Ras/Erk Signaling Is Essential for Activation of Protein Synthesis by Gq Protein–Coupled Receptor Agonists in Adult Cardiomyocytes

Lijun Wang, Christopher G. Proud

Abstract—The Gq protein–coupled receptor agonists phenylephrine (PE) and endothelin-1 (ET-1) induce cardiac hypertrophy and stimulate protein synthesis in cardiomyocytes. This study aims to investigate how they activate mRNA translation in adult cardiomyocytes. PE and ET-1 do not activate protein kinase B but stimulate Ras and Erk, and their ability to activate protein synthesis was blocked by inhibition of Ras or MEK and by rapamycin, which inhibits mTOR (mammalian target of rapamycin). These agonists activated ribosomal protein S6 kinase 1 (S6K1) and induced phosphorylation of eIF4E-binding protein-1 (4E-BP1) and its release from eIF4E. These effects were blocked by inhibitors of MEK. Furthermore, adenovirus-mediated expression of constitutively-active MEK1 caused activation of S6K1, phosphorylation of 4E-BP1, and activation of protein synthesis in a rapamycin-sensitive manner. Expression of N17Ras inhibited the regulation of S6K1 and protein synthesis by GqPCR agonists. These data point to a signaling pathway involving Ras and MEK that acts, with mTOR, to control regulatory translation factors and activate protein synthesis. This study provides new insights into the mechanisms underlying the stimulation of protein synthesis by hypertrophic agents in heart. (Circ Res. 2002;91:821-829.)

Key Words: cardiac hypertrophy ■ MEK ■ mRNA translation ■ S6 kinase 1 ■ eIF4E-binding protein-1

Control of mRNA translation plays key roles in regulating gene expression and cell growth.1,2 In mammalian cells, rapid activation of translation is mediated through the regulation of mRNA, by phosphorylation, of the activities and functions of components of the translational machinery, including initiation3 and elongation4 factors and ribosomal protein S6.5 Regulation of several of these components is sensitive to rapamycin linking them to the mammalian target of rapamycin (mTOR).6,7

Many agents that activate protein synthesis stimulate S6 kinase 1 (S6K1) and thus S6 phosphorylation. S6K1 is thought to upregulate translation of mRNAs that possess a 5′-cap structure of eukaryotic mRNAs and prevents it from forming initiation complexes by occluding the binding site of eIF4E for the scaffold protein eIF4G. 4E-BP1 contains at least 6 sites of phosphorylation.11 In response to agents such as insulin, 4E-BP1 undergoes increased phosphorylation and is released from eIF4E allowing formation of eIF4F complexes containing eIF4E, eIF4G and eIF4A. Rapamycin prevents the phosphorylation and release of 4E-BP1 identifying 4E-BP1 as a downstream target of mTOR signaling.

The signaling events involved in the regulation of both S6K and 4E-BP1 remain to be fully established. However, several studies, mostly using insulin, suggested a role for phosphatidylinositol (PI) 3-kinase and protein kinase B (PKB, also termed Akt).12–14 Another well-understood signaling pathway involved in cellular responses to growth stimuli is the classical MAP kinase (Erk) pathway involving the small G-protein Ras and the kinase cassette Raf/MEK/Erk.15

Although regulation of protein synthesis is crucial for cell growth, early studies suggested that Raf/MEK/Erk pathway was not involved in regulating S6K1 and 4E-BP1.16–18 Hypertrophy, eg, of cardiomyocytes, is a condition in which increased rates of protein synthesis are of profound importance. Cardiomyocytes are regarded as terminally differentiated cells in which adaptive hypertrophic growth in-
volves increase in protein content and cell size and changes in myofibrillar organization and gene expression. Phenylephrine (PE, an α1-adrenergic agonist) and the vasoactive peptide endothelin 1 (ET-1), both Gq protein–coupled receptor (GqPCR) agonists, exert hypertrophic effects and stimulate protein synthesis in cardiomyocytes. Increasing evidence suggests that this, and the activation of protein synthesis, requires Ras and MAP kinase signaling, in particular the MEK/Erk cascade. In this study, we used primary adult rat ventricular cardiomyocytes (ARVCs) to investigate the mechanisms underlying the activation of protein synthesis by these hypertrophic agents. We showed previously that, in ARVCs, PE/ET-1 do not activate PKB but potently stimulate Erk. In the present study, we demonstrate that activation of protein synthesis in ARVCs by these agonists is blocked by inhibition of MEK, raising the important question of how they stimulate protein synthesis via MEK. Investigation of the signaling events involved here is important for overall understanding of the molecular processes involved in cardiac hypertrophy, a condition that may ultimately lead to cardiac arrhythmia, heart failure and death.

