Cross-talk between the ERK and p70 S6 Kinase (S6K) Signaling Pathways

MEK-DEPENDENT ACTIVATION OF S6K2 IN CARDIOMYOCYTES*

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The α1-adrenergic agonist phenylephrine (PE) and insulin each stimulate protein synthesis in cardiomyocytes. Activation of protein synthesis by PE is involved in the development of cardiac hypertrophy. One component involved here is p70 S6 kinase 1 (S6K1), which lies downstream of mammalian target of rapamycin, whose regulation is thought to involve phosphatidylinositol 3-kinase and protein kinase B (PKB). S6K2 is a recently identified homolog of S6K1 whose regulation is poorly understood. Here we demonstrate that in adult rat ventricular cardiomyocytes, PE and insulin each activate S6K2, activation being 3.5- and 5-fold above basal, respectively. Rapamycin completely blocked S6K2 activation by either PE or insulin. Three different inhibitors of MEK1/2 abolished PE-induced activation of S6K2 whereas expression of constitutively active MEK1 activated S6K2, without affecting the p38 mitogen-activated protein kinase and JNK pathways, indicating that MEK/ERK signaling plays a key role in regulation of S6K2 by PE. PE did not activate PKB, and expression of dominant negative PKB failed to block activation of S6K2 by PE, indicating PE-induced S6K2 activation is independent of PKB. However, this PKB mutant did partially block S6K2 activation by insulin, indicating PKB is required here. Another hypertrophic agent, endothelin 1, also activated S6K2 in a MEK-dependent manner. Our findings provide strong evidence for novel signaling connections between MEK/ERK and S6K2. p70 S6 kinase 1 (S6K1)* phosphorylates ribosomal protein S6 and is activated in response to hormones and mitogens. S6K1 is thought to regulate the translation of a subset of mRNAs that are characterized by the presence of their 5'-terminal tract of pyrimidines (5'-TOP mRNAs) and generally encode ribosomal proteins and elongation factors (1, 2). According to this model, activation of S6K1 leads to up-regulation of ribosome biosynthesis and increases the translational capacity of the cell. The control of S6K1 has been the subject of detailed investigations in several laboratories. Activation of S6K1 involves its phosphorylation at multiple serine/threonine residues probably catalyzed by several upstream kinases. Recent studies have shown that at least two signaling pathways influence S6K1. One pathway involves PI 3-kinase, and its downstream effector, PDK1 (which phosphorylates a site in the catalytic domain of S6K1, T229/252 in the cytoplasmic domain of S6K1, respectively (3, 4)), and perhaps also PKB, as demonstrated by a variety of molecular biological and pharmacological analyses (3–6). An additional pathway essential for S6K1 activation involves the mammalian target of rapamycin (mTOR) as revealed using the immunosuppressant rapamycin, which inhibits mTOR and activation of S6K1 by all stimuli tested and using specific mutants of mTOR (7, 8). Other phosphorylation sites involved in the activation of S6K1 lie in its C-terminal domain and are followed by Pro residues, suggesting they may be targets for proline-directed kinase(s). However, although S6K1 can be phosphorylated in vitro by MAP kinase (a proline-directed enzyme; see Ref. 9), early studies indicated that S6K1 resides on a signaling pathway distinct from the Ras/MAP kinase pathway (10).

S6K2 is a recently identified homolog of S6K1 that phosphorylates S6 in vitro and is highly homologous to S6K1 in the core kinase and linker regulatory domains but differs from S6K1 in its N- and C-terminal regions and is differently localized being primarily nuclear because of a C-terminal nuclear localization signal not found in S6K1 (11–14). Studies of exogenous or endogenous S6K2 in other cell systems (cell lines) imply that S6K2 is regulated by PI 3-kinase signaling and by mTOR. In these respects it is thus similar to S6K1 (13–15), but regulation of S6K2 may differ from that of S6K1, because its C terminus, which exhibits only 25% identity, probably plays a critical role in S6K2 regulation, in particularly in response to weak PI 3-kinase agonists (16).

