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Original article

p90^{RSK}s mediate the activation of ribosomal RNA synthesis by the hypertrophic agonist phenylephrine in adult cardiomyocytes

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ABSTRACT

Cardiac hypertrophy involves the growth of heart muscle cells and is driven by faster protein synthesis which involves increased ribosome biogenesis. However, the signaling pathways that link hypertrophic stimuli to faster ribosome production remain to be identified. Here we have investigated the signaling pathways which promote ribosomal RNA synthesis in cardiomyocytes in response to hypertrophic stimulation. We employed a new non-radioactive labeling approach and show that the hypertrophic agent phenylephrine (PE) stimulates synthesis of 18S rRNA (made by RNA polymerase I) and 5S rRNA (produced by RNA polymerase III) in adult cardiomyocytes. In many settings, rRNA synthesis is driven by rapamycin-sensitive signaling through mammalian target of rapamycin complex 1 (mTORC1). However, the activation of rRNA synthesis by PE is not inhibited by rapamycin, indicating that its regulation involves other signaling pathways. PE stimulates MEK/ERK signaling in these cells. Inhibition of this pathway blocks the ability of PE to activate synthesis of 18S and 5S rRNA. Furthermore, BI-D1870, an inhibitor of the p90^{RSK}s, protein kinases which are activated by ERK, blocks PE-activated rRNA synthesis, as did a second p90^{RSK} inhibitor, SL0101. BI-D1870 also inhibits the PE-stimulated association of RNA polymerase I with the rRNA promoter. These findings show that signaling via MEK/ERK/p90^{RSK}, not mTORC1, drives rRNA synthesis in adult cardiomyocytes undergoing hypertrophy. This is important both for our understanding of the mechanisms that control ribosome production and, potentially, for the management of cardiac hypertrophy.

1. Introduction

In the adult myocardium, growth is mediated through changes in the size of the cardiomyocytes. A major driver of cell growth is the rate of protein synthesis, reflecting the fact that the majority of dry cell mass is protein. Consistent with this, agents that induce cardiomyocyte growth (hypertrophy) activate protein synthesis (reviewed in [1]). Cardiac hypertrophy (CH) occurs in response, e.g., to increased load, and is initially adaptive. However, in conditions of continued stress, CH leads to loss of cardiomyocytes and to tissue fibrosis, becoming a major risk factor for heart failure. A better understanding of the molecular mechanisms that link pro-hypertrophic stimuli to increased protein synthesis in cardiomyocytes is needed to develop therapeutic strategies for CH.

Cellular protein synthesis rates are determined both by the overall levels of ribosomes and translation factors ('translational capacity') and by their intrinsic activities ('translational efficiency'). The hypertrophic agonist PE rapidly stimulates protein synthesis in ARVC [2]. This effect is markedly inhibited by rapamycin, indicating that it is mediated through the mammalian target of rapamycin complex 1, mTORC1. Activation of mTORC1 by hypertrophic stimuli such as PE in ARVC is mediated by MEK/ERK signaling [3–6]. Early studies demonstrated that ribosome content increases in hypertrophying rat heart and that increased ribosome synthesis is an early event in hypertrophy [7,8]. However, it is unclear how hypertrophic stimuli promote ribosome biogenesis in cardiomyocytes.

Production of new ribosomes requires synthesis of four ribosomal RNAs (rRNAs) and about 80 ribosomal proteins (Rps) [9]. The three larger rRNAs are made in the nucleolus by RNA polymerase I (Pol I)

Abbreviations: 4SU, 4-thiouridine; ARVC, adult rat ventricular cardiomyocytes; CH, cardiac hypertrophy, ChIP, chromatin immunoprecipitation; ERK, extracellular ligand-regulated kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; p90^{RSK}, 90 kDa ribosomal protein S6 kinase; PE, phenylephrine; PKB, protein kinase B (also termed Akt); Pol I (or Pol III), RNA polymerase I/III; Rp, ribosomal protein; rRNA, ribosomal RNA; UBF, upstream binding factor.