In the present study, we demonstrate that GqPCR-agonists activate S6K1 and 4E-BP1, in a Ras-/MEK-dependent manner that is dependent on mTOR signaling. These effects are important for overall activation of protein synthesis by these stimuli.

Materials and Methods

Reagents
Reagents used are listed in the expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org.

Cell Culture and Adenovirus Infection of ARVCs
ARVCs were isolated from hearts of adult male Sprague-Dawley rats (250 to 300 g) (Charles River, Margate, Kent, UK). Details of isolation, culture, treatments, and extraction are provided in the online data supplement. For adenovirus infection, ARVCs were cultured for 2 hours after isolation before infection was performed. ARVC cultures were washed and incubated in 1 mL (for 60-mm dishes) or 3 mL (for 100-mm dishes) M199 medium containing recombinant adenoviruses for 2 to 3 hours at 37°C at the indicated multiplicity of infection (moi) as described in the figure legends. Cultures were then given fresh M199 medium and incubated for 36 hours before further treatments. We also employed an adenovirus-encoding LacZ as control and to assess infection efficiency. Infectivity was >95% based on β-galactosidase staining of cells.

Activated Ras Affinity Binding Assay
The assay was performed essentially as described in the online data supplement.

mGTP-Sepharose Chromatography
To assess the interaction of eIF4E with 4E-BP1 or eIF4G, we performed mGTP-Sepharose chromatography. Briefly, 20 μL of a 50/50 slurry of mGTP-Sepharose CL-4B was rotated with 1 mg of cell lysate protein for 1 hour at 4°C. The mGTP-Sepharose was pelleted by centrifugation, and washed thrice in extraction buffer. For SDS-PAGE, proteins bound to mGTP-Sepharose were removed by boiling in sample buffer.

Treatment of S6K1 and 4E-BP1 From ARVCs by Erk2 In Vitro
Endogenous S6K1 and 4E-BP1 were immunoprecipitated from untreated ARVCs and treated in vitro with activated Erk2. Details of experiments performed are described in the online data supplement. Other methods including SDS-Polyacrylamide Gel Electrophoresis (PAGE) and immunoblotting, in vitro kinase assays, and measurement of protein synthesis are provided in the online data supplement.

Results

Stimulation of Protein Synthesis by PE Requires MEK Activity
To study the regulation of protein synthesis by GqPCR agonists, we initially examined the effects of signaling inhibitors on incorporation of [35S]methionine into newly synthesized protein. Treatment with PE for 1 hour increased the rate of incorporation of label into protein by 60.5±7.9% versus untreated controls. This activation was inhibited by the MEK inhibitors PD184352 or U0126 implying it is dependent on MEK. PD184352 also significantly reduced basal [35S]methionine incorporation (to 76.3% of controls that received vehicle only). This may be due to the effects of this compound on the basal activity or phosphorylation of the components of translational machinery, such as S6K1 and 4E-BP1, in control cells (see below). When corrected for this effect, PD184352 almost completely inhibited activation of protein synthesis by PE (95.8% inhibition). U0126 did not affect basal [35S]methionine incorporation but again profoundly inhibited protein synthesis by PE (80.3% inhibition).

Rapamycin also markedly, although incompletely, inhibited activation of protein synthesis by PE (53.2% inhibition), indicating that mTOR signaling also plays an important role here. These data imply the existence of cross-talk between MEK- and mTOR-dependent signaling in the activation of translation. In contrast, our previous report showed that insulin, which potentiate activates PI 3-kinase/PKB pathway but only stimulates Erk1/2 very weakly in ARVC, activates protein synthesis almost independently of MEK.

Activation of S6K1 by PE and ET-1 Involves MEK
Because the above data indicated important roles for MEK and mTOR in PE-induced activation of protein synthesis, we focused on the effects of PE and ET-1 on translational regulators that are targets of mTOR. Both agents activated S6K1. Activation was first observed 30 to 60 minutes after PE treatment and generally reached a maximum (3.76±0.25-fold above controls) at about 1 hour (Figure 1A). S6K1 activity remained elevated for at least 4 hours after PE treatment (Figure 1A). Activation of S6K1 by ET-1 was maximal at 30 minutes (2.82±0.25-fold) and gradually declined thereafter (Figure 1A).