The α1-adrenergic receptor agonist phenylephrine (PE), which is a G protein-coupled receptor (GPCR) agonist, activates protein synthesis and exerts hypertrophic effects in cardiomyocytes (17, 18). Although previous reports suggested that this involves MAP kinase pathways (18–20), the signaling events through which PE activates protein synthesis or which steps in mRNA translation it affects are still poorly understood. Given the importance of S6 kinases in the regulation of protein synthesis and the fact that PE and insulin each activate S6K1 in cardiomyocytes (21, 22), we considered it important to ex-

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‡ The abbreviations used are: S6K, ribosomal S6 kinase; ERK, extracellular signal-regulated kinase; ET-1, endothelin 1; GPCR, G protein-coupled receptor; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein-ERK kinase; mTOR, mammalian target of rapamycin; PDK1, phosphatidylinositol-dependent protein kinase 1; PE, phenylephrine; PI, phosphatidylinositol; PKB, protein kinase B; 5'-TOP mRNAs, 5'-terminal tract of pyrimidines mRNA; MAP, mitogen-activated protein; GST, glutathione S-transferase; ARVC, adult rat ventricular cardiomyocytes; PAGE, polyacrylamide gel electrophoresis; GSK, glycogen synthase kinase.
plore the mechanisms regulating the novel S6 kinase, S6K2, in primary adult cardiomyocytes. Our data show that regulation of S6K2 by PE is dependent on MEK/ERK signaling. Another GPCR agonist, endothelin 1 (ET-1), which is also a hypertrophic agent (23), again activates S6K2 in a MEK-dependent manner. Furthermore, we show that the expression of a constitutively active mutant of MEK1 causes activation of S6K2 in cardiomyocytes. Activation of S6K2 by insulin is also partially dependent upon MEK/ERK signaling. This study provides strong evidence for novel signaling connections between MEK/ERK and S6K2, which might be involved in the control of mRNA translation in primary adult cardiomyocytes.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Other Materials—**[32P]ATP and ECL reagents were purchased from Amersham Pharmacia Biotech. Microcystin LR, wortmannin, rapamycin, and PD98059 were from Calbiochem. U0126 was obtained from Promega. PD184352 was provided by the Division of Signal Transduction Therapy (DSTT) in Dundee, Scotland. Bovine serum albumin (fatty acid-free) was from Roche Molecular Biochemicals. Adult male Harlan Sprague-Dawley rats (250–300 g) were obtained from Charles River, United Kingdom. A vector encoding GST-c-Jun (amino acids 1–135) was kindly provided by Professor P. H. Sugden (London, UK) (24). The recombinant protein was expressed in Escherichia coli by Ian Baines. All other chemicals or biochemicals (unless stated otherwise) were obtained from Sigma.

**Isolation, Culture, Treatment, and Extraction of Adult Rat Ventricular Cardiomyocytes (ARVC)—**Ventricular myocytes were isolated from hearts of adult rats as described previously (22). After isolation, cells were washed, seeded onto laminin-coated dishes, and cultured as described previously (22). Details of treatments are provided in the figure legends. Cells were extracted as described previously (22). Protein concentrations were determined by the Bradford method.

**Adenoviral-mediated Gene Transfection—**The recombinant adenovirus vector carrying constitutively active MEK1 (Ser218 and Ser222 to Glu; AxMEKCA) was kindly provided by Dr. S. Tanaka (Tokyo, Japan) (25). The adenovirus vector encoding epitope (FLAG)-tagged dominant-negative PKB (Thr308 and Ser473 to Ala; AxPKB-AA) was kindly provided by Dr. M. Kasuga (Kobe, Japan) (26). ARVC were cultured in 60-mm plates for 2 h after isolation before the infection was carried out. ARVC cultures were washed and incubated in 1 ml of M199 medium containing recombinant adenoviruses for 2–3 h at 37°C at the indicated multiplicity of infection (m.o.i.) as described in the figure legends. Cultures were then given fresh M199 medium and incubated for another 36 h before further treatments.

**SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting—**SDS-PAGE and Western blotting were performed as described previously (22). The anti-S6K2 antibody was prepared as described previously (11). Anti-phospho-ERK1/2, anti-phospho-GSK3, anti-phospho-p38 mitogen-activated protein kinase, anti-phospho-PKBSer473 and anti-phospho-PKBThr308, and anti-PKB (total) antibodies were supplied by New England Biolabs. Anti-ERK2 and anti-MEK1 antibodies were kindly provided by the Division of Signal Transduction Therapy (DSTT) in Dundee, Scotland. Anti-phospho-hsp27 monoclonal antibody was provided by Dr. R. A. Quinlan (Dundee, Scotland). Anti-FLAG antibody (M2) was from Sigma.

