Original article

p90RSKs mediate the activation of ribosomal RNA synthesis by the hypertrophic agonist phenylephrine in adult cardiomyocytes

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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]. Early studies demonstrated that ribosome content increases in hypertrophying rat heart and that increased ribosome synthesis is an early event in hypertrophy [4,5]. 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) [6]. The three larger rRNAs are made in the nucleolus by RNA polymerase I (Pol I)
as a precursor, processing of which yields the mature 5.8S, 18S and 28S rRNAs. The 5S RNA 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 p90rsk, kinases downstream of ERK [14]. Thus, in cardiomyocytes stimulated with a hypertrophic agonist, MEK/ERK/p90rsk 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 Bohringer 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 Medicon International. One mL pastettes were from Alpha Laboratories. Vacuum filter units Stericup sterile membranes (0.22 μm pore size) were 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 Tocris Bioscience. L-[35S]methionine and [3H]thymidine, [3H]uridine and BrdU 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 Tocris Bioscience. 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However, although PE activated synthesis of both 18S rRNA and 5S rRNA by 24 h, there was no detectable change in levels of UBF or Brf1 (Fig. 1D). Thus, stimulation of rRNA synthesis by PE in ARVC does not require increased levels of UBF or Brf1, but likely involves activation of pre-existing components of the Pol I/III machinery.

Before its incorporation into new RNA molecules, 4SU must enter the cells. It was therefore important to test whether relevant signaling inhibitors affected uridine uptake [26,27]. Linear uptake of [3H]uridine into ARVC was observed up to 90 s (Fig. 2A); linear rates of net uptake are not maintained at longer times, because the nucleoside transporters are equilibrative, not concentrative. PE did not affect the rate of uridine uptake (Fig. 2B). Because a previous report indicated that very high concentrations of rapamycin (10 μmol/L) inhibited uptake of [3H]uridine into human K562 cells [26], we first assessed what concentration of rapamycin was required for maximal inhibition of mTORC1 signaling in ARVC, as judged from the phosphorylation of S6 at Ser240/244, specific substrates for the mTORC1-activated S6 kinases [28]. 100 nmol/L was required to block this completely (Fig. 2C) and this concentration was used in subsequent experiments. At this concentration, rapamycin reduced the uptake of [3H]uridine into ARVC (Fig. 2B).

Importantly for the work described below, neither the MEK inhibitor AZD6244 [29] (Fig. 2D) nor the p90RSK inhibitor BI-D1870 [30] (Fig. 2E) significantly affected uridine uptake. They can therefore be used to study the regulation of rRNA synthesis without interference from effects on nucleoside transport.

3.2. Rapamycin does not block PE-stimulated rRNA synthesis

To examine whether activation of rRNA synthesis by PE requires mTORC1, we studied the effect of rapamycin. Rapamycin decreased both basal and PE-stimulated synthesis of 18S and 5S rRNA (Fig. 3A,B). The reduced basal rate of labeling likely reflects the partial impairment of uridine uptake observed in Fig. 2B. Importantly, PE still enhanced rRNA labeling in the presence of rapamycin, and to at least the same extent as the control.
extent as in its absence. Importantly, given that 100 nmol/L rapamycin completely blocks PE-induced S6 phosphorylation (Fig. 2C), these findings demonstrate that the PE-induced activation of rRNA synthesis is not mediated via rapamycin-sensitive functions of mTORC1 and thus, in this case, does not involve the S6 kinases which have been suggested to promote rRNA synthesis [reviewed [31]]. Thus, the control of rRNA synthesis by PE in ARVC differs from other settings, where mTORC1 signaling does play a major role [9].