Pretreatment of ARVCs with the MEK inhibitor PD184352 dramatically reduced S6K1 activation in response to PE or ET-1, inhibition being 92.2±7.2% and 95.7±15.6%, respectively, when taking into account that this compound also slightly decreased basal S6K1 activity (Figure 1B). Similar results were obtained with another MEK inhibitor, U0126 (not shown). Activation of S6K1 involves its phosphorylation.
Ras Is Required for Activation of S6K1 by PE and ET-1

To gain insight into the receptor-proximal intracellular signaling events that mediate the effects of GqPCR activation of S6K1, we studied the role of Ras. Low levels of Ras·GTP were detected in control myocytes. PE and ET-1 rapidly increased Ras·GTP loading, which was maximal within 2 to 3 minutes and sustained for at least 10 minutes (Figure 2A, top blot). We used two approaches to perturb Ras signaling: (1) the widely-used inhibitor of farnesyl transferase (FT Inh III33) and (2) a recombinant adenovirus expressing the dominant-negative mutant, N17Ras. FT Inh III dose-dependently inhibited PE-induced Ras activation (Figure 2B, upper panel) and inhibited Erk activation, as demonstrated by immunoblotting using anti-phospho-Erk, which detects activated forms of Erk1/2 (Figure 2B, bottom panel). Although inhibition of Ras activation by FT Inh III was complete at 50 μmol/L, traces of phosphorylated Erk1/2 were still detectable (Figure 2B). Expression of N17Ras dose-dependently prevented activation of Erk1/2 by PE (Figure 2C, upper blot). Inhibition was almost complete at the highest dose (moi 200), whereas, as we reported earlier,26 infection of ARVC with the negative control LacZ virus did not affect Erk activation. Similar results were obtained with ET-1 (not shown). Ras activity is therefore required for activation of Erk1/2 by PE and ET-1 in ARVCs.

Consistent with this, activation of S6K1 by PE was markedly reduced by FT Inh III or N17Ras, inhibition being 76.2±14.2% and 64.0±11.8%, respectively (Figure 2D). The smaller inhibition of S6K1 activation compared with that caused by PD184352 or U0126 (Figures 1B and 1C) may reflect incomplete inhibition of Erk activation (Figures 2B and 2C). These results suggest that activation by PE of Ras, which probably in turn activates c-Raf and thus MEK, contributes to stimulation of S6K1 in ARVCs.

PE- and ET-1–Induced Phosphorylation of 4E-BP1 and Its Release From eIF4E Depend on MEK/Erk Signaling

Phosphorylation of 4E-BP1 was judged by its migration on SDS-PAGE. In unstimulated cells, 4E-BP1 was present as the least phosphorylated) and (2) a recombinant adenovirus expressing the dominant-negative mutant, N17Ras. FT Inh III dose-dependently inhibited PE-induced Ras activation (Figure 2B, upper panel) and inhibited Erk activation, as demonstrated by immunoblotting using anti-phospho-Erk, which detects activated forms of Erk1/2 (Figure 2B, bottom panel). Although inhibition of Ras activation by FT Inh III was complete at 50 μmol/L, traces of phosphorylated Erk1/2 were still detectable (Figure 2B). Expression of N17Ras dose-dependently prevented activation of Erk1/2 by PE (Figure 2C, upper blot). Inhibition was almost complete at the highest dose (moi 200), whereas, as we reported earlier,26 infection of ARVC with the negative control LacZ virus did not affect Erk activation. Similar results were obtained with ET-1 (not shown). Ras activity is therefore required for activation of Erk1/2 by PE and ET-1 in ARVCs.

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phosphorylation (Figure 3B and data not shown). PE and ET-1 also caused 4E-BP1 to dissociate from eIF4E (Figure 3C), as assessed by Western blotting of proteins bound to mGTP-Sepharose, and this was again blocked by inhibition of mTOR or MEK (Figure 3C).

Figure 3. Role of mTOR and MEK in the regulation of 4E-BP1 and eIF4E by PE and ET-1. A, ARVCs were treated with PE (10 μmol/L) or ET-1 (0.1 μmol/L) for the times shown. Cells were harvested and the activated Ras affinity binding assay was performed (see Materials and Methods). GTP-bound form of Ras was detected after affinity binding assay by immunoblotting with anti-pan-Ras antibody (top blot). Ras levels in cell lysates were assessed by immunoblotting with the same antibody (bottom blot). B, ARVCs were preincubated with FT inh III at the indicated concentrations for 24 hours before treatment with PE (10 μmol/L), for 5 minutes. Samples of extract were processed to assess Ras-GTP formation (top two blots, as in A) or phosphorylation of Erk1/2 (by immunoblotting; bottom two panels: upper one developed with anti–phospho-Erk1/2; lower one with anti-Erk2, loading control). Positions of p-Erk1/2 and Erk2 are indicated. C, ARVCs were infected, where indicated, with adenovirus encoding N17Ras (AxN17Ras) at the indicated moi for 36 hours. Cells were then treated with PE (10 μmol/L) for 5 minutes, where indicated, and lysed. Extracts were analyzed by immunoblotting using anti-phospho-Erk1/2 or anti-Erk2. Data in panels A through C are representative of 4 experiments performed. D, ARVCs were either treated with FT inh III (50 μmol/L for 24 hours) or infected with adenovirus AxN17Ras at moi of 40 pfu/cell as in C. In some cases, PE cells were treated with this hormone (10 μmol/L) for 1 hour. Samples of cell lysate were processed for measurement of S6K1 activity. Data are expressed as mean±SD, and are derived from 6 separate experiments. *P<0.01 vs PE treatment alone.
Binding of 4E-BP1 to eIF4E prevents eIF4E from binding eIF4G to form eIF4F complexes. PE markedly increased the association of eIF4G with eIF4E (Figure 3D), indicating increased eIF4F formation. This effect was blocked by inhibition of mTOR or PD184352 (Figure 3D). These data demonstrate that, in ARVCs, GqPCR stimulation causes the phosphorylation of 4E-BP1 and its dissociation from eIF4E, allowing eIF4E to form eIF4F complexes, and that MEK/Erk signaling plays an essential role in this.

Although LY294002 Blocks S6K1 Activation, Wortmannin Has Little, If Any, Inhibitory Effect on PE-Induced S6K1 Activation and 4E-BP1 Phosphorylation

As shown in Figures 1B and 1C, LY294002 blocked activation of S6K1 by PE or ET-1, although these agents do not stimulate PKB or, by implication, PI 3-kinase. To clarify the role of PI 3-kinase signaling in the regulation of translation factors studied presently, we also examined the effect of a structurally different, more potent, PI 3-kinase inhibitor, wortmannin. The data in Figure 4 show, first, that LY294002 inhibited the phosphorylation (activation) of S6K1 in a dose-dependent manner (Figure 4A). In contrast, wortmannin failed to inhibit either S6K1 activation or 4E-BP1 phosphorylation induced by PE, even at concentrations as high as 1 μmol/L (Figure 4B). LY294002 also blocked PE-induced phosphorylation of 4E-BP1 (Figure 4B, bottom blot). The differing effects of these two PI 3-kinase inhibitors are surprising. One possibility is that the wortmannin concentrations used failed to inhibit PI 3-kinase in ARVCs. However, these concentrations of wortmannin did completely block the insulin-induced phosphorylation of PKB (Figure 4C). These results suggest that the inhibitory effect of LY294002 on the actions of PE does not reflect inhibition of PI 3-kinase, but rather an effect on another cellular component(s). In vitro, LY294002 inhibits mTOR autokinase activity at concentrations similar to those at which it inhibits PI 3-kinase, whereas wortmannin is less potent against mTOR than against PI 3-kinase. It is thus possible that the inhibitory effects of LY294002 on S6K1 or 4E-BP1 are due to its ability to inhibit mTOR.

**MEK1 Adenovirus Induces Activation of S6K1, Phosphorylation of 4E-BP1, and Its Dissociation From eIF4E**

To confirm the role of MEK signaling in regulating S6K1 and 4E-BP1, we considered it essential to use an additional approach and therefore examined the effect of constitutively active mutant of MEK1, the upstream activator of Erk1/2. Infection with adenovirus expressing activated MEK1 (AxMEKCA) resulted in a substantial increase in MEK1 protein and activated Erk1/2, without affecting the level of Erk2 (Figure 5A). Infection of ARVCs with an adenovirus-expressing β-galactosidase (AxLacZ) (same moi) as negative control did not affect Erk1/2 activation (Figure 5A). We previously showed that overexpression of MEK1CA does not activate p38 MAPK or JNK in ARVCs.

