included in the immunoprecipitation experiments. The immunoprecipitated proteins along with total lysates were resolved on SDS-PAGE and Western blotted with anti-mTOR, PKC ϵ , pAkt (S473) and Akt antibodies. (C) Cell lysates were prepared from adult cardiomyocytes stimulated with $\pm$ 100 nM insulin and used for immunoprecipitation with anti-Akt or anti-Rictor antibody. For non-specific binding of the proteins to the beads, cell extracts without the addition of antibody (Lys

+Protein A/G beads alone) were also included in the immunoprecipitation experiments. The immunoprecipitated proteins along with total lysates were resolved on SDS-PAGE and Western blotted with anti-Rictor, PKC ϵ and Akt antibodies. All antibodies used for the immunoprecipitation and Western blot analyses were raised in rabbit where some of them serve as internal controls to show the specificity of the immune complexes.