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as a precursor, processing of which yields the mature 5.8S, 18S and 28S rRNAs. The 5S rRNA is made by Pol III. Studies in cell lines have revealed that mTORC1 can regulate Pol I and Pol III [9–11].

Previous work on ribosome biogenesis in cardiomyocytes used neonatal cardiomyocytes (e.g., [12]), which are far easier to make and work with than adult cardiomyocytes. Here, we have studied the regulation of rRNA synthesis in primary adult rat cardiomyocytes, where PE drives cell growth [13]. We show that PE stimulates the synthesis of the 18S and 5S rRNAs, implying that it activates Pol I and Pol III, respectively. These effects are blocked by inhibition of MEK, indicating a requirement for MEK/ERK signaling. However, PE-activated rRNA synthesis is not blocked by rapamycin indicating that, unusually, mTORC1 does not mediate the activation of rRNA production in this setting. Instead, the stimulation of rRNA synthesis by PE requires the activity of $p90^{RSK}$s, kinases downstream of ERK [14]. Thus, in cardiomyocytes stimulated with a hypertrophic agonist, MEK/ERK/$p90^{RSK}$ signaling, and not mTORC1, appears to be the major driver of activated rRNA synthesis.

2. Materials and methods

2.1. Materials

Chemicals for cardiomyocyte isolation were purchased from BDH-Merck (Poole, UK) or Sigma-Aldrich unless otherwise stated. Bovine serum albumin (BSA, fraction V) was from Boehringer Mannheim and Collagenase (type II) from Worthington Biochemical, New Jersey. Nylon monofilament filter cloth was from Cadish Precision Meshes. Dialysis tubing of size 5 (MW cut-off: 12–14000 Da) was from Medicell International. One mL pastettes were from Alpha Laboratories. Vacuum filter unit Stericup sterile PES membrane (0.22 μm pore size) was from Millipore.

All chemicals and biochemicals for RNA isolation, including 4-thiouridine (4SU), and for the chromatin immunoprecipitation assay were obtained from Sigma-Aldrich unless otherwise stated. Tissue culture reagents were provided by Invitrogen. Trizol was from Invitrogen and EZ-Link Biotin-HPDP was from Pierce. Magnetic Porous Glass (MPG) Streptavidin beads were purchased from PUREBiotech. Actinomycin-D, PP242 and rapamycin were supplied by Calbiochem, PD184352 from Toronto Research Chemicals, and AZD6244 from Selleck Chemicals. BI-D1870 was obtained from the Division of Signal Transduction Therapy, College of Life Sciences, University of Dundee (UK). A769662 and SL0101 were from TOCRIS Bioscience. L-[^{35}S]methionine and [^{3}H] uridine were from PerkinElmer. Econofluor scintillation liquid was from Packard Instruments. Real Time-PCR primers and 2× SYBR Green qPCR mastermix were purchased from PrimerDesign. 3MM filter paper was from Whatman International and nitrocellulose transfer membrane (0.45 μm pore size) from Bio-Rad.

All phosphospecific antibodies and anti-ribosomal protein S6 were from Cell Signaling Technology. Anti-α-tubulin and anti-UBF (H-300) were from Santa Cruz Biotechnology, as were antibodies for IP of Pol I (sc-46699) and Pol II (sc-47701). Appropriate marker proteins were included on all gels and immunoblots, and were used to ensure that the antibodies did indeed detect the protein bands of the expected sizes.

2.2. Preparation, maintenance and treatment of ARVC

Adult rat ventricular cardiomyocytes were isolated and maintained in culture as described [15,16]. Adult male Sprague–Dawley rats (250–300 g) were from the Biomedical Research Facility, University of Southampton.

2.3. Assay of uridine uptake into ARVC

Uridine transport was monitored in cultured ARVC cells in medium either containing or lacking Na^+ ions, using a method based on those used in earlier studies [17–19].