Infection of cells with AxMEKCA increased S6K1 activity (Figure 5B) to a similar extent to that caused by PE (Figures 1A and 1B). This was inhibited by PD184352 (Figure 5B) or U0126 (not shown), demonstrating that activated MEK/Erk signaling itself is sufficient to drive activation of S6K1. LY294002 blocked the stimulation of S6K1 by activated MEK1, perhaps reflecting nonspecific inhibition of mTOR.
We also examined the phosphorylation of 4E-BP1. Activated MEK1 increased 4E-BP1 phosphorylation (Figure 5C, top panel), whereas infection of ARVCs with control virus (AxLacZ) had no effect on 4E-BP1 phosphorylation (Figure 5C, top panel). As observed for S6K1, inhibitors of MEK, PI 3-kinase, or mTOR blocked the effect of activated MEK1 on 4E-BP1 phosphorylation (Figure 5C, top panel).

In agreement with the data showing that activated MEK1 induced phosphorylation of 4E-BP1, this active mutant also caused dissociation of 4E-BP1 from eIF4E when compared with either uninfected cells or cells infected with control virus (Figure 5C, bottom panel). This effect, like 4E-BP1 phosphorylation, was diminished by inhibition of mTOR or MEK activity (Figure 5C, bottom panel). These data strongly suggest that activated MEK/Erk signaling in ARVC is sufficient to drive phosphorylation of 4E-BP1 and its release from eIF4E.

These data implied that a MEK-dependent input plays a key role in the regulation of S6K1 and 4E-BP1 by PE and ET-1. Because the only known in vivo substrates for MEK1/2 are the closely related MAP kinases, Erk1 and Erk2, we tested whether activated Erk2 stimulated S6K1 activity or 4E-BP1 phosphorylation in vitro. As shown in the online Figure (available at http://www.circresaha.org), treatment with Erk2 led only to partial activation of S6K1 and a shift of 4E-BP1 to the γ form, indicating that, although Erk can regulate these proteins, additional inputs are required, especially for activation of S6K1.

**Ras/Erk Signaling Is Required for Activation of Protein Synthesis in ARVCs**

Because GqPCR activation stimulates targets of mTOR that could contribute significantly to activation of mRNA translation, we investigated the roles of Ras and MEK in the regulation of protein synthesis by GqPCR activation. Treatment of ARVCs with PE and ET-1 (2 hours) led to substantial activation of protein synthesis, by 94.4 ± 17.5% and 65.7 ± 10.8%, respectively (Figure 6A). Consistent with the initial data mentioned, the stimulation of protein synthesis by PE or ET-1 was completely inhibited by preincubation of ARVCs with PD184352. FT Inh III and rapamycin also markedly inhibited this activation (Figure 6A). In order to rule out the possibility that the treatments could affect the rate of metabolism of methionine (to cysteine), we also used a nonmetabolizable amino acid, leucine, as a radioactive label. The data in Figure 6B clearly shows that the increased incorporation of [3 H]leucine elicited by PE was similarly affected by the MEK, mTOR, and Ras inhibitors. These data indicate that the activation of protein synthesis by PE involves signaling via MEK, mTOR, and Ras. Furthermore, expression of N17Ras substantially decreased PE-induced activation of protein synthesis compared with AxLacZ-infected cells at the same moi (Figure 6C). Thus, inhibition of Ras activity or MEK/Erk signaling is closely associated with inhibition of overall protein synthesis, consistent with the finding that Ras/Erk signaling plays critical roles in regulating S6K1 and 4E-BP1.

AxMEK CA infection increased [35 S]methionine incorporation by 95.4 ± 34.7% compared with control virus–infected
cells (Figure 6D). PD184352 or rapamycin inhibited MEK1-driven activation of protein synthesis, PD184352 reducing the rate to levels seen in cells infected with control virus (Figure 6D). Importantly, the marked inhibition by rapamycin clearly demonstrates that MEK1 activates protein synthesis, in part, via translation regulators dependent on mTOR (Figure 6D).

