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Translational activation of 5'-TOP mRNA in pressure overload myocardium

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■ **Abstract** The present study was conducted to determine the magnitude and duration of ribosomal protein translation in response to pressure overload and determine if additional, paracrine events associated with mechanical transduction, such as integrin activation using a bioactive peptide ligand, RGD or endothelin stimulation lead to ribosomal protein translation. Polysome analysis of ventricular tissue samples obtained from an in vivo model of right-ventricular pressure overload (RVPO) showed a significant shift in the proportion of a 5'-terminal oligopyrimidine (5'-TOP) mRNA, rpL32, associated with the polysomal fraction when compared with non-5'-TOP mRNAs, β -actin and β -myosin heavy chain (β -MHC), in the early stages of the hypertrophic response (24–48 h). Furthermore, this increase in polysome-bound rpL32 mRNA was accompanied by the phosphorylation of mammalian target of rapamycin (mTOR), p70 S6 kinase (S6K1), and S6 ribosomal protein. In our in vitro studies, treatment of primary cultures of adult feline cardiomyocytes (cardiocytes) with 100 nM endothelin, 9 mM RGD, 100 nM insulin, or 100 nM TPA activated mTOR via distinct signaling pathways and resulted in an increased proportion of polysome-bound rpL32 mRNA. Pre-treatment of cardiocytes with the mTOR inhibitor rapamycin blocked the agonist-induced rpL32 mRNA mobilization to polysomes. These results show that mechanisms that regulate ribosomal biogenesis in the myocardium are dynamically sensitive to pressure overload. Furthermore, our in vitro studies indicate that distinct pathways are operational during the early course of hypertrophic growth and converge to activate mTOR resulting in the translational activation of 5'-TOP mRNA.

■ **Key words** cardiac hypertrophy – signal transduction – 5'-TOP mRNA – ribosome – mTOR

Introduction

When exposed to an increase in afterload such as aortic stenosis or chronic hypertension, the myocardium undergoes the compensatory process of

cardiac hypertrophy. Hypertrophic growth is characterized by the coordinate activation of several pathways that mediate both protein synthesis and cardiomyocyte survival [6, 23, 24, 35]. Several studies have shown that translational control

mechanisms play a critical role in facilitating a sustained increase in protein synthesis rates [28, 30]. Additional studies have shown that increased synthesis of translational components such as ribosomal proteins and other translation factors are regulated by translational mechanisms [1, 7]. Indeed, a significant proportion of mRNA that encode for components of the translational apparatus possess a unique 5'-terminal oligopyrimidine (5'-TOP) sequence in the 5'-untranslated region (5'-UTR) [2, 18]. This structural element is thought to confer a degree of translational specificity such that under steady-state conditions, a significant fraction of 5'-TOP mRNAs is found in translationally inactive messenger ribonucleoprotein (mRNP) particles. However, upon anabolic stimulation, 5'-TOP mRNAs are rapidly mobilized into translationally active polysomes. The mobilization was thought to be controlled to a large extent by the activity of p70 S6 kinase (S6K1), a kinase, that has been implicated in hypertrophic growth and ventricular remodeling [17]. This conclusion has been challenged by recent studies showing that translation of 5'-TOP mRNA is unaffected in S6K1^{-/-}/S6K2^{-/-} double knockout mice [27] or in unphosphorylatable Ala-to Ser knockin rpS6 P^{-/-} mice [36]. It has been shown that activation of mammalian target of rapamycin (mTOR), the major upstream kinase for S6K1, is required for increased translational efficiency of 5'-TOP mRNAs. Whether additional translational related S6K1 substrates, such as eukaryotic initiation factor 4B (eIF4B), eukaryotic elongation factor 2 kinase (eEF2 kinase), or S6K1 Aly/REF-like target (SKAR) individually or in concert play a role in 5'-TOP mRNA translation is not known [31, 32].

mTOR can be described as a master switch in which signaling pathways, including the phosphatidylinositol-3-OH kinase (PI3K), the protein kinase A (PKA) and the protein kinase C (PKC) pathway, ultimately converge to control cellular events including protein synthesis, cell growth and cell survival [5, 9, 41]. Furthermore, a broad range of stimuli, such as amino acid availability acting through class III PI3K or changes in intracellular ATP levels acting through AMP kinase are known to influence mTOR and its effectors [9, 11, 25]. Most significantly, in terms of sensing physical changes brought on by increased mechanical loading, Malik and Parsons [19] have shown in fibroblast, a robust activation of S6K1 in response to integrin activation that is blocked by the inhibitors of PI3K and mTOR.

In adult myocardium, increases in mechanical load can also serve as a potent initial signal in the hypertrophic growth response with the interface between integrins and the extracellular matrix (ECM) serving as an important conduit [34, 40]. Indeed, several

studies have demonstrated that increases in mechanical loading can act as the primary stimulus for additional autocrine/paracrine mechanism that enhance the hypertrophic growth response [37, 39]. As a specific, van der Laarse and colleagues have shown that endothelin, which primarily signals through a PKC dependent pathway, serves as a paracrine mediator during stretch-induced cardiomyocyte hypertrophy [44].

We have shown in prior studies that S6K1 activation, in response to pressure overload, occurs as early as 1 h and was dependent upon PKC [15]. In subsequent studies, we demonstrated that PKC-mediated S6K1 activation and mTOR phosphorylation requires the involvement of c-Raf/MEK/ERK pathway [9]. Additional studies conducted in our laboratory demonstrated that engagement of integrins with the ECM cleaved peptide product RGD (Arg-Gly-Asp) resulted in both mTOR and S6K1 activation via a PKC-independent pathway [3]. Therefore, we initiated a study to characterize the temporal relationship between activation of mTOR and 5'-TOP mRNA translation in vivo, and to determine if the hypertrophic agonist endothelin and RGD peptide can increase the translational activity of 5'-TOP mRNA by measuring the mobilization of rpL32 mRNA into polysomes.

Our studies demonstrate, specific temporal changes in cardiac 5'-TOP mRNA polysomal content in response to pressure overload. Specifically, dramatic increases in the translational activation of 5'-TOP mRNA occur within the first 48 h of right-ventricular pressure overload (RVPO), indicating that the myocardium can increase translational capacity rapidly in response to overload. In addition, we show that endothelin, a paracrine mediator of hypertrophic growth, can promote the translational activation of 5'-TOP mRNA in a PI3K independent manner and finally, we reveal a novel mechanism of 5'-TOP mRNA translation activation that is induced through integrin activation with the ECM cleaved peptide, RGD.

Experimental methods

■ Animal models

Adult cats weighing ~3 kg were used for RVPO by partial occlusion of the pulmonary artery as previously described [21, 22]. Systemic arterial and chamber pressures were recorded at time of sacrifice. The left ventricle from each cat served as a normally loaded internal control in the same animal. Additional controls include RV and LV from non-surgical animals as well as sham-operated ani-

mals that were subjected to thoracotomy and pericardiotomy without any arterial occlusion. The care of the animals and all experiments were conducted in accordance with the US National Institute of Health guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1985) and the Institutional Animal Care and Use Committee of the Medical University of South Carolina.