**In Vitro Kinase Assays—**S6K2 activity was assayed using a specific peptide substrate (as used for S6K1) after immunoprecipitation with S6K2 antibody, as described previously (11, 27). JNK activity was assayed using GST-c-Jun (amino acids 1–135) as substrate as described previously (24).

**RESULTS**

**S6K2 Is Activated by PE and Insulin—**To study whether S6K2 is regulated by PE and insulin in ARVC, we treated ARVC with PE and insulin for differing times. Significant activation of S6K2 was first observed 30 min after PE treatment and reached a maximum after 60 min, assessed both by activity measurements (using a peptide substrate) after immunoprecipitation of the enzyme (Fig. 1A) and by Western blot analysis, which showed that activation of S6K2 is accompanied by a reduction in its mobility on SDS-PAGE (Fig. 1B), presumably because of its increased phosphorylation at multiple sites. The extent of this shift correlated well with alterations in S6K2 activity (Fig. 1, A and B). Such behavior is also a well documented characteristic of S6K1. The activity of S6K2 remained elevated above basal for at least 4 h after PE treatment but declined toward control level at later times (Fig. 1, A and B). Maximal activation of S6K2 by insulin required ~30 min, and activity subsequently declined (Fig. 1, C and D).

**PKB Is Not Activated by PE in ARVC—**Previous studies have suggested that regulation of p70 S6 kinases involves PI 3-kinase and PKB (3–6). Our earlier studies indicated that this is also so for the regulation of S6K1 by insulin in ARVC (22). To assess whether PE activated PI 3-kinase signaling in ARVC, we studied the phosphorylation of PKB, an effector of PI 3-kinase signaling (28). Activation of PKB involves its phosphorylation at Ser473 in the C-terminal tail and Thr308 in the catalytic domain (PKBα). Western blots using an antibody that specifically recognizes phospho-Ser473 of PKB showed no detectable phosphorylation at this site in cells treated with PE over a 60-min period (Fig. 2A, upper blot). The total amount of PKB extracted from the cells did not change under different condi-

![FIG. 1. Activation of S6K2 by PE and insulin.](http://www.jbc.org/doi/fig.png)
rapid activation of both ERK1 and ERK2 in ARVC, as demonstrated by increased phosphorylation of these kinases (Fig. 2B). Activation was maximal within 5 min after addition of PE, after which it declined substantially but was still apparent up to 2 h (Fig. 2B). Separate experiments showed that ERK phosphorylation was still elevated above the very low level seen in untreated cells up to at least 8 h after PE treatment (data not shown). Maximal activation of ERK1/2 by insulin was also seen at 5 min, as revealed by extended exposure of the immunoblot (Fig. 2C), but was much lower than that caused by PE (see Fig. 2D for a direct comparison). The MEK1/2 inhibitor U0126 (30) completely abolished ERK activation (Fig. 2D). Similar results were obtained when other two structurally distinct inhibitors of MEK1/2, PD98059 (31, 32) and PD184352 (33), were used (data not shown). As expected, the PI 3-kinase inhibitor LY294002 did not affect ERK activation (Fig. 2D).

Inhibition of MEK Activity Abolishes Activation of S6K2 by PE and Partially Blocks Insulin Stimulation of S6K2—Because PE activates ERK1/2 but not PKB in ARVC, we next examined whether the MEK/ERK pathway played a role in the regulation of S6K2. To do this, we made use of MEK inhibitors, because they effectively block the activation of ERK1/2 by PE in ARVC (Fig. 2D, and data not shown). Pretreatment of ARVC with U0126 prior to addition of PE abolished the ability of PE to induce activation of S6K2, as assessed by both activity measurements (Fig. 3A) and by Western blot analysis, which showed that these compounds blocked the characteristic reduction in mobility of this protein on SDS-PAGE normally seen after PE treatment (Fig. 3B). Similar results were obtained when the other MEK inhibitors PD98059 and PD184352 were applied (data not shown). These data suggested that the MEK/ERK pathway plays a critical role in the regulation of S6K2 by PE.

Despite the fact that PE did not appear to activate PKB, and thus by implication PI 3-kinase, we also tested the effect of the PI 3-kinase inhibitor LY294002 on the activation of S6K2 by PE. Surprisingly, this compound completely blocked the activation of S6K2 by PE (Fig. 3, A and B), suggesting that PI 3-kinase activity is nonetheless important for the activation of S6K2 by this agent.

