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# p70 S6 Kinase Is Activated by Sodium Arsenite in Adult Rat Cardiomyocytes: Roles for Phosphatidylinositol 3-Kinase and p38 MAP Kinase

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**p70 S6 kinase (p70 S6k) is important in regulating a variety of cellular functions including mRNA translation and cell cycle progression and is activated by mitogens and hormones. Unexpectedly, we have found that, in adult rat cardiomyocytes, arsenite, which generally induces stress responses, markedly and rapidly activates p70 S6k. This activation of p70 S6k is completely blocked by rapamycin but only partially prevented by inhibitors of phosphatidylinositol 3-kinase. In trying to delineate the mechanism underlying this effect, we found that arsenite did not activate protein kinase B, JNK or MAP kinase, but did activate p38 MAP kinase in cardiac myocytes. A specific inhibitor of p38 MAP kinase (SB203580) partially attenuated the stimulation of p70 S6k by arsenite. These data indicate that the activation of p70 S6k by arsenite involves p38 MAP kinase and phosphatidylinositol 3-kinase but not PKB.** 

The protein kinase termed p70 S6 kinase was first identified by its ability to phosphorylate ribosomal protein S6. It is activated by a variety of stimuli, generally those which stimulate protein synthesis and induce proliferative responses [1]. It is now believed to play a role in the regulation of a number of cellular processes including transcription [2] and several events during mRNA translation [3-10]. p70 S6k plays an important role in the progression of cells from G1 to S phase of the cell cycle [11-14].

A valuable tool for studying the role of the signalling pathway which regulates p70 S6k is the immunosuppressant, rapamycin, which completely blocks the activation of p70 S6k in response to all stimuli so far studied [1,12,14-17]. This compound binds to a protein termed FKBP12 and the FKBP12.rapamycin complex in turn interacts with a protein called RAFT or FRAP in mammals (a homologue of phosphatidylinositol 3-kinase and of the yeast target of rapamycin, TOR) [18-20]. Rapamycin blocks a range of hormone and growth factor-induced effects [1] including the phosphorylation of the regulatory protein 4E-BP1 (which modulates translation initiation [6-10]) and the inactivation of elongation factor-2 kinase (which controls peptide-chain elongation [3]).

Activation of p70 S6k involves its phosphorylation at multiple sites and rapamycin blocks at least some of these phosphorylation events [21-26]. Although the kinases responsible for phosphorylating p70 S6k remain to be identified, the upstream signalling events leading to its activation are somewhat better characterised [1]. Phosphatidylinositol 3-kinase (PI 3-kinase) appears to be required for activation of p70 S6k by a variety of stimuli and inhibitors of PI 3-kinase (e.g., wortmannin, LY294002) block the activation of p70 S6k [27-29]. Furthermore, expression of a constitutively active PI 3-kinase leads to increased p70 S6k activity in intact cells [30]. Recent data also point to a role for protein kinase B (PKB), which is regulated by PI 3-kinase [31,32], in the activation of p70 S6k [31].

Relatively little work has been carried out to examine the regulation of kinase cascades in primary adult mammalian cells. In this paper we have studied this in adult rat cardiomyocytes and we report that arsenite activates p70 S6k in these cells. These effects are blocked by rapamycin and partially inhibited by wortmannin and LY294002, suggesting that arsenite activates p70 S6k via a similar pathway to that through which insulin and mitogens activate this enzyme. SB203580, a specific inhibitor of the p38 MAP kinase, which is activated by arsenite, partially attenuated the activation of p70 S6k, suggesting that this pathway plays a role in the activation of p70 S6k by arsenite. The present report showing that arsenite activates p70 S6 kinase in adult rat cardiomyocytes is of considerable potential importance given the current high level inter-

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est in stress-activated signalling pathways and their roles in the control of cell function and cell death (apoptosis).