■ Adult cardiomyocyte primary culture

Adult feline cardiomyocytes were isolated from normal adult cats and cultured on laminin coated four-well culture trays (Nunc) at a concentration of 1.2×10^6 cells/tray. Isolated cardiomyocytes were maintained in M-199 media at 37°C in humidified air with 5% CO₂ [13]. Following pre-treatment with 20 nM rapamycin or 100 nM wortmannin for 30 min, cardiomyocytes were stimulated with either 200 nM endothelin for 1 h, 9 mM RGD peptide for 2 h, or 100 nM insulin for 1 h. RGD (Gly-Arg-Gly-Asp-Ser) peptide was synthesized at the Medical University of South Carolina and purified on Superdex-peptide gel-filtration column (Pharmacia Biotech Inc).

■ Preparation of extracts for polysomal analysis

Preparation of primary cultured cardiomyocytes were carried out as described previously [10]. Tissue samples were treated in similar fashion except that 100 mg of free-wall LV and RV were directly homogenized in 1.5 ml of polysomal resuspension buffer (PRB), (10 mM Tris, pH 7.5, 250 mM KCl, 2 mM MgCl₂, 0.5% Triton X-100) supplemented with 100 µg/ml cyclohexamide, 2 mM DTT, and 140 U/ml RNasin.

■ Polysome preparation and analysis

To obtain polysome profiles, 1.2 ml of the post-mitochondrial supernatant was layered onto 9.6 ml of a 15–50% linear sucrose gradient and centrifuged for 100 min at 35,000 rpm using a Beckman SW-41 rotor. Eighteen 0.6 ml fractions were collected by upward displacement and their respective RNA content was constantly monitored at A₂₆₀ with an Isco absorbance detector. Fractions were pooled into two groups—Fractions 1–8 sub-polysomal (translational inactive) and fractions 9–18 polysomal (translationally active). Fractionated RNA from each pooled group was extracted using Trizol (Invitrogen) according to the manufacturer's instructions.

■ Real-time RT-PCR to determine mRNA mobilization

Pellets of RNA obtained from polysome fractionation were re-solubilized in 100 µl RNase-free water. RNA samples were treated with DNase for 30 min at 37°C. Each RNA sample was heated for 10 min at 90°C to inactivate enzymatic activity. Real-time RT-PCR was performed with the iCycler iQ Real Time PCR detection system (BIO-RAD) using the Quantitect SYBR Green RT-PCR kit (Qiagen). A 70-point post-amplification dissociation melt curve 95–20°C was performed to verify the presence of a single amplified product. Further verification was obtained by running aliquots of RT-PCRs on a 2% agarose gel. For each set of primers, a no-RT (reverse transcriptase) and no-template control was included. Primer sets: L32 For 5'-AAC CAAGCACATGCTGCCTAG-3' and L32 Rev 5'-ATTGT GAGCAATCTCAGCACA-3', β-actin For 5'-CTCTCCC TCACGCCATCC and β-actin Rev 5'-CACGCACGATTT CCCTCTC-3', β-MHC For 5'-AACCGTATGGCTGTG AG-3' and β-MHC Rev 5'-GATGGCGATGTTCTCC TTC-3' 18S For 5'-TATGGTTCCTTTGGTCGCTC and 18S Rev 5'-GGTTGGTTTTGATCTGATCTGATAAAT-3'.

■ Calculations for mRNA polysomal mobilization

Changes in polysomal mRNA distribution were determined by relative quantification using the mathematical model developed by Pfaffl [29].

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta\text{CP}} \text{target}^{\text{(control-sample)}}}{(E_{\text{reference}})^{\Delta\text{CP}} \text{reference}^{\text{(control-sample)}}}$$

where target = rpL32, β-actin or β-MHC, reference = 18S, E = PCR efficiency, ΔCP = threshold crossing difference, control = LV and sample = RV. Ratios were determined for the primary cultured models in a similar manner, however, crossing point differences of target (rpL32 and β-actin) and reference (18S) were calculated by subtracting treated sample from control quiescent cells. Values for each threshold crossing are the mean of three reactions.

■ Western blot analysis

Left and right ventricular tissues were homogenized in 2% SDS lysis buffer (30 mM Tris-HCl, pH 7.4, 2% SDS, 10 mM β-glycerolphosphate, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mM E-64, 0.5 mM PMSF, 1 mM sodium orthovanadate, 0.02 mM okadaic acid, 0.5 mM EGTA). Following determination of protein content by BCA protein assay, 20 µg of indicated extract were resolved by SDS-PAGE, and proteins were transferred electrophoretically to Immobilon-P

membranes for Western blot analysis with the following antibodies, phospho-mTOR (Ser2448), S6K1, phospho-S6K1 (Thr389), S6 ribosomal protein, phospho-S6 ribosomal protein (Ser235/236), (Cell Signaling Technology), and mTOR (BD Bioscience).

Statistical analysis

Data are presented as means \pm SEM. When comparing groups, one-way ANOVA was used to test for significance. A value of $P < 0.05$ was considered significant.