The ability of insulin to activate S6K2 was completely inhibited by LY294002, as demonstrated by both activity measurements and the mobility of this enzyme on SDS-PAGE (Fig. 3, C and D). This was expected as insulin strongly activates PI 3-kinase signaling/PKB in ARVC (22). Inhibition of MEK activity also partially impaired S6K2 activation (and to a small extent, its phosphorylation) in response to insulin (Fig. 3, C and D). Insulin-induced S6K2 activation was inhibited by 34.3% by U0126 (Fig. 3C) (*, p < 0.01). These results suggest a partial involvement of the MEK/ERK pathway in the effects of insulin on S6K2, consistent with the modest ability of insulin to activate ERKs in ARVC (Fig. 2, C and D). Stimulation of S6K2 by PE or insulin was completely blocked by the mTOR inhibitor rapamycin (Fig. 3, A–D) confirming that, like S6K1, regulation of S6K2 requires mTOR in ARVC.

Expression of a Constitutively Active MEK1 Mediated by Adenovirus in ARVC Activates S6K2—To investigate further the role of MEK/ERK in the regulation of S6K2, we examined the effect of a constitutively active mutant of MEK1, the specific upstream activator of ERK1/2. The activated MEK1 mutant was expressed in ARVC using an adenovirus vector (AxMEKCA). As indicated by Western blotting using an antibody specific for MEK1 (Fig. 4A, top blot), infection of ARVC with AxMEKCA at an m.o.i. of 1–5 resulted in a dose-dependent expression of MEK1 in ARVC and concomitantly activated ERK1 and ERK2, as shown by increased phosphorylation of these enzymes (Fig. 4A, top and middle blots). The level of
caused by infection of cells with AxMEKCA at an m.o.i. of 10. Activation of ERK1/2 (LacZ) (m.o.i. of 10) as a control had no effect on MEK1 expression of S6K2 by PE and insulin.

Isolated ARVC were cultured overnight and were then treated with PE (10 μM) for 30 min (A and C) or insulin (INS; 10 nM) for 30 min (C and D) prior to treatment with the inhibitors mentioned in the legend for Fig. 2 or rapamycin (Rap; 100 nM for 30 min), and extracts were prepared. A and C, activity of S6K2 was assayed after immunoprecipitation as described in the legend for Fig. 1. Data are the means ± S.D. of four independent experiments performed in duplicate. The bar labeled * shows significant difference (p < 0.01) compared with insulin treatment alone (INS; C, B and D, immunoblots developed using an antiserum to S6K2. Arrows labeled p and pp indicate increasingly highly phosphorylated forms of S6K2. Data are representative of three experiments.

ERK2 protein was not affected (Fig. 4A, bottom blot). Infection of ARVC with an adenovirus expressing β-galactosidase (AxLacZ) (m.o.i. of 10) as a control had no effect on MEK1 expression or ERK1/2 activation (Fig. 4A). Activation of ERK1/2 caused by infection of cells with AxMEKCA at an m.o.i. of 10 plaque-forming units/cell was significantly blocked by U0126, whereas, as expected, LY294002 and rapamycin did not inhibit this (Fig. 4B, top and middle panels). Infection of cells with AxMEKCA at an m.o.i. of 10 plaque-forming units/cell induced a marked increase in S6K2 activity and phosphorylation (Fig. 4, C and D), to a similar extent of that caused by PE (Fig. 3, A and B). This activation was almost completely inhibited by U0126 (Fig. 4, C and D). Similar inhibitory effects were observed using the other two MEK inhibitors PD98059 or PD184352 (data not shown). The PI 3-kinase inhibitor LY294002 completely blocked the activation of S6K2 by activated MEK, again suggesting a requirement for PI 3-kinase signaling activity in the activation of S6K2 (Fig. 4, C and D). Rapamycin also completely inhibited S6K2 activation by insulin in Chinese hamster ovary and 3T3-L1 cells (6).