Since some mTOR functions are resistant to rapamycin [32–35], we used PP242 [32] which, unlike rapamycin, directly inhibits mTOR’s kinase activity and blocks all functions of mTOR. As expected, PP242 blocked PE-induced phosphorylation of S6 at Ser235/236 and Ser240/244 (Suppl. Fig. S1A) without affecting ERK phosphorylation. PP242 partially inhibited basal rate 18S and 5S rRNA synthesis (Suppl. Fig. S1B). PP242 also substantially inhibited the PE-activated synthesis of both 18S rRNA and, to a lesser extent, 5S rRNA synthesis (Suppl. Fig. S1B). Nonetheless, and especially for 5S rRNA synthesis, PE was still able to stimulate rRNA transcription even in the presence of PP242, indicating that events independent of mTOR are involved. PP242, like rapamycin, inhibited [3H]uridine uptake by 40–50% in both PE-treated and control conditions (Suppl. Fig. S1C). Hence, its inhibitory effect on basal 4SU-labeling of rRNA likely reflects impaired uptake of 4SU, while the partial inhibition of the stimulatory effect of PE may reflect a genuine impairment of rRNA transcription or increased decay of new rRNA given that inhibition of mTOR affects rRNA processing [17]. Such effects could be mediated via mTORC2 or rapamycin-insensitive functions of mTORC1; the lack of a specific mTORC2 inhibitor precludes us from studying this further.

3.3. PE enhances rRNA synthesis via MEK signaling

As shown here and previously [2–4], PE activates MEK/ERK signaling in ARVC and this mediates its short-term (1–3 h) stimulatory effects on protein synthesis. To test the role of this pathway in rRNA synthesis, we used the specific MEK inhibitor, AZD6244 [29], which completely blocked PE-induced ERK activation (phosphorylation; Fig. 4A). Consistent with earlier data obtained using other MEK inhibitors [2–4], AZD6244 also largely inhibited activation of mTORC1 signaling as manifested by decreased phosphorylation of S6 (Ser240/244).

AZD6244 did not significantly affect basal 18S rRNA transcription (Fig. 4B) but completely blocked the stimulation by PE, indicating that PE activates Pol I via MEK/ERK signaling (independently of mTORC1, which is also activated by MEK/ERK in ARVC). AZD6244 did not elicit activation of AMPK (which can negatively regulate the
Pol I machinery [36]), which is caused by some other MEK inhibitors [37,38] (Fig. 4A).

A second MEK inhibitor, PD184352 [39], also completely blocked PE-induced 5S and 18S rRNA synthesis, without affecting basal synthesis rates, confirming the importance of MEK in this response (Suppl. Fig. S2). However, because PD184352 is one of the MEK inhibitors that can activate AMPK, we did not use this compound further in this study.

3.4. Enhances rRNA synthesis via p90RSKs

To assess which signaling components mediate activation of Pol I and Pol III downstream of MEK/ERK, we used BI-D1870, a potent and selective inhibitor of the four p90RSK isoforms [30]. Earlier data (discussed in [40]) indicate that p90RSK2/3 are the main isoforms in ARVC. Our data (Fig. 5A) confirm this and that their levels are not altered by PE (Fig. 5A). p90RSKs are activated by ERK [14] and we previously showed that PE rapidly activates p90RSKs in ARVC [4], and that BI-D1870 blocks signaling through p90RSKs in these cells [41], as it also does in neonatal cardiomyocytes [42]. BI-D1870 did not impede activation of ERK by PE (Fig. 5B); rather, it potentiated it, likely because it blocks an inhibitory feedback loop, as reported earlier [30,41]. Extended treatment of ARVC with BI-D1870 (for 24 h) caused a decrease in the levels of p90RSK2, but not p90RSK3 (Fig. 5A). This may reflect a destabilizing effect of BI-D1870 on its target, the p90RSK2 protein, but could reflect an additional effect of this compound. This effect was not observed in earlier studies where cells were only treated for shorter periods with this compound (e.g., [30]).

BI-D1870 did not affect basal labeling of 5S or 18S rRNA, but completely blocked the stimulation by PE (Figs. 5C,D), indicating that activation of Pol I and Pol III by PE requires an obligatory input from p90RSKs. One potential caveat is that BI-D1870 can also interfere with activation of PKB [41,43]; however, PE does not activate PKB in ARVC [2] and Figs. 1A,5B).