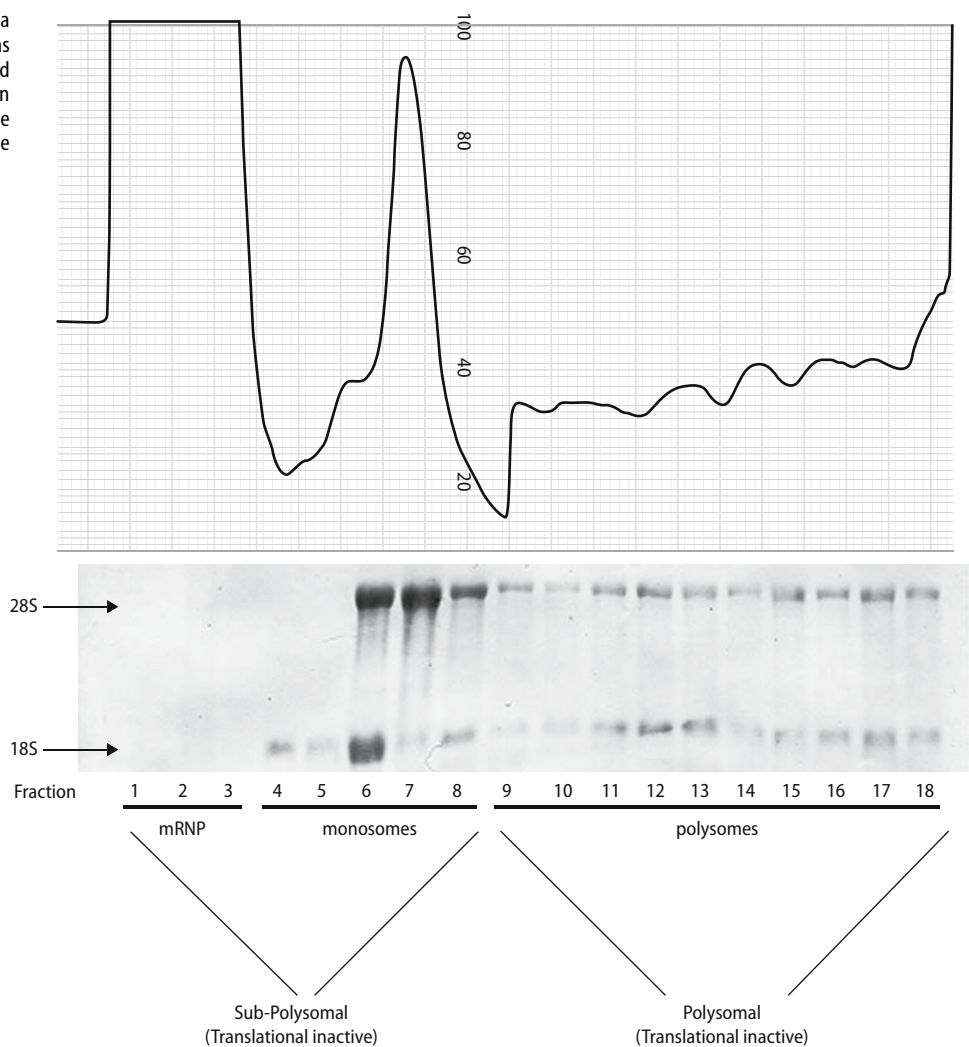
Results

Polysome fractionation and real-time RT-PCR to measure translational control

In order to quantitate changes in translational efficiency of 5'-TOP mRNAs, we used a highly sensitive

polysomal mobilization detection technique that couples polysome fractionation with real-time RT-PCR. This technique allowed us to detect shifts of a specific 5'-TOP mRNA from translationally inactive fractions (mRNP particles and monosomes) into the translationally active polysomal fractions. Figure 1 shows the distribution of ribosomes and polysomes resolved on a linear sucrose gradient. Of the 18 separate fractions collected, the first eight fractions contained (mRNP) (1-3) and monosomes (4-8) and were designated as the sub-polysomal (translationally inactive) pool. Fractions 9-18, which constitute the heavier (translationally active) polysomes, were collected as the polysomal pool. Throughout the experimentation, although subtle changes were observed in terms of the profile tracings, indicating increased accumulation of sub-polysomal and/or polysomal components, the separation of these distinct fractions remained constant. Following preparation of mRNA as outlined in experimental procedures, aliquots of

Fig. 1 Distribution of RNA in a representative polysome profile: RNA was extracted from each of the 18 fractions and subsequently transferred onto a nylon membrane. Staining of the membrane with methylene blue indicates the distribution of 28S and 18S rRNA



the pooled samples were tested for the presence of rpL32 mRNA (5'-TOP), β -actin mRNA (non-5'-TOP), and 18S rRNA (experimental control) by real-time RT-PCR.

RVPO induction and characterization

Male cats were subjected to RVPO by means of partial occlusion of the right pulmonary artery. At 24 h, banding of the animals resulted in a significant increase in several hypertrophic growth indices including RV weight/LV weight ratio, RV weight/tibia length ratio, and RV weight/liver weight ratio (Table 1). We also measured markers of mTOR activity that are associated with cardiac hypertrophy, including S6K1 activation (measured as Thr 389 phosphorylation), rpS6 protein accumulation, and rpS6 phosphorylation (Fig. 2A–C). At 24 h, RVPO induced a significant increase in the amount of phosphorylated Thr 389 as compared to controls. A significant increase in Thr 389 phosphorylation was observed after 48 h and 1 week of RVPO, however, it was significantly less than in 24 h. S6 protein content, as measured by Western blot, coincided with several hypertrophic indices as accumulation of ventricular mass increases significantly following 24 h of RVPO (Table 1).

Effects of PO on polysomal mobilization of L32

To determine the effects of pressure overload on the translational activation of 5'-TOP mRNAs, samples from surgical models of 24 h, 48 h, 1 week and 4 week (RVPO) were examined for rpL32 mRNA. Figure 3A shows that in the RV control, under basal conditions, approximately 25% of the rpL32 mRNA is found in polysomes. Following 24 h and 48 h of RVPO, the proportion of rpL32 mRNA significantly increased to approximately 58% and 44%, respectively. At 1 week and 4 week, the percentage of rpL32 mRNA associated with polysomes returned to control values, indicating that a new steady-state had been

achieved. During the course of RVPO, the proportion of rpL32 mRNA found in the same animal LV controls remained approximately the same, indicating that these shifts in mobilization are due to pressure overload. To determine if this distributional shift into polysomes was indeed a specific increase in the translational activation of 5'-TOP mRNA, the proportion of β -actin mRNA associated with polysomes was measured and taken as a control for mRNA that does not have a 5'-TOP (Fig. 3B). As an additional control, β -MHC, whose mRNA expression levels increase dramatically in response to pressure overload were also examined. Unlike the changes observed with rpL32 mRNA, a major proportion (approximately 75–85%) of β -actin mRNA and β -MHC mRNA was associated with polysomes and remained constant at all time points of pressure overload.

While the percentage of rpL32 mRNA associated with polysomes increased transiently during the early stages of RVPO, these proportions do not indicate the functional significance in terms of the actual increase in rpL32 mRNA mobilized into polysomes. Therefore, using the same data, we calculated the relative amount of rpL32 mRNA, β -actin mRNA and β -MHC mRNA found in each sub-polysomal and polysomal fraction by dividing the values obtained in the RV by the value of the same animal LV. As shown in Fig. 4A, the control and sham-operated animals displayed little variability, as changes in rpL32 mRNA content between the RV and LV remained virtually the same. However, with 24 h of RVPO, polysome bound rpL32 mRNA increased approximately 7-fold compared to control and sham-operated animals. When compared to the 24 h time point, a steady reduction in rpL32 mRNA at 48 h and 1 week was observed, but still remained at significantly higher levels (4-fold and 2-fold, respectively) than controls. By 4 week after banding, rpL32 mRNA mobilization values return to control and sham levels, indicating that a new steady-state level of ribosomal protein synthesis had been achieved. Figure 4B and C shows the corresponding relative quantification of β -actin and β -MHC mRNA, where 24 h of RVPO resulted in a significant increase