Expression of a Dominant Negative Mutant of PKB in ARVC Fails to Block the Activation of S6K2 by PE but Partially Blocks S6K2 Activation by Insulin.—To assess whether PKB activity is required for the regulation of S6K2 by PE and insulin, we next examined the effect of a dominant negative mutant of PKB, a downstream effector of PI 3-kinase, on the PE- and insulin-induced activation of S6K2. Infection of ARVC with an adenovirus vector encoding FLAG-tagged dominant negative PKB (AxPKB-AA) led to dose-dependent expression of this mutant (PKB-AA) as demonstrated by Western blots using an anti-FLAG antibody (Fig. 5A, upper blot). A blot showing the level of endogenous ERK2 was also carried out to verify the same loading of all samples (Fig. 5A, lower blot). The dominant negative effect of PKB-AA was confirmed by examining the phosphorylation of GSK3, a downstream effector of PKB, in PKB-AA-transfected ARVC in response to insulin. Expression of PKB-AA in ARVC inhibited the phosphorylation of GSK3 induced by insulin in a dose-dependent manner (Fig. 5B), indicating that this kinase-dead mutant interferes with the function of the endogenous PKB. This result is consistent with a previous report in that expression of this PKB mutant inhibited the activation of endogenous PKB by insulin in Chinese hamster ovary and 3T3-L1 cells (6).

Infection of AxPKB-AA at an m.o.i. of 10 plaque-forming units/cell did not affect the activation or phosphorylation of S6K2 by PE in ARVC (Fig. 5, C and E) but did impair its activation by insulin in these cells, activation of S6K2 by insulin being inhibited by 33.4% (Fig. 5D, *, p < 0.01, and E). These results suggest that, in ARVC, PKB is not required for activation of S6K2 by PE but that it is involved in the stimulation of S6K2 by insulin.

ET-1 Also Activates S6K2 in a MEK-dependent Manner.—To explore further the possibility that other GPCR agonists act similarly to PE in ARVC, we tested the effect of ET-1 on the regulation of S6K2. Like PE, ET-1 strongly activated ERK1/2 (Fig. 6A) and did not induce phosphorylation of PKB (data not shown). Treatment of ARVC with ET-1 resulted in marked activation of S6K2, as shown by both kinase activity and mobility shift (Fig. 6, B and C). Again, this stimulation was abolished by the MEK inhibitor U0126. LY294002 and rapamycin also completely inhibited activation of S6K2 by ET-1 (Fig. 6, B and C). These results imply that, in ARVC, these GPCR agonists regulate S6K2 activity in a similar manner, which depends on MEK/ERK signaling.

Other MAP Kinase Pathways Are Not Involved in the Activation of S6K2 by Activated MEK1.—It was clearly possible that the stimulation of S6K2 by expression of activated MEK1 might also be mediated, in whole or part, by other MAP kinase pathways, which could be switched on as a consequence of high level expression of activated MEK1. To investigate other pathways, we first studied the ability of activated MEK1 to activate the p38 MAP kinase subfamily. We assessed this by examining the phosphorylation of these enzymes and of hsp27, a substrate for mitogen-activated protein kinase APK-2, which is itself activated by p38 MAP kinase (7) and hsp27 (Fig. 7A). A single immunoreactive band was seen in untreated ARVC when using the anti-phospho-p38 MAP kinase antibody. Infection of ARVC with AxMEKCA at an m.o.i. of 10 plaque-forming units/cell had no effect on the phosphorylation of p38 MAP kinase or hsp27 (Fig. 7A, top and middle blots, and data not shown for other
sites in hsp27; see legend). We also tested whether overexpression of MEK1 activated JNK by an in vitro kinase assay using GST-c-Jun (amino acids 1–135) as substrate (Fig. 7B). Again, no activation of this pathway was observed (Fig. 7B). As a positive control, we treated human embryonic kidney 293 cells with arsenite, and this showed strong activation of JNK (Fig. 7B). These data apparently rule out the possibility that these pathways might contribute to the activation of S6K2 caused by MEK1 expression. It seems likely that this activation reflects a role for MEK itself or ERK1/2 (or other downstream kinases) in the regulation of S6K2.

To examine whether the p38 MAP kinase or JNK pathways are involved in the activation of S6K2 by PE or ET-1, we first tested whether PE or ET-1 activated these kinases. As shown in Fig. 7A, p38 MAP kinase was activated by PE and, to a lesser extent, ET-1, as indicated by Western blotting for phosphorylated p38 MAP kinase (Fig. 7A, top blot). Both agonists also increased the phosphorylation of hsp27, in agreement with the above data showing activation of p38 MAP kinase (Fig. 7A, middle blot). However, neither PE nor ET-1 activated JNK, as assessed by kinase assay using GST-c-Jun as substrate (Fig. 7B). Similar findings have been obtained by Antony Davies in this laboratory.2