To extend these data, we also used an additional p90RSK inhibitor, SL0101 [44] although this compound is a much less potent inhibitor of p90RSKs as judged from in vitro data [45]. We therefore employed it at a higher concentration than for BI-D1870 (50 μM, the concentration generally used in other studies). As shown in Figs. 5E,F, SL0101 strongly inhibited the activation of 18S and 5S rRNA synthesis caused by PE, but without affecting basal synthesis rates. The latter observation indicates it very unlikely to affect uridine uptake, while the observed inhibition of the effect of PE supports the conclusion that p90RSKs play a key role in mediating the activation of rRNA synthesis in response to PE. The incomplete inhibition likely reflects the lower potency of this inhibitor, and is consistent with the observation (Suppl. Fig. S3) that, while BI-D1870 strongly inhibits the PE-induced...
phosphorylation of the p90RSK substrate GSK3, SL0101 only does so to a lesser extent. Given we already have to use a relatively high concentration of SL0101, we were reluctant to test it at even higher levels, where it may well exert off-target effects.

An earlier study suggested that p90RSK may regulate the activity of Pol I by phosphorylating TIF-1A [46]. While this may explain the ability of BI-D1870 to inhibit 18S rRNA synthesis, it does not explain the equally strong inhibition of 5S synthesis (Fig. 5D). Since the assembly of new ribosomes requires all four rRNAs, three of which are derived from the same precursor as 18S rRNA, it was possible that inhibition of the activation of Pol I indirectly affected the stability of new 5S rRNA, perhaps because it cannot be incorporated into new ribosomes without the other rRNAs. To test this possibility, we configured the 4SU labeling experiment as a pulse-chase protocol (Fig. 6A). The data clearly show that BI-1870 causes the decay of new 18S rRNA and 5S rRNA. This effect is similar to the effects of rapamycin-induced inhibition of rRNA synthesis in HeLa cells, which also promotes decay of new rRNA [17] and may explain the observed inhibition of the accumulation of new 5S rRNA seen here.

It is potentially relevant that p90RSKs are reported to regulate mTORC1 signaling [47-49]. However, our earlier data revealed that BI-D1870 does not interfere with activation of mTORC1 by PE in ARVC [41]. Thus, p90RSKs control PE-stimulated rRNA transcription in ARVC directly, rather than via mTORC1. Interestingly, PE induces nuclear translocation of p90RSK in neonatal cardiomyocytes [50].

Earlier work implied that p90RSKs phosphorylate Ser649 in the C-terminus of TIF-1A [46]; however, there is no consensus phosphorylation site for the N-terminal kinase domain of these enzymes in this region of TIF-1A, leading those authors to suggest that phosphorylation was catalysed by the C-terminal kinase domain (which functions to activate the N-terminal kinase domain, which in turn transphosphorylates other proteins). However, BI-D1870 inhibits the N-terminal, not the C-terminal, domain of p90RSKs [30], ruling out this explanation. While human, mouse and rat TIF-1A sequences contain
the proposed ERK site at Ser633 (human numbering), the local sequence around the proposed p90RSK-regulated site does not match the consensus for these kinases and differs significantly between these species (see Fig. 4B of [46]). It seems likely that p90RSKs regulate Pol I by phosphorylating another substrate. Identifying it requires substantial additional work, which is unfeasible in ARVC. Our observation that BI-D1870 completely inhibits the PE-activated synthesis of both 18S and 55 rRNA, while also causing hyperactivation of ERK, shows that the p90RSKs play a dominant role in controlling RNA synthesis in ARVC.