Table 1 Postmortem analysis of right ventricular pressure overloaded felines

Cats	n	BW (kg)	RVW (g)	LVW (g)	RV/LV	TL (cm)	RV/TL	LW (g)	LW/BW	ASP	ADP	RVSP	RVEDP
Con	3	3.3 ± .12	2.0 ± .17	7.4 ± .18	.27 ± .01	9.8 ± .37	.20 ± .02	98.1 ± 3.2	29.7 ± 4.3	133.0 ± 10.3	101.1 ± 6.8	23.5 ± 1.8	3.6 ± .80
Sham	3	3.6 ± .23	2.0 ± .12	8.6 ± .46	.23 ± .05	10.7 ± .30	.19 ± .05	100.2 ± 9.5	27.8 ± 5.2	129 ± 7.7	93.1 ± 5.2	30.3 ± 2.2	4.0 ± 1.4
24 h	5	3.5 ± .18	2.9 ± .27*	8.4 ± .65	.34 ± .01*	10.3 ± .33	.28 ± .03*	101.1 ± 4.0	28.8 ± 1.4	132.5 ± 12.5	87.2 ± 7.5	41.5 ± 3.2*	11.0 ± 3.0*
48 h	4	3.8 ± .18	3.0 ± .15*	8.2 ± .48	.38 ± .01*	10.4 ± .19	.29 ± .02*	94.1 ± 3.5	24.7 ± 1.4	126.7 ± 4.8	83.2 ± 3.2	39.8 ± 1.4*	14.5 ± 1.4*
1 week	4	3.2 ± .19	2.9 ± .04*	6.9 ± .26	.41 ± .01*	10.3 ± .45	.28 ± .01*	105.8 ± 9.0	33.1 ± 1.4	138.9 ± 10.8	90.7 ± 15.9	47.2 ± 3.5*	9.0 ± 1.5*
4 week	4	3.7 ± .40	4.3 ± .47*	9.2 ± 1.4	.47 ± .05*	10.7 ± .38	.39 ± .07*	98.2 ± 11.5	26.5 ± 1.6	140 ± 5.3	98.8 ± 4.4	67.0 ± 8.1*	12.2 ± 2.0*

Results are presented as means ± standard errors of means. BW, body weight; RVW, right ventricle weight; LVW, left ventricle weight; TL, tibia length; LW, liver weight; ASP, aortic systolic pressure; ADP, aortic diastolic pressure; RVSP, right ventricle systolic pressure; RVEDP, right ventricle end diastolic pressure. *, $P < 0.05$ compared to Con

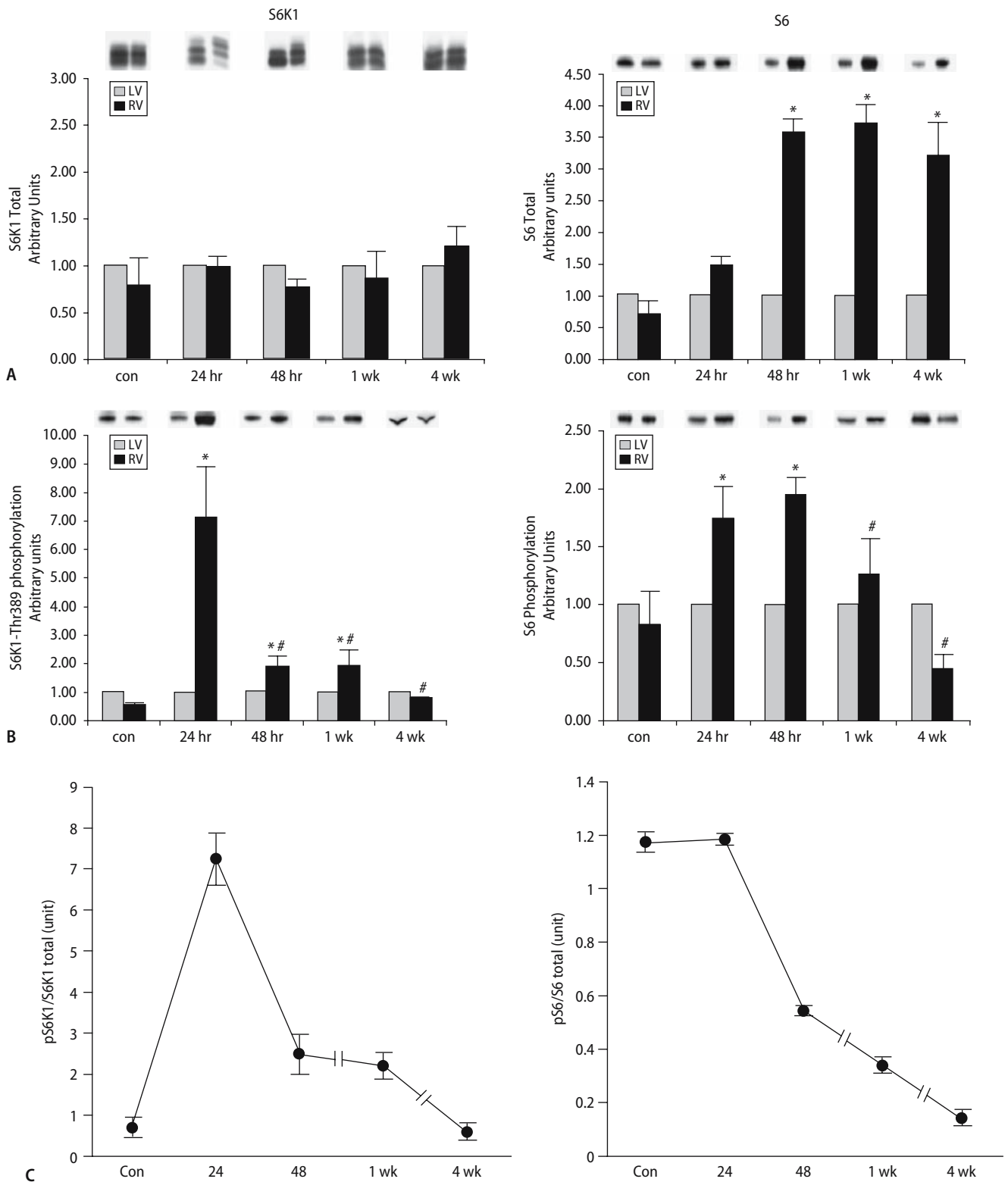
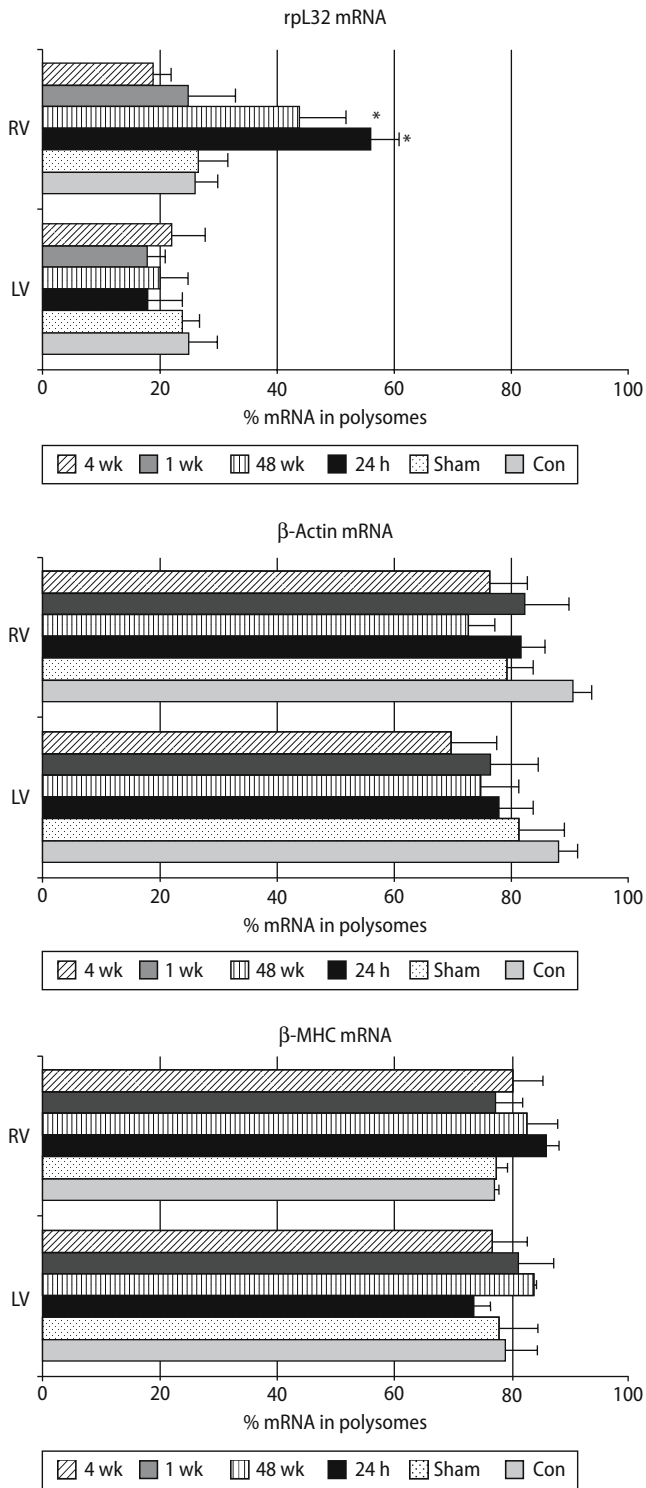