Thus, we could rule out a role for JNK in the activation of S6K2 by these agonists. However, it remained possible that p38 MAP kinase αβ played a role in these effects. To assess this, we employed two structurally distinct inhibitors of these enzymes, SB203580 and SB202190. Each of these compounds did partially block the activation of S6K2 by PE (Fig. 7C). This could indicate a role for p38 MAP kinase αβ. However, SB203580 has also been reported to inhibit PDK1 (35), the enzyme that phosphorylates the T-loop threonine residues in S6K1 (T229) (3, 4) and PKB (Thr308 in the α isoform) (36, 37). The residue corresponding to Thr229 (Thr228) and its flanking sequence are conserved in S6K2 (11–13, 15), and PDK1 has been indeed reported to regulate S6K2 in transfected 293 cells (13, 15). Because phosphorylation of this residue is required for activation of the kinase, an inhibitory effect of SB203580 on PDK1 in ARVC would result in impairment of activation of S6K2. To assess whether SB203580 did affect PDK1 activity in ARVC, we examined whether it affected the phosphorylation of Thr308 in PKBα, which is the major isoform stimulated by insulin in ARVC (22). SB203580, and also SB202190, which had not been tested previously, each markedly inhibited the phosphorylation of PKBα at Thr308 (Fig. 7D). Both compounds therefore seem likely to inhibit PDK1 in ARVC, and it is therefore possible that the effects of these agents on the activation of S6K2 by PE are because of inhibition of PDK1 rather than of p38 MAP kinase αβ. We cannot distinguish between these possibilities, and, because this aspect was not central to our present studies, we have not investigated this issue any further.

**DISCUSSION**

The data presented here show that PE (and ET-1) and insulin each activate the novel p70 S6 kinase, S6K2, in primary adult cardiomyocytes. This effect presumably contributes to
effects.
showed that PE or insulin did not activate MEK5 or ERK5 in ARVC (data not shown). Furthermore, expression of constitutively active MEK1 in ARVC activates S6K2 to similar extent to PE. These results thus strongly imply that the MEK1/ERK1/2 cascade plays a critical role in the signaling mechanisms that lead to the activation of S6K2. We cannot distinguish whether this input is via ERK or whether MEK itself exerts the effect, although given that several phosphorylation sites in the C terminus of S6K2 lie in Ser/Thr-Pro motifs, it is possible that they are targets for direct phosphorylation by ERK. Earlier studies suggested that ERK might play a role in the regulation of S6K1 as it is a substrate for ERK in vitro (9). However, subsequent work, mostly involving insulin, which often activates ERK only weakly, indicated that S6K1 lies on a signaling pathway distinct from MAP kinase (10, 40). The present observation that MEK/ERK signaling does play an important role in the activation of S6K2 may reflect the differences between the C termini of S6K1 and S6K2, which contain several phosphorylation sites important in activation of these isoenzymes (41, 16). Indeed, while this paper was in preparation, Blenis and colleagues (16) published data using the MEK inhibitor U0126, which suggested that MEK/ERK signaling was important for the activation of S6K2 by EGF in 293 cells. Although the input from MEK/ERK clearly depends on mTOR signaling (as S6K2 activation is abolished by rapamycin), it is not clear whether MEK/ERK signaling acts via, or in parallel to, the input from mTOR.

PI 3-kinase and its downstream effectors are generally recognized as important regulators of S6K1 (3–6). However, PE and ET-1 elicited no detectable activation of PKB in ARVC, implying a minimal activation of PI 3-kinase by these agents. However, our results do imply that PI 3-kinase activity is still essential for regulation of S6K2 by PE or ET-1, as LY294002 blocks S6K2 activation. This may reflect a requirement for basal activity of PI 3-kinase or PI 3-kinase signaling complexes for S6 kinase activation, as suggested earlier by Conus et al. (42), who found that PI 3-kinase activity was required for activation of S6K1 in response to Ca2+ ions even though they did not significantly activate PI 3-kinase. However, we cannot rule out the possibility that LY294002 could act nonspecifically on mTOR (34).

In summary, the present study provides new evidence for the mechanisms by which G protein-coupled receptor agonists, such as PE and ET-1, and insulin activate S6K2. PE and ET-1 regulate S6K2 via mechanisms that involve MEK, and perhaps ERK, signaling. This is the first report that investigates the linkage between the MEK/ERK signaling and the regulation of S6K2 in response to hypertrophic agents in primary cells. Moreover, the cell type used is a physiologically and pathologically important model for the study of cardiac hypertrophy and other cardiomyopathies.

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