**Discussion**

The data presented here show that in ARVCs, the GqPCR agonists PE and ET-1 activate components of the translational machinery linked to mTOR. For example, PE and ET-1 activate S6K1. We have previously shown that PE and ET-1 activate a related enzyme, p70 S6 kinase 2 (S6K2), in ARVCs.26 Because S6Ks are believed to promote translation of mRNAs for components of the translational machinery, their activation by PE and ET-1 may be particularly important for their hypertrophic effects, which involve sustained increases in protein synthesis. PE and ET-1 also elicit phosphorylation of 4E-BP1 and its release from eIF4E, allowing increased formation of the eIF4F translation factor complexes, which is important for recruitment of ribosomes to the mRNA.

An important finding of the present study is that the effects of PE and ET-1 on S6K1 and 4E-BP1 and overall protein synthesis are blocked by inhibition of Ras or MEK. Although Ras and MEK/Erk have been implicated in mediating signal events linking GqPCR to hypertrophic responses for years,20,21 their roles in the activation of protein synthesis have not previously been established. We show here, using pharmacological approaches as well as adenovirus-mediated gene transfer techniques, that activation of Ras/MEK signaling is necessary and sufficient for stimulation of S6K1 and 4E-BP1 and of protein synthesis in ARVCs in response to GqPCR agonists.

The control of S6K1 and 4E-BP1 in response to GqPCR activation in ARVCs is blocked by rapamycin, indicating it is mediated through mTOR and thus implying convergence between mTOR and MEK-dependent signaling pathways. Consistent with this, activation of protein synthesis by PE or ET-1 was blunted by rapamycin or MEK inhibitors. This study thus reveals important roles for MEK signaling in activation of overall protein synthesis and of specific, mTOR-dependent, translational regulator proteins. How is MEK signaling linked to the control of S6K1 and 4E-BP1 at the molecular level? Earlier studies suggested that Erk might play a role in the regulation of 4E-BP1: in vitro 4E-BP1 is a good substrate for Erk,34,35 but subsequent studies, often involving insulin, which generally activates Erk only weakly, if at all, indicated Erk was not involved in its control in vivo.17,36,37 Even in cases where Erk is activated, 4E-BP1 phosphorylation was not affected by PD098059.18 Although Erk phosphorylates 4E-BP1 efficiently, it cannot phosphorylate 4E-BP1 bound to eIF4E.36 This suggests that MEK/Erk signaling may bring about phosphorylation of 4E-BP1 by phosphorylating unbound 4E-BP1. Thus, as eIF4E/4E-BP1 complexes transiently dissociate within the cell, and free 4E-BP1 is phosphorylated by Erk, the amount of 4E-BP1 bound to

![Figure 6](http://circres.ahajournals.org/)[Figure 6. Signaling pathways involved in activation of protein synthesis by PE, ET-1, or activated MEK1. A and B, Overnight cultures of ARVCs were treated with PE (10 μmol/L) or ET-1 (0.1 μmol/L) for 1.5 hours, as indicated. Where indicated, cells were preincubated with PD184352, U0126, rapamycin, or FT Inh III as in Figures 1 and 2 before addition of agonists. Protein synthesis was assayed as described in Materials and Methods. Data are presented as percent of untreated control (100 ± SD; n=6 for A, n=4 for B. C, ARVCs were infected with adenoviruses AxLacZ or AxN17Ras at moi 40 pfu/cell (or left uninfected, control). After 36 hours, PE was added for 1.5 hours to where indicated, and protein synthesis was assessed as described in Materials and Methods. Data are presented as percent of infected control (100 ± SD; n=4. D, ARVCs were left uninfected or infected with adenovirus AxLacZ or AxMEK CA at an moi of 10 pfu/cell. After 36 hours, PD184352 (PD, 5 μmol/L, 2 hours) or rapamycin (Rap, 100 nmol/L, 2 hours) was added to some dishes. Protein synthesis rates were then measured as described in Materials and Methods. Data are shown as percent of uninfected control ± SD; n=4. *P<0.01 vs PE, ET-1 treatments, or MEK1 infection alone, respectively.]
elF4E will decrease, allowing increased formation of elF4E/elF4G complexes, as seen here.