Fig. 2 Activation of S6K and S6 in response to RVPO: Triton X-100 extracts from left and right ventricles (LV and RV) obtained from indicated time points were subjected to Western blot with indicated antibody. **(A)** Total protein levels of S6K1 and S6. **(B)** Phosphorylation state of S6K1 and S6. **(C)** Ratio of

phosphorylation to total protein. Graphs show data generated from at least three experiments per group. *, $P < 0.05$ compared to RV control. #, $P < 0.05$ compared to RV 24 h PO



in both sub-polysomal and polysomal fractions, indicating that unlike the specific translational activation of rpL32 mRNA, both general transcription and translational mechanisms are activated for β-actin and β-MHC.

Fig. 3 Effects of RVPO on rpL32 mRNA polysomal association: Adult cats were used for right-ventricular pressure overload (RVPO) by partial occlusion of the pulmonary artery. Non-operative (Con) and sham operated (Sham) animals serve as controls. Post-operative times are indicated in the figure. Ventricular tissue samples were processed for polysomal fractionation as described in the *methods* section. Values are shown as percentage of rpL32 5'-TOP mRNA (top panel), and the non-5'-TOP mRNA β-actin (middle panel) and β-MHC (bottom panel) found in corresponding polysomal fraction compared to total fractions (sub-poly+poly). Graphs show data generated from at least three experiments per group. *, $P < 0.05$ compared to RV control

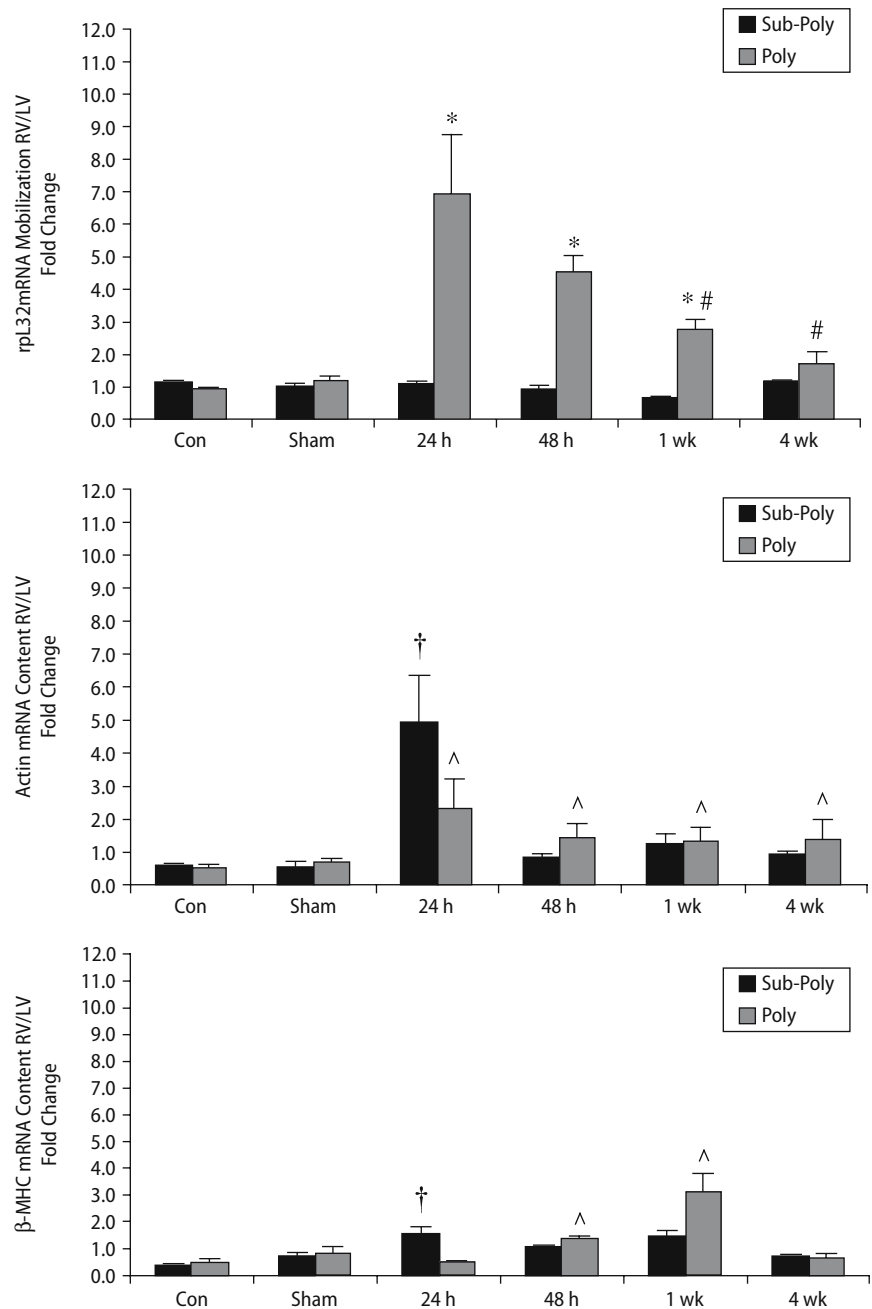
Endothelin stimulates L32 mRNA polysomal mobilization in primary culture of adult cardiocytes

Previous work has shown that S6K1 activation and S6 phosphorylation observed in the early stages of pressure overload were dependent on the PKC pathway [9]. Based on this result and the observation that endothelin act as an early paracrine activator as the result of mechanical deformation [43], we sought to test if endothelin treatment could drive rpL32 mRNA into the translationally active polysome fractions. As shown in Fig. 5A, endothelin significantly induced the mobilization of rpL32 mRNA into the translationally active polysome fraction as compared to quiescent control cardiocytes. Insulin, which is well established as a potent activator of translation, significantly increased the amount of rpL32 mRNA associated with polysomes. Pre-treatment with the mTOR inhibitor, rapamycin, almost completely blocked both endothelin and insulin stimulated mobilization. Pre-treatment with the PI3K inhibitor, wortmannin, also completely blocked insulin stimulated rpL32 mRNA mobilization, however, wortmannin only partially blocked endothelin stimulated rpL32 mRNA mobilization.

RGD stimulates L32 polysomal mobilization in primary culture of adult cardiocytes

ECM degradation as a result of tissue remodeling during cardiomyocyte growth is a well-characterized phenomena [8, 20]. Indeed, studies in our laboratory have shown specific increases in the RGD containing ECM constituents, fibronectin and vitronectin, during the early stages of hypertrophic growth [16]. Based on previous work that shows that RGD peptide can evoke mTOR signaling in adult cardiocytes through a process involving endocytosis [3], we asked whether RGD can trigger an up-regulation of ribosomal protein translation and therefore act as a possible secondary stimulant during pressure overload. Figure 6 shows that treatment of cardiocytes with RGD peptide resulted in a significant increase in the amount of rpL32 mRNA found in the polysomal fraction when compared to quiescent controls. This translational activation was specific for 5'-TOP mRNA as β-actin mRNA polysome

Fig. 4 Quantitative measurement of sub-polysomal and polysomal rpl32 mRNA in response to RVPO: Same data generated from real time RT-PCR to determine the effects of RVPO on rpl32 and β -Actin mRNA polysomal association was used to calculate the respective relative amounts by the equation described in *methods*. Values in top panel (rpl32 mRNA), middle panel (β -actin mRNA), and bottom panel (β -MHC mRNA) are shown as the ratio of RV versus same tissue LV in sub-polysome fraction (Sub-Poly) and polysome fraction (Poly). All data normalized to recovered 18S content. Graphs show data generated from at least three experiments per group. (Top panel), *, $P < 0.05$ compared to control L32 (Poly). #, $P < 0.05$ compared to 24 h L32 (Poly). (Middle panel), †, $P < 0.05$ compared to Con β -actin (Mono). ^, $P < 0.05$ compared to Con β -actin (Poly). (Bottom panel), †, ^, $P < 0.05$ compared to Con β -MHC (Mono). ^, $P < 0.05$ compared to Con β -MHC (Poly)



content remained unchanged compared to controls. Furthermore, the translational activation of 5'-TOP in response to RGD treatment is comparable to the increases seen with 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent activator of PKC and mTOR.

■ mTOR activation in RVPO and primary culture of adult cardiocytes

To further support our contention that the mTOR-mediated pathway regulates 5'-TOP mRNA transla-

tional activation during RVPO and with endothelin and RGD treatment in vitro, we wanted to test directly if mTOR activity is increased in response to these hypertrophic stimuli. As shown in Fig. 7A, 24 and 48 h of RVPO resulted in increased mTOR phosphorylation when compared to control LV or RV, and this phosphorylation returned to baseline level in 1- and 4-week RVPO. Treatment of cardiocytes with endothelin and RGD, but not a control peptide RGE (Gly-Arg-Gly-Glu-Ser) resulted in increased mTOR phosphorylation as compared to untreated controls

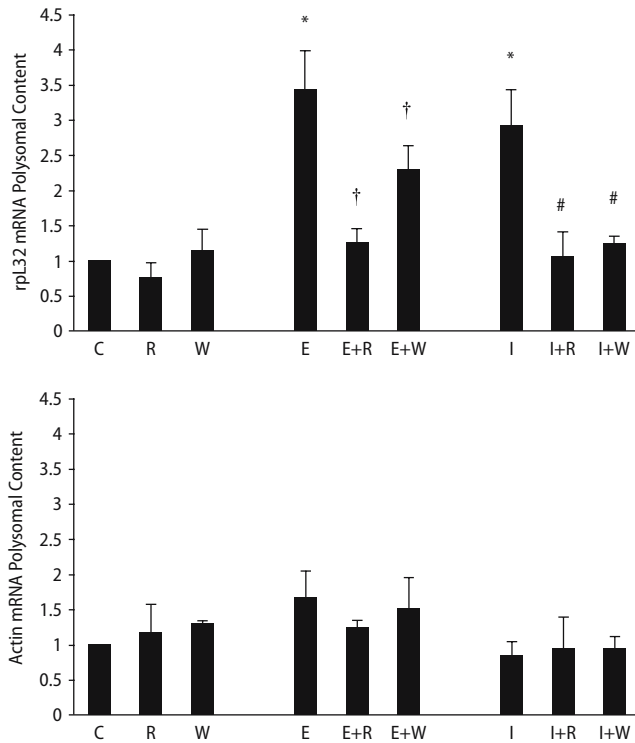


Fig. 5 Quantitative changes of L32 mRNA distribution following endothelin stimulation: Primary cultures of adult feline cardiocytes were left as untreated controls (C), or treated for 30 min with either 20 nM rapamycin (R) or 100 nM wortmannin (W). Additional cardiocytes pretreated in this manner were used for subsequent stimulation for 1 h with either 200 nM endothelin (E) or 100 nM insulin (I). Cells were processed for polysomal separation and the polysome-bound rpl32 mRNA (top panel) and β -actin mRNA (bottom panel) were then analyzed. Values are given as fold change difference of treated versus untreated controls. All data normalized to recovered 18S content. Graphs show data generated from at least three experiments per group. *, $P < 0.05$ compared to rpl32 control. †, $P < 0.05$ compared to rpl32endothelin. #, $P < 0.05$ compared to rpl32 insulin

(Fig. 7B). Stimulants such as TPA and insulin also resulted in increased mTOR phosphorylation.

Discussion

To compensate for an increase in load, the cardiomyocyte must activate the translational machinery required to achieve higher rate of protein synthesis for hypertrophic growth. Clearly, translational efficiency mechanisms, such as eIF4E phosphorylation, eIF4F complex formation, 4EBP phosphorylation, and mTOR/S6K1 activation, are key processes involved in hypertrophic growth [23, 42, 45]. These mechanisms increase the efficiency of existing translational components, but what of translational capacity mechanisms in the face of sustained pressure overload? Capacity requires coordinate increases in rRNA syn-

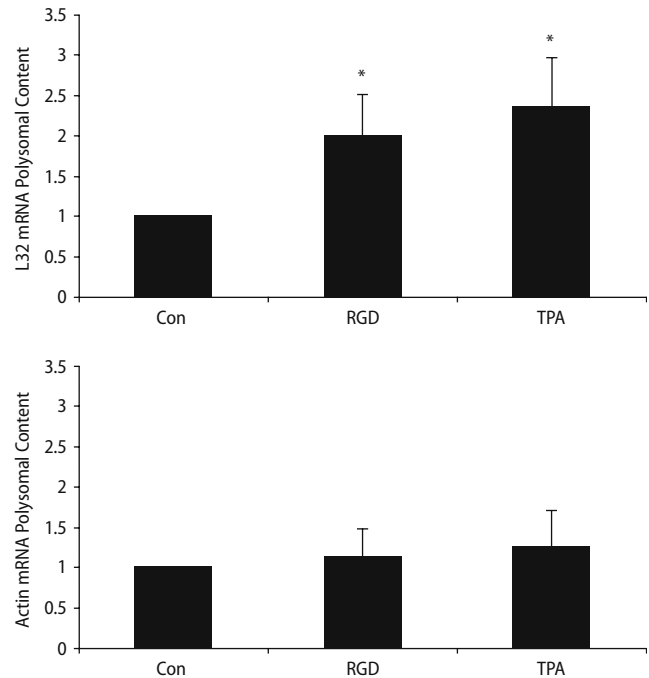


Fig. 6 Quantitative changes of L32 mRNA distribution following RGD treatment: Primary cultures of adult feline cardiocytes were left as untreated controls (Con), or treated with 9 mM RGD for 2 h (RGD), or 100 nM TPA for 1 h. Values are given as fold change difference of treated versus untreated control of rpl32 mRNA (top panel) and β -actin mRNA (bottom panel). All data normalized to recovered 18S content. Graphs show data generated from at least three experiments per group. *, $P < 0.05$ compared to rpl32 Control

thesis and the synthesis of ribosomal proteins in order to expand the pool of ribosomes.

The regulation of 5'-TOP mRNA for ribosomal expansion has been documented in a variety of tissues and cell lines, but to our knowledge not in cardiac tissue or cardiocytes [4, 32]. The choice of rpl32 mRNA as an endogenous marker of 5'-TOP translational activation was 3-fold. First, of the approximately 200 or so 5'-TOP family members, rpl32 has a relatively short 5'-UTR which in our opinion reduces the likelihood of additional cis-trans regulation occurring in the 5'-UTR. Second, reports using other tissue or cell system models, have used rpl32 mRNA in their studies characterizing 5'-TOP regulation, thereby providing a standard to compare our results [12, 46]. Third, although other 5'-TOP candidates, such as poly A binding protein (PABP), rpS16, and p40, demonstrated similar patterns of translational activation, rpl32 exhibited the best PCR efficiency and therefore provided the most accurate read-out. While it is generally accepted that increases in ribosomal biogenesis precede hypertrophic growth, the temporal relationship over 4 week has not been attempted nor has a technique as sensitive as real-time RT-PCR been used to document these changes. In

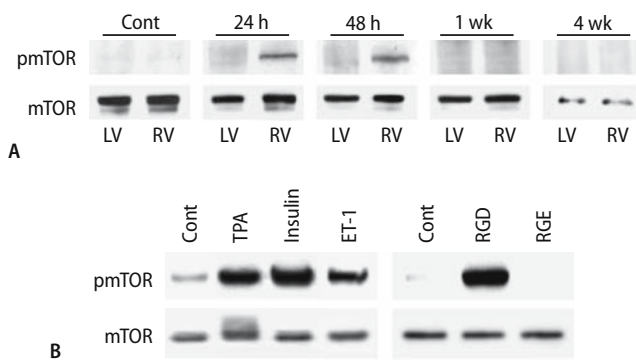


Fig 7 mTOR phosphorylation in response to RVPO and in vitro stimulation of cardiocytes with agonists: Triton X-100 soluble cell extracts were analyzed by Western blotting using mTOR and pmTOR antibodies. **(A)** Extracts from left and right ventricles (LV and RV) obtained from indicated time points. **(B)** Primary culture of adult feline cardiocytes were left as untreated controls (Con), or treated for 1 h as follows: 100 nM TPA, 100 nM insulin, 200 nM endothelin, and 9 mM RGD or RGE and used for Triton X-100 extraction. Results confirmed in at least two individual experiments

addition, many reports using either in vivo models or primary culture to study hypertrophic growth mechanisms have used S6K1 activation and ribosomal protein S6 phosphorylation as a marker for ribosomal biogenesis. However, recent studies have now shown that 5'-TOP regulation can occur via a mechanism that is mTOR dependent yet does not require S6K1 and S6 activation. Therefore, the goal of this study was to examine the translational regulation of 5'-TOP mRNA in both pressure overloaded myocardium and agonist stimulated adult cardiomyocytes and determine whether these hypertrophic stimuli contribute to ribosomal biogenesis through the activation of a common mediator, namely mTOR.

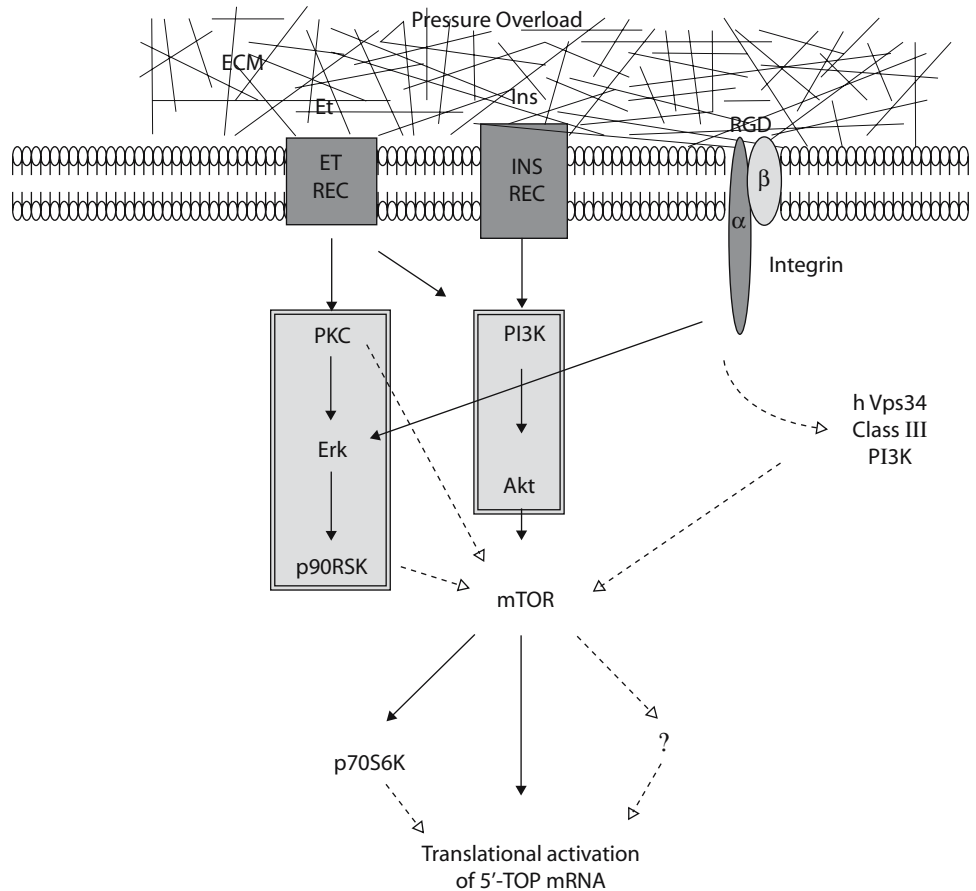
Several studies have shown that the regulation of 5'-TOP translation can be controlled by a variety of metabolic effectors including growth factors, mitogenic stimulation, amino acid sufficiency, and the energy status of a cell [5, 11]. Here, we show that pressure overload is a potent growth stimulus that facilitates the mobilization of 5'-TOP mRNA. We come away with two important conclusions regarding the in vivo model: 1) the heart possesses a remarkable ability to respond to pressure overload and that it does so rapidly in the early stages of hemodynamic overload. Indeed, significant shifts in polysomal distribution of rpL32 mRNA were observed at 24 h and 48 h (Fig. 3). Interestingly, after 1 week, and out to 4 weeks, rpL32 mRNA distribution resembled what was observed in the controls, demonstrating that even though the heart is subjected to a sustained overload the signaling events that occur early on have a significant impact on eventual hypertrophic growth. 2) the magnitude of the translational activation of rpL32 mRNA during the acute phase of hypertrophic growth

is striking. We found that rpL32 mRNA accumulation into the translationally active polysomes is 7-fold higher than what is found in controls (Fig. 4). Furthermore, protein expression levels of the ribosomal protein S6, itself a member of the 5'-TOP mRNA class and therefore serves as a ribosomal protein expression marker, accumulates to a 3-fold increase over controls at 48 h and beyond (Fig. 2A). As this accumulation of rpS6 progresses during RVPO, a corresponding reduction in the phosphorylation of mTOR, S6K1, and S6 (displayed as a ratio of phosphorylation/total protein content, Fig. 2C) was observed. These results further underscore the concept that the heart utilizes translational efficiency mechanisms during the acute phase of pressure overload which over a period of time give way to capacity mechanisms as more translational components are generated to normalize wall stress.

Next we examined whether 5'-TOP translational activation can occur in cardiomyocytes upon stimulation with hypertrophic agents that mediate their effects through multiple signaling pathways. Since our previous work demonstrates that mTOR and S6K1 activation can be induced via PI3K-dependent and independent mechanisms [9], we used a panel of stimulants that were found to activate mTOR and S6K1 pathways in adult cardiomyocytes. For this, we used the following agents: (i) endothelin, which is known to activate mTOR via a PKC dependent and PI3K independent pathway (unpublished observation), (ii) insulin, which activates mTOR and S6K1 via a PI3K/Akt pathway, and (iii) RGD peptide, that activates mTOR and S6K1 via a process that involves activation of $\beta 3$ integrin and endocytosis of RGD. Stimulation of cardiomyocytes with all of these agents resulted in the translational activation of 5'-TOP indicating that these agents on their own, or in concert, may have an impact on ribosomal biogenesis during pressure overload hypertrophy. Furthermore, based on this study and our previous studies, these effects are dependent upon mTOR, as mTOR was found to be phosphorylated (active) under these conditions and the mTOR inhibitor, rapamycin, blocks the translational activation of rpL32 mRNA.

With respect to the individual agonist, we show here that endothelin can significantly increase the mobilization of 5'-TOP mRNA. Indeed, the increases seen with endothelin were equal to what we see with insulin, considered to be one of the most potent stimuli of translational regulation. Of particular interest were the differential effects of the mTOR inhibitor, rapamycin and the PI3K inhibitor, wortmannin on endothelin stimulated rpL32 mRNA mobilization. Rapamycin completely blocked endothelin and insulin stimulated mobilization, indicating that both mitogens converge at mTOR to induce

Fig 8 Schematic diagram illustrating proposed pathways that connect pressure overload and associative paracrine events with mTOR mediated 5'-TOP translational activation: Pressure overload acts as the primary hypertrophic growth stimulus that may trigger additional paracrine effectors such as insulin, endothelin and RGD/integrin activation. In turn, these anabolic stimuli utilize distinct signaling pathways that ultimately converge at mTOR to regulate 5'-TOP mRNA translation. Endothelin predominately works through the PKC mediated pathway. Insulin (Ins) predominately uses the PI3K/Akt pathway. RGD/integrin association results in mTOR and 5'-TOP mRNA translational activation that is wortmannin sensitive, yet proceeds without AKT activation without AKT activation suggesting that an alternative class of PI3K may mediate 5'-TOP mRNA translational activation. Solid arrows indicate a direct interaction, dotted arrows indicate putative interactions



5'-TOP mobilization. Wortmannin, on the other hand, resulted in the complete inhibition of insulin stimulated L32 mRNA mobilization but only partial inhibition of endothelin stimulated L32 mRNA mobilization, indicating that endothelin activation utilizes additional pathways other than PI3K for L32 mRNA mobilization. This observation stands in contrast to what has been reported in other cell types such as mouse embryonic fibroblast (MEF) and PC12 cells in which translational activation of 5'-TOP mRNA was shown to be fully reliant on the PI3K pathway [38]. A viable candidate for the PI3K independent activation of 5'-TOP mRNA translation could be a PKC isoform that directly interacts with mTOR. Precedent exists in which PKC δ has been shown to co-immunoprecipitate with mTOR in 293T cells [14], however this association has yet to be established in cardiomyocytes. Another possible candidate could be p90 Rsk. Rolfe et al. [33] has shown in adult rat ventricular cardiomyocytes (ARVC) that p90 Rsk through PE stimulated Erk activation was able to phosphorylate distinct sites of tuberous sclerosis complex 2 (TSC2), thereby alleviating mTOR inhibition.

Our result showing that RGD treatment of adult cardiomyocytes increased the translational activation

of rpL32 mRNA, while novel, was not unexpected. Based in conjunction with previous results [3], our contention that the translational activation of 5'-TOP mRNAs seen with RGD treatment is regulated by mTOR is supported by the following two observations. First, mTOR itself is phosphorylated in response to RGD stimulation, and its activity, as measured by S6K1 Thr389 phosphorylation, is blocked by rapamycin. Second, the mobilization of L32 mRNA in response to RGD stimulation is blocked by rapamycin (data not shown). In addition, the translational activation of 5'-TOP mRNA through RGD stimulation appears to occur through a process that is wortmannin sensitive yet AKT/PKB independent, since our earlier studies indicate that RGD stimulated mTOR/S6K1 activation occurred independent of Akt activation but was still sensitive to wortmannin. Subsequent work by Nobukuni et al. [26] has now shown that amino acids signaling to mTOR occurs through hVps34, a class III PI3K. Together, these results suggest that amino acid stimulation and RGD stimulation, triggered through integrin activation, may utilize a similar pathway that lead to increased 5'-TOP translational activation. As depicted in Fig. 8, our studies demonstrate that

pressure overload is a potent stimulant of 5'-TOP translational activation and that distinct hypertrophic agonist utilize multiple pathways that ultimately converge to activate mTOR and increase the translation of 5'-TOP mRNA.

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