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Original article

Rheb activates protein synthesis and growth in adult rat ventricular cardiomyocytes

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ABSTRACT

The mammalian target of rapamycin complex 1 (mTORC1), a key regulator of protein synthesis, growth and proliferation in mammalian cells, is implicated in the development of cardiac hypertrophy. Ras homolog enriched in brain (Rheb) positively regulates mTORC1. We have studied whether Rheb is sufficient to activate mTOR signaling and promote protein synthesis and cardiac hypertrophy in adult rat ventricular cardiomyocytes (ARVC). Rheb was overexpressed via an adenoviral vector in isolated ARVC. Overexpression of Rheb in ARVC activated mTORC1 signaling, several components of the translational machinery and stimulated protein synthesis. Our direct visualization approach to determine ARVC size revealed that overexpression of Rheb also induced cell growth and indeed did so to similar extent to the hypertrophic agent, phenylephrine (PE). Despite potent activation of mTORC1 signaling, overexpression of Rheb did not induce expression of the cardiac hypertrophic marker mRNAs for brain natriuretic peptide and atrial natriuretic factor, while PE treatment did markedly increase their expression. All the effects of Rheb were blocked by rapamycin, confirming their dependence on mTORC1 signaling. Our findings reveal that Rheb itself can activate both protein synthesis and cell growth in ARVC and demonstrate the key role played by mTORC1 in the growth of cardiomyocytes.

1. Introduction

Cardiac hypertrophy (CH) is a major risk factor for heart failure and involves increased cell size rather than cell number. The principal factor driving cardiomyocyte growth is accelerated protein synthesis [1]. The mammalian target of rapamycin (mTOR) is a key regulator of protein synthesis (mRNA translation) [2,3]. mTOR exists mainly in two types of complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 signaling is generally blocked by the immunosuppressant drug, rapamycin, although some events involving mTORC1 are not sensitive to this drug [4]. mTORC2's acute signaling effects are insensitive to rapamycin. Rapamycin (also called sirolimus) is used clinically to prevent graft rejection and cardiovascular disorders such as restenosis after angioplasty [5]. Rapamycin analogs are in clinical trials as anti-cancer drugs [6]. mTORC1 regulates cell growth and proliferation [3] and is well-known for its ability to promote protein synthesis by regulating components of the translational machinery [2].

Most relevant for the present investigation are the observations that rapamycin can prevent or even reverse CH, *in vitro* and *in vivo* (see, e.g., [7–9]). These data indicate that mTORC1 signaling plays an indispensable role in the development and/or maintenance of the hypertrophic state, but do not inform us, for example, whether mTORC1 signaling is sufficient to drive cardiomyocyte growth. A second important issue, in animal models, is whether rapamycin's effects are actually on the cardiomyocytes themselves or are exerted indirectly, e.g., on other cell types and mediators involved in CH.

We have previously explored the role of mTOR signaling in controlling protein synthesis in isolated adult rat ventricular cardiomyocytes (ARVC). We showed that the activation of protein synthesis by the hypertrophic agents phenylephrine (PE) and endothelin-1 (ET-1) was substantially blocked by rapamycin [10]. This strongly implies that mTORC1 signaling plays a key role in controlling protein synthesis in myocytes in response to hypertrophic stimuli. PE and ET-1 activate mTORC1 via Ras/MEK/ERK signaling in ARVC (see, e.g., [10]). Activation of this pathway likely leads to the inhibition of the tuberous sclerosis complex (comprising the proteins TSC1 and TSC2). The TSC1/2 complex acts as a GTPase-activator protein for the small GTPase Rheb, and Rheb GTP activates mTORC1 signaling (for review see [11]). Thus, inactivation of the TSC1/2 complex leads to the stimulation of mTORC1 signaling. As discussed above, the TOR pathway and its downstream targets promote cell growth [3], at least in part by stimulating protein synthesis.

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mTORC1 controls the phosphorylation and function of three types of proteins linked to the short-term control of the translational machinery, i.e., the ribosomal protein S6 kinases, the eukaryotic initiation factor 4E-binding proteins (4E-BPs, typified by 4E-BP1) and the kinase that phosphorylates and inhibits eukaryotic elongation factor 2 (eEF2), the protein that mediates the movement of ribosomes along the mRNA (for a review see [2]). mTORC1 signaling activates the S6 kinases and inhibits 4E-BP1 and eEF2 kinase. Since the latter two proteins negatively regulate translation, mTORC1 functions in each case to activate the translational machinery. mTORC1 also promotes ribosome biogenesis by increasing rRNA synthesis [12]. This increases the cellular capacity for protein synthesis and can enhance protein synthesis rates in the longer term.

ARVC provide a valuable system for investigating the control of protein synthesis in differentiated primary cells, rather than the immortalized cell lines (e.g., fibroblasts) that have been used in most studies. For example, α1-adrenergic (hypertrophic) agonists markedly activate protein synthesis (by roughly 2–3 fold) in ARVC [10], which contrasts with the much smaller effects of insulin generally observed in immortalized/transformed cell lines (see, e.g., [13]). ARVC are thus particularly valuable for studying the intracellular signaling events that underlie this disease.

CH is also associated with changes in gene expression and remodelling of the heart [14]. Two genes which are upregulated in CH and which are widely used as markers of CH and/or remodelling are those for atrial natriuretic factor (ANF [15]) and brain natriuretic peptide (BNP [16]). Although BNP was first described from pig brain, subsequent studies have shown that it, and ANF, are primarily made in cardiac myocytes.