## MATERIALS AND METHODS

*Chemicals and biochemicals.* Reagents were obtained from BDH (Poole, Dorset, UK) or Sigma Chemical Co (Poole, Dorset, UK), respectively, unless otherwise indicated. Specialised research materials were obtained as described earlier [10,33]. Wortmannin was from TCS and LY294002 from Sigma. Antisera were obtained as follows: anti-eIF4E (Dr Andrea Flynn, this laboratory), anti-p70 S6k (from Dr Emily Foulstone, University of Bristol; anti-PKB (from Dr Dario Alessi, University of Dundee), anti-MAP kinase (Dr Jeremy Tavaré, University of Bristol) and anti-4E-BP1 (Dr Tricia Diggle and Professor R M Denton, University of Bristol). Synthetic peptides were prepared by Dr Graham Bloomberg (University of Bristol) apart from 'Cross-tide' which was kindly provided by Darren Cross (University of Dundee). SB203580 was kindly provided by Dr John Lee (Smith Kline Beecham).

*Isolation, incubation and extraction of cardiomyocytes.* Ventricular myocytes were isolated from adult male rat hearts by the collagenase perfusion technique as described previously [34]. After isolation, cells were washed (3 × 20 min) in buffer A (from method in [34]) supplemented with 2% bovine serum albumin and 50$\mu$M $Ca^{2+}$-ions. Incubations were performed at 37°C in the same solution. Where included, inhibitors were added to the cells for a 20 min preincubation period prior to the addition of agonist (e.g., arsenite). Controls containing the vehicle for the inhibitors used (generally dimethylsulfoxide, final concentration 0.1% (v/v)), were performed, as well as controls without vehicle. After incubation, cells were spun down at 2000 rpm in a microcentrifuge for 10s. The cell pellets were immediately frozen in liquid nitrogen and stored at −80° until required. Pellets were quickly thawed and then extracted in the buffer described in [33] which contains a cocktail of proteinase and protein phosphatase inhibitors.

*Isolation of eIF4E and 4E-BP1.* eIF4E and associated proteins were isolated from heart cell extracts by affinity chromatography on 7-methyl GTP-Sepharose as described previously [33,35]. Immunoprecipitation of 4E-BP1 was as described by Diggle *et al.* [36].

*Gel electrophoresis and western blotting.* Gel electrophoresis and western blotting were carried out as described previously using the BioRad Mini Protean II system [37]. In general, about 200$\mu$g protein were applied to each lane. Protein was determined by the method of Bradford [38]. Western blots were visualised by enhanced chemiluminescence (ECL).

*Protein kinase assays.* p70 S6k and MAP kinase were assayed as described previously [39] using appropriate peptide substrates. Both enzymes were immunoprecipitated prior to assay [39]. Protein kinase B was assayed as described earlier [40]. p38 MAP kinase (measured indirectly as the activity of MAP kinase-activated protein kinase-2; MAPKAPK-2) and JNK were measured as previously described using hsp25 [41] (kind gift from Dr Matthias Gaestel, Berlin) or a GST-c-Jun(1-169) fusion protein [42] (generously provided by Ian Baines, Kent) as substrates, respectively.

## RESULTS AND DISCUSSION

### *Arsenite Activates p70 S6k in Adult Cardiomyocytes*

Isolated rat cardiomyocytes were treated with a range of concentrations of sodium arsenite and the cells were extracted. p70 S6 kinase activity was measured against a synthetic peptide substrate after immunoprecipitation with anti-p70 S6k antiserum. The results (Fig. 1A) indicated that arsenite treatment caused a marked activation of p70 S6k. The extent of activation by arsenite was typically 2-4 fold (mean 3.14 ± 0.20 fold over control, n = 7). For most subsequent experiments a time of 15 min was chosen. When examined over a time course, activation was apparent by 5 min and persisted up to 25 min (Fig. 1B). For most subsequent experiments a time of 15 min was chosen and a concentration of 1nM sodium arsenite was used.

Samples were also analysed by gel electrophoresis and western blotting using an antibody directed against p70 S6k. The results (Fig. 1C) indicated that arsenite treatment induced a retardation in the migration of the protein, which is characteristic of its activation. The time course of this mirrored that for activation of the kinase activity itself (cf. Fig. 1B).