Our data show that Erk signaling is essential for activation of S6K1 in vivo, revealing similarity to the control of S6K2 by PE and ET-1 in ARVCs.** Although Mukhopadhyay et al** reported that recombinant S6K1 was a substrate for Erk in vitro, they did not reproducibly observe activation. Our data suggest that Erk can partially activate S6K1, possibly by phosphorylating Ser/Thr-Pro sites in its C-terminus that are known to be important for activation.

Moreover, because activation by Erk2 in vitro was less than that induced by PE or ET-1 in ARVCs, other regulatory events must also contribute to activation of S6K1 in vivo. Regulation of S6K1 involves interplay between numerous phosphorylation sites and we have not studied this further.

Because rapamycin only partially blocked activation of protein synthesis by PE or ET-1, whereas interference with MEK completely inhibited it, other mTOR-independent effects must be involved in the activation of protein synthesis. These could include contributions from regulation of eEF2, which is partly mediated via mTOR-independent mechanisms and perhaps from other factors such as the regulatory guanine nucleotide-exchange factor elF2B, and can be regulated in a MEK-dependent manner.

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**References**


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Chemicals and Other Materials
[γ-32P]ATP, L-[35S]methionine, L-[3H]leucine, m7GTP-Sepharose, glutathione-Sepharose 4B and ECL reagents were purchased from Amersham Pharmacia Biotech. LR-microcystin, LY294002, PD098059, rapamycin and FT Inh III were from Calbiochem. U0126 was obtained from Promega. PD184352 was provided by the Division of Signal Transduction Therapy (DSTT) in Dundee. BSA (fatty acid free) was from Boehringer Mannheim. Collagenase (type 2) was from Worthington Biochemical, New Jersey. All other chemicals or biochemicals (unless otherwise stated) were obtained from Sigma. Recombinant adenoviruses expressing a dominant-negative mutant form of Ras (N17Ras), or activated MEK1 (Ser218/222 to Glu, AxMEKCA), were kindly provided by Drs. B. Kahn, Boston¹ and S. Tanaka, Tokyo.²

Isolation, Culture and Treatment of Adult Rat Cardiomyocytes
Ventricular myocytes were isolated from hearts of adult rats. After isolation, cells were washed, seeded onto laminin-coated dishes and cultured.³ Details of treatments are provided in the figure legends. Cells were extracted either as described previously³ or as in the Activated Ras Affinity Binding Assay.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting
SDS-PAGE and Western blotting were performed as described previously⁴,⁵ or in the Activated Ras Affinity Binding Assay. Anti-phospho-Erk1/2 and anti-phospho-PKB (Ser473) antibodies was supplied by New England Biolabs. Mouse monoclonal antibody to pan-Ras was from Transduction Laboratories. Anti-Erk2 and anti-MEK1
antibodies were provided by DSTT in Dundee. Western blots of S6K1, eIF4G1, eIF4E and 4E-BP1 were performed as described previously.3-5

Activated Ras Affinity Binding Assay

The assay was performed essentially as described by Chiloeches et al.6 The GST-RBD (Ras-binding domain of c-Raf, aa51-131) expression vector used here was kindly provided by Prof. P. Sugden (London). Recombinant GST-RBD was expressed in E. coli strain BL21(DE3)pLysS and purified by affinity chromatography using glutathione-Sepharose 4B. Details of the assay are provided here. ARVC cultured in 60 mm plates were exposed to agonists in medium 199. The medium was aspirated and the cells were washed three times with ice-cold PBS and extracted by scraping into 200 µL of buffer A (20 mmol/L Tris-HCl pH 7.4, 2 mmol/L EDTA, 100 mmol/L KCl, 5 mmol/L MgCl2, 5 mmol/L NaF, 0.2 mmol/L Na3VO4, 2 µmol/L LR-microcystin, 10% (v/v) glycerol, 1 % (v/v) Triton X-100, 0.5% (v/v) 2-mercaptoethanol, 10 mmol/L benzamidine, 0.2 mmol/L leupeptin, 0.3 mmol/L phenylmethylsulfonyl fluoride). Lysates were centrifuged (10,000 x g, 10 min, 4°C), and the supernatants (equalized for protein) were incubated with mixing at 4°C for 2 h with GST-RBD that had been previously bound to glutathione-Sepharose beads resuspended in buffer A. Beads were washed four times with buffer A and boiled with SDS-PAGE sample buffer. The eluted proteins were resolved on 15% polyacrylamide gels and transferred to PVDF membranes. Immunoblotting was carried out with antibody against pan-Ras.
In Vitro Kinase Assays