2.4. Analysis of RNA dynamics using 4SU labeling

The synthesis and decay of labeled RNA were isolated and measured as previously described [20,21]. Briefly, 4SU is incorporated into newly-synthesized RNA; following cell lysis, 4SU tagged RNA is covalently coupled to biotin, and isolated on streptavidin beads. After reverse-transcription to cDNA, the quantity of any RNA of interest can be determined by real-time PCR using appropriate primers.

2.5. qChIP analysis

Chromatin immunoprecipitation (ChIP) was performed as described previously with modifications [22,23]. Cross-linking was achieved with 1% formaldehyde for 5 min at an ambient temperature and the cells were sonicated eighteen times for 30 s.

Primers for qPCR analysis of ChIP reactions were β-actin (Primerdesign, Southampton, UK), ENH (sense: AGGAGGCCGGGCA AGCA, antisense: CCTCCTTGTTAGAGACCGTCCTTAA), UCE (sense: AGTTGTTCCTTTGAGGTCCGGT, antisense: AGGAAAGTGACAGGCCAC AGAG) and CORE (sense: AGTTGTTCCTTTGAGGTCCGGT, antisense: CAGCCTTAAATCGAAAGGGTCT). The relative level of DNA binding was analysed using primers specific for the indicated regions of the Pol I promoter by qRT-PCR. Average values of precipitated DNA were normalized to input control.

3. Results and discussion

3.1. PE stimulates the synthesis of new rRNAs in ARVC

We applied a non-radioactive procedure to tag and quantify newly-made rRNA whereby cells are incubated with 4SU which is incorporated into new RNA molecules. Following cell lysis, labeled RNA molecules are covalently linked to biotin and then isolated. After reverse transcription (RT), quantitative real-time PCR (RT-qPCR) is used to measure the levels of any RNA molecule of interest.

To assess whether transferring the cells to medium containing 4SU affected their response to PE, we incubated ARVC for 30 min in medium containing or lacking 4SU to treatment with PE for times up to 1 h. As expected from our earlier studies [2], PE rapidly induced the phosphorylation (activation) of ERK (Fig. 1A). The presence of 4SU did not affect this. To assess activation of mTORC1 signaling, we looked at the phosphorylation of Rp S6 (Fig. 1A). As reported earlier [2], PE increased S6 phosphorylation and neither the timing nor the extent of this response was affected by 4SU (Fig. 1A).

Activation of rRNA transcription has previously been shown to be slow in neonatal cardiomyocytes [12]. Therefore, to assess whether PE activates rRNA synthesis, ARVC were treated with PE for 20 h prior to the addition of 4SU. In some cases, cells were treated with actinomycin D (2μg/mL), which inhibits transcription by Pol I, Pol II and Pol III, prior to addition of 4SU. In the absence of actinomycin D, we observed labeling of transcripts made by each of these polymerases (18S rRNA, Pol I; actin, Pol II; and 5S rRNA, Pol III; Fig. 1B). Incorporation of 4SU into each of them was enhanced by PE, indicating that PE stimulates the transcription of each of these genes, including rRNAs. Importantly labeling was completely blocked by actinomycin D (Fig. 1B), confirming that this method only detects newly-synthesized RNAs.

By 12 h of PE treatment, a small increase in the labeling of 18S rRNA was observed (Fig. 1C), but no significant changes in 5S or actin were apparent (Fig. 1C). After 24 h treatment, much more robust increases in all three were observed (Fig. 1C); we therefore selected this as the time-point for further experiments.

Previous work, using neonatal rat cardiomyocytes [12,24], indicated that hypertrophic stimuli increase the cellular levels of upstream binding factor (UBF; a component of the Pol I machinery) and Brf1 (a component of TFIIIB, which is a Pol III-specific transcription factor). UBF levels were also increased in vivo after one week of