Although inhibition of mTORC1 signaling by rapamycin can impair the growth of heart cells, it is not clear whether mTORC1 signaling is itself sufficient to promote adult myocyte growth or whether other signaling events are required. It is also unclear what role mTORC1 signaling plays in the changes in gene expression that accompany CH. Here, we show that activation of mTORC1 signaling (induced by Rheb) itself promotes the marked growth of ARVC, but does not induce the expression of ANF or BNP.

2. Materials and methods

2.1. Chemicals, antisera, and other materials

L-[^{35}S]methionine, L-[2,3,4,5,6-^{3}H]phenylalanine, 7-methylGTP (m^7GTP)-Sepharose CL-4B, and protein G-Sepharose were from GE Healthcare. Rapamycin was from Calbiochem. BSA (fraction V) was from Roche Molecular Biochemicals. Collagenase (type II) was from Worthington. Fura-2/AM was from Invitrogen. Anti-eIF4G was kindly provided by Dr. Simon Morley (Sussex, UK). Anti-phospho-Ser235/6 ribosomal protein S6, anti-S6 and anti-phospho-Ser65 4E-BP1 antibodies were from Cell Signaling Technology. Anti-4E-BP1, anti-eEF2 and anti-phospho-Thr56 eEF2 antibodies were prepared as described [17]. Other chemicals or biochemicals were from Sigma unless otherwise indicated.

2.2. Isolation, culture, treatment and lysis of cardiomyocytes

ARVC were isolated from adult male Sprague–Dawley rats (250–300 g; Animal Care Centre, UBC) as described [18]. After isolation, ARVC were washed and seeded onto laminin-coated tissue culture dishes, and cultured [10]. After treatment, cells were lysed as described [18]. Protein concentrations were determined as in [19]. All experiments were conducted in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals (volume 1, 1993) and approved by the local (UBC) Animal Care Committee.

2.3. Construction and use of adenoviral vectors

The Adeno-X Expression System 1 (BD Biosciences) was employed to construct all adenoviruses. Adenovirus-mediated gene transfer was performed after ARVC had attached to the dishes [10]. Cultured ARVC were incubated with recombinant adenoviruses in minimal volumes of M199 medium (37 °C, 3 h). After removing the adenovirus, fresh M199 medium was supplied to the cells. ARVC were cultured for 40 h to allow expression of the encoded proteins before further treatment.

2.4. m^7GTP-Sepharose chromatography, gel electrophoresis and western blotting

Purification by affinity chromatography of eIF4E and associated proteins was conducted as described [10]. SDS-PAGE and western blot analysis were carried out as described [20]. Western blots were quantitated using ImageJ (http://rsb.info.nih.gov/ij/).

2.5. Measurement of protein synthesis

Fresh M199 medium was supplied to cultured ARVC 20 h before treatment with rapamycin (100 nM, 30 min). Cardiomyocytes were then stimulated with phenylephrine (10 μM, 45 min) prior to addition of L-[^{35}S]methionine (5 μCi/ml, 45 min) or 2,3,4,5,6-[^{3}H]phenylalanine (1 μCi/ml, 180 min). The much lower specific radioactivity of the tritiated phenylalanine meant that longer incubation times had to be used than for methionine. After removing the medium, cells were washed twice with ice-cold PBS and lysed with extraction buffer. Protein was applied to 3MM filter paper (Whatman) before precipitation with cold 5% (w/v) trichloracetic acid (TCA) [10]. Incorporated radioactivity was measured using a MicroBeta Trilux liquid scintillation counter (Perkin Elmer).

2.6. L-[^{35}S]methionine labeling of ARVC

Forty hours after adenovirus infection, ARVC were transferred to methionine-free M199 medium for 2 h. Cells were treated with or without rapamycin (100 nM, 30 min) prior to stimulation with phenylephrine (10 μM, 45 min). L-[^{35}S]methionine (20 μCi/ml) was added 6 h before lysis. After removing the medium, cells were washed and lysed as described [18]. SDS-PAGE [20] were carried out. The SDS-PAGE gel was fixed (50% (v/v) methanol and 10% (v/v) acetic acid, 30 min) and dried onto 3MM filter paper (80 °C, 90 min). Radiolabeled proteins were detected using a Typhoon™ phosphorimager.

2.7. Determination of cell area, length, width, and volume

For microscopy-based estimates of cell cross-sectional area and volume, ARVC were seeded on laminin-coated glass coverslips. After adenovirus infection, ARVC were treated with phenylephrine (10 μM) or rapamycin (100 nM) as indicated. Area and volume estimates were performed after 40 h. Images were obtained using a CoolSNAP-HQ camera (Roper Scientific, Tucson, AZ) attached to a Zeiss 200 M microscope (Carl Zeiss, Thornwood, NY). Image acquisition and analysis were controlled by the Slidebook™ 4.2 software (Intelligent Imaging Innovations Inc., Denver, CO). To determine average ARVC area, length, and width, brightfield images were taken using a 10× objective. Each cell in a given field was manually traced and the area of the resulting masks was automatically calculated in Slidebook™. The small (<5%) proportion of the ARVC that appeared misshapen (see Fig. 3A below) was rigorously excluded from the analysis.

Correlated cell area and volume estimates were performed on a cell-by-cell basis. ARVC were labeled either by infection with adenovirus encoding GFP or by loading with the cell-permeable fluorescent Ca^{2+} dye Fura-2-AM (excited at 380 nm). Individual cells were imaged using a 20× objective. The fluorescent labeling permitted the automatic generation of a mask that delineated the cell and formed the basis for morphological quantifications. The cross-sectional area was estimated at the center plane of the cell. Subsequently a 50–60 μm thick stack of images was collected at