A number of cellular stresses are now known to elicit the activation of responses which include activation of kinase cascades involving the JNK (Jun-N-terminal kinase); also termed SAP (stress-activated protein) kinase) family of enzymes and p38 MAP kinase. These stresses include heat shock and treatment of cells with hydrogen peroxide, but, unlike arsenite, these conditions led to decreased activity of p70 S6k in cardiac myocytes (heat shock at 43°: 36±6% of control; 3mM hydrogen peroxide: 60±7% of control, after 20 min exposure to the stress).

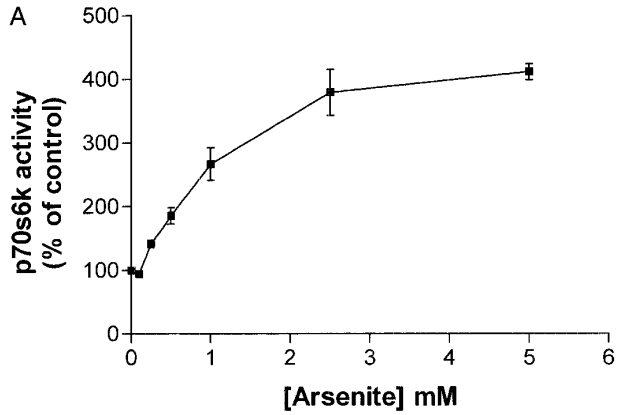
### *Effect of Arsenite on Stress-Activated Kinase Cascades in Heart Cells*

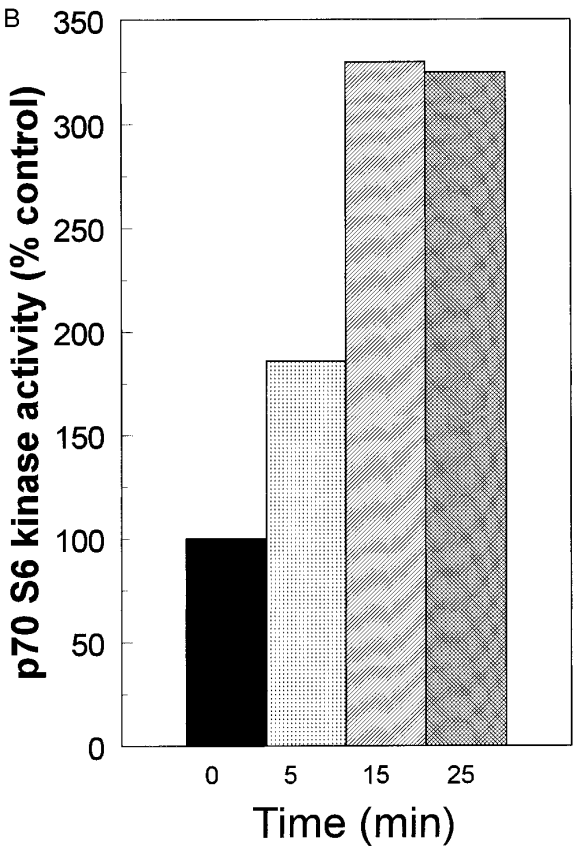
JNK activity was barely detectable in extracts from either control or arsenite-treated cardiomyocytes (data not shown), suggesting that these cells contain only very low levels of this enzyme family. It has previously been shown that cell stresses do activate JNK in neonatal (as opposed to adult) rat cardiac myocytes [43]. Arsenite also failed to activate the 'classical' MAP kinase (Erk) pathway. In contrast, MAPKAPK-2 activity was readily seen in extracts from control or arsenite treated cells, arsenite typically eliciting a 5-6 fold activation (as assessed by image analysis of the autoradiographs from assays with hsp25 as substrate, data not shown). As expected, the specific p38 MAP kinase inhibitor, SB203580 [41], eliminated the activation of MAPKAPK-2 by arsenite (data not shown).