S6K1 activity was assayed using specific peptide substrate after immunoprecipitation, as described previously. 7

Treatment of S6K1 and 4E-BP1 from ARVC by Erk2 in vitro

S6K1 was immunoprecipitated from untreated ARVC and then incubated in vitro with activated Erk2 (from Dr. C. Armstrong, Dundee) and unlabelled ATP (0.1 mmol/L, with 10 mmol/L MgCl₂) for 1h at 30°C. S6K1 assays were then performed using radiolabelled ATP and peptide substrate. Controls were incubated without Erk2. Similarly, immunoprecipitation reactions were performed on lysates from untreated ARVC using anti-4E-BP1 (+ α-BP1 IP) or, as a negative control, without antibody (-α-BP1 IP). Samples were then incubated with activated Erk2 for 2h (25°C) using radiolabelled or unlabelled ATP and analysed by SDS-PAGE/autoradiography, where appropriate.

Measurement of Protein Synthesis

Cultured ARVC were preincubated with fresh medium 199 with or without signalling inhibitors. Cells were then stimulated with PE or ET-1 before the addition of L-[³⁵S]methionine (10 μCi/mL) or L-[³H]leucine (10 μCi/mL) for a further 30 min. For adenovirus-infected cells, fresh medium 199 was added after 36 h infection and the cells were either incubated with L-[³⁵S]methionine (10 μCi/mL) for a further 30 min or stimulated with PE or ET-1 for 1.5 h before the addition of L-[³⁵S]methionine. Cells were washed three times with ice-cold PBS and then were lysed with extraction buffer. Proteins were then collected by filtration on 3MM filters (Whatman) before
precipitation with 10 % (w/v) TCA and measurement of incorporated radiolabel by scintillation counting.

Erk2 Can Phosphorylate 4E-BP1 and Partially Activate S6K1 in vitro

Endogenous S6K1 was immunoprecipitated from unstimulated cultured ARVC and incubated with activated Erk2 and ATP-MgCl₂. In the second stage of the assay, the peptide substrate for S6K1 and radiolabelled ATP were added. The data clearly show that preincubation with Erk2 increases the activity of S6K1, suggesting that Erk2 can phosphorylate S6K1 at sites that contribute to its activation (Fig. S1, Panel A). However, activation is clearly much less than is seen following PE-treatment of ARVC. Treatment of 4E-BP1 immunoprecipitated from ARVC with Erk2 resulted in its phosphorylation as manifested by incorporation of label from [γ³²P]ATP and retardation of its mobility on SDS-PAGE (Fig. S1, Panel B). Erk2 has previously been shown to phosphorylate several sites in recombinant 4E-BP1,⁸,⁹ the major site being S64 (S65 in the human protein). From the data in Fig. 5 it seems likely that the MEK-dependent input to regulation of S6K1 and 4E-BP1 may involve their direct phosphorylation by Erk, although additional inputs are also likely to be involved, especially for S6K1. This may reflect requirements for phosphorylation of other sites in S6K1, which are needed for its full activation.
References


7. Moule SK, Edgell NJ, Welsh GI, Diggle TA, Foulstone EJ, Heesom KJ, Proud CG, Denton RM. Multiple signalling pathways involved in the stimulation of fatty


**Figure S1.** *In vitro* effects of Erk2 on S6K1 and 4E-BP1 from ARVC. (A) S6K1 was immunoprecipitated from untreated ARVC and then incubated *in vitro* with activated Erk2 (10U/mL) prior to assay for S6K1 activity as described in the Methods. Data are corrected for the low level of phosphorylation of the S6 substrate peptide by this amount of Erk2 and are shown as mean ± SD (n=4). (B) Immunoprecipitated 4E-BP1 from ARVC was incubated with Erk2 (amounts as indicated) and [γ-32P]ATP (upper panel) or unlabelled ATP (lower panel). Analysis was performed by SDS-PAGE followed by autoradiography (top) or western with anti-4E-BP1/ECL (bottom).
Fig. S1

A

S6K1 activity (% of control) vs. control and Erk2.

B

Western blot analysis of 4E-BP1 and Erk2 expression under different conditions.