Since BI-D1870 clearly inhibits the induction of rRNA synthesis by PE, we asked whether it also interfered with the rapid activation of protein synthesis by PE. At times up to about 2 h, we have already shown that this effect is mediated by MEK/ERK signaling [2,5,6]. ARVC were incubated with or without PE for 24 h; [32P]methionine was added 30 min after PE (where added). BI-D1870 clearly does not impede PE-stimulated protein synthesis; in fact, if anything, it causes an increase (Suppl. Fig. 4A), perhaps reflecting its ability to enhance ERK phosphorylation, first reported in [30] and observed in Suppl. Figs. 4B and 5B. Thus, at relatively short times up to 2 h, the activation of protein synthesis by PE presumably reflects control of the activities of components of the translational machinery (e.g., the initiation and elongation factors which are rapidly stimulated by PE [2,5]), rather than increased ribosome content. Indeed, the present data show that rRNA synthesis is only activated slowly in ARVC. Ribosome levels do increase at longer times in hypertrophying neonatal cardiomyocytes [51,52] or in vivo [7] and this effect is presumably important for the sustained faster rates of protein synthesis that lead to cardiac hypertrophy.

3.5. BI-D1870 inhibits the PE-induced recruitment of Pol I to the rRNA promoter

Since BI-D1870 clearly inhibits the PE-induced synthesis of new 18S rRNA, we used ChIP analysis to ask whether PE promoted the association of Pol I with the rDNA promoter and whether BI-D1870 affected this. We immunoprecipitated Pol I and used three different sets of primers (Fig. 7A) to study its association with different parts of the rDNA promoter. Since PE does not increase UBF levels (Fig. 1D), this is the first report of Pol I binding at rDNA sequences was analysed by qPCR using primers specific for the indicated regions of the Pol I promoter, and the values were normalized to the input control. Data are shown as mean ± SEM (n = 3). The inset shows an ethidium bromide-stained agarose gel of CORE and GAPDH products amplified after 20 PCR cycles.

4. Concluding remarks

This is the first study of the signaling events that control rRNA synthesis in adult cardiomyocytes. It shows that the hypertrophic agonist PE stimulates the synthesis of rRNA in adult rat cardiomyocytes, consistent with much earlier data showing that hypertrophy is associated with increased cardiac ribosome content and faster ribosome synthesis [7]. We show that PE stimulates the synthesis of both 5S rRNA, made by Pol III, and 18S rRNA, made by Pol I. It is important to note that the proposed ERK site at Ser633 (human numbering), the local sequence around the proposed p90RSK-regulated site does not match the consensus for these kinases and differs significantly between these species (see Fig. 4B of [46]). It seems likely that p90RSKs regulate Pol I by phosphorylating another substrate. Identifying it requires substantial additional work, which is unfeasible in ARVC. Our observation that BI-D1870 completely inhibits the PE-activated synthesis of both 18S and 55 rRNA, while also causing hyperactivation of ERK, shows that the p90RSKs play a dominant role in controlling RNA synthesis in ARVC.

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that our data indicate that MEK/ERK/p90RSK signaling, rather than signaling through mTORC1, drives rRNA synthesis in response to hypertrophic stimulation of ARVC. This finding both differentiates the control of rRNA synthesis from other settings where mTORC1 appears to play a major role [9] and also raises the possibility that this pathway may also promote rRNA synthesis in other situations, e.g., cancer cells where the corresponding pathway is activated due to oncogenic mutations in Ras or Raf. We note that p90RSK regulates the sodium/proton exchanger (NHE-1) in cardiomyocytes (reviewed in [53]) and that the NHE-1 is implicated hypertrophy ([54]). It will be important to investigate the possible role of NHE-1 in regulating rRNA synthesis, e.g., via alterations in intracellular pH.

Interestingly, just prior to submission of this article, p90RSK was shown to be required for cardiac hypertrophy in neonatal cardiomyocytes and in vivo in response to pressure overload [55], although the molecular mechanism(s) underlying its contribution to this process was not determined. Here, we demonstrate that p90RSK plays a key role in the activation of rRNA synthesis in response to hypertrophic stimulation. Increased ribosome biogenesis is likely to be important for the sustained increases in protein synthesis that underlie cardiomyocyte growth. Our findings from other groups that p90RSK inhibitors hyperblock in block neonatal myocytes [42,50,55]. It is now clearly important to establish how p90RSK activates rRNA synthesis in ARVC, insights into this may provide opportunities for intervention to prevent or reverse cardiac hypertrophy.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2013.03.006.

Disclosures
The authors have no conflicts of interest to declare.

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