### *Activation of p70 S6k by Arsenite Is Sensitive to Rapamycin and Inhibitors of PI 3-Kinase*

Rapamycin completely blocked the activation of p70 S6k as assessed by kinase activity measurement (Fig. 2).

To investigate the possible role of PI 3-kinase in the activation of p70 S6k by arsenite, the inhibitors wortmannin and LY294002 were employed. As shown in Fig. 2, both compounds inhibited the activation of p70



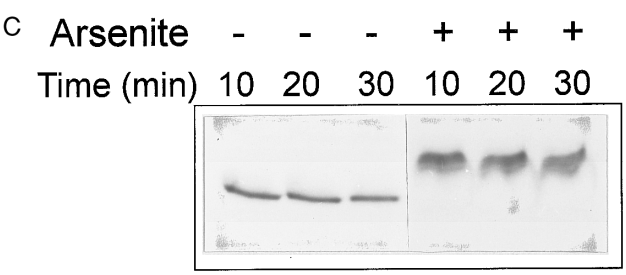




**FIG. 1.** A: Arsenite activates p70 S6k in isolated cardiomyocytes. Freshly isolated cardiomyocytes were treated with sodium arsenite at the concentrations (panel A) or for the times (panels B and C) indicated and extracts were prepared and analysed either by immunoprecipitation with anti-p70 S6k antibody followed by assay for this enzyme using a synthetic peptide substrate (panels A and B) or SDS–PAGE followed by western blotting with anti-p70 S6k antibody (panel C). For the dose–response (panel A), incubations were for 20 min. For the time course (panels B and C), 1mM sodium arsenite was used. For panel A, data are mean±SEM for six experiments. In panel B, results are the average of duplicate determinations from one experiment which was typical of four similar experiments performed with different batches of cells.

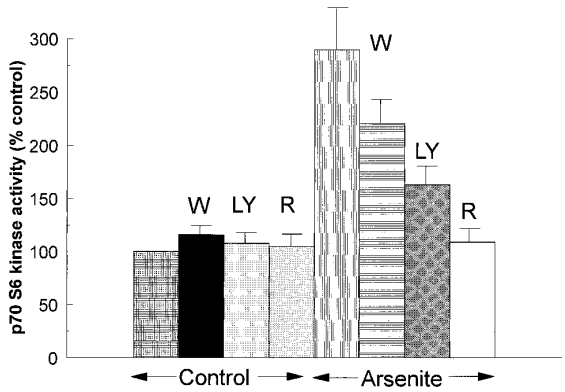



**FIG. 2.** Effects of rapamycin and PI 3-kinase inhibitors on the activation of p70 S6k by arsenite. Freshly isolated cardiomyocytes were preincubated with rapamycin (100nM), wortmannin (100nM) or LY294002 (30$\mu$M) for 20 min and then treated with or without sodium arsenite (1 mM) for 20 min and extracts were prepared. They were analyzed for p70 S6k activity using a synthetic peptide substrate, after immunoprecipitation with anti-p70 S6k serum. Data are in ± SEM for five separate experiments on different batches of heart cells.

S6k although the effect was only partial, even though we used concentrations of the drugs which are maximally effective against activation of p70 S6k by other agents (100nM wortmannin; 30$\mu$M LY294002). This inhibitory effect was also apparent when p70 S6k activation was assessed by SDS-PAGE (data not shown).

Since recent findings have suggested that PKB may play a role in the activation of p70 S6k [31], we also studied whether arsenite activated this enzyme. PKB exists as three isoenzymes in mammals [44-47]. Immunoblotting using isoform-specific antisera revealed that cardiac myocytes contain only the $\alpha$-isoenzyme (data not shown). Arsenite was unable to induce the shift in migration which is generally observed when PKB is activated [48] (data not shown). To rule out that activation was occurring without an observable band shift, PKB activity was also measured directly, following immunoprecipitation using a peptide substrate based on the N-terminus of GSK-3 ('Cross-tide', [40,49]). No increase in PKB activity was observed following treatment of the cells with arsenite: if anything a small decrease was seen (control, 100%; plus 1mM arsenite, 80.6±7.0%, n = 6). It thus appears that the very substantial arsenite-induced increase in p70 S6k activity thus occurs without a detectable change in PKB activity.

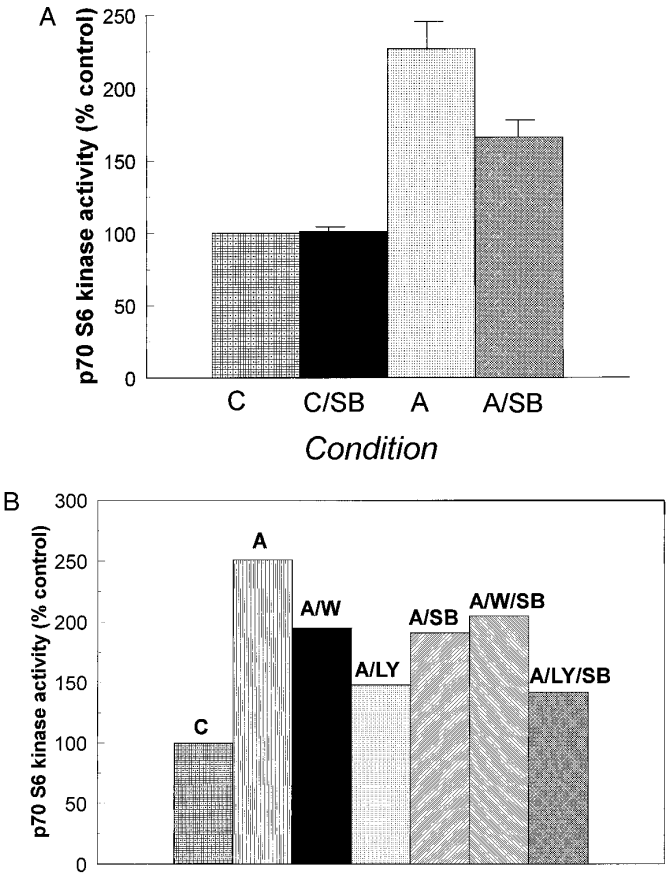



**FIG. 3.** Effects of SB203580 on the activation state of p70 S6k. Panel A: p70 S6k activity was measured after immunoprecipitation from extracts of heart cells pretreated (30min) with or without SB203580 (25$\mu$M) and then incubated (20min) with or without 1mM arsenite. Data are mean ± SEM from six separate experiments. Panel B: p70 S6k activity was measured after immunoprecipitation from extracts of heart cells pretreated with or without wortmannin (W; 100nM), LY294002 (LY; 30$\mu$M) or SB203580 (SB; 25$\mu$M) and then incubated with or without sodium arsenite (A; 1mM). Data are the average of two separate experiments.

### *Role of p38 MAP Kinase*

We assessed the role of p38 MAP kinase in the activation of p70 S6k by arsenite using the selective inhibitor SB203580 at a concentration which completely blocks activation of the downstream kinase MAPKAPK-2. Unexpectedly, it partially prevented the arsenite-induced activation of p70 S6k as assessed by kinase activity measurements (Fig. 3A). This inhibition of the arsenite-induced activation of p70 S6k was only partial even when submaximally effective concentrations of arsenite were used (data not shown). The partial inhibition by SB203580 was not additive with the inhibition caused by agents which block PI 3-kinase, indicating that these agents interfere with similar inputs into p70 S6k activation (Fig. 3B). It has recently been shown that MAPKAPK-2 can phosphorylate and partially activate PKB$\alpha$ *in vitro* [49]. However, we could not detect any activation of PKB is response to arsenite in our study, indicating the SB203580 sensitive input into to p70 S6k is not mediated through the action of MAPKAPK-2 on PKB.

### *Arsenite Causes Increased Phosphorylation of 4E-BP1*

The recently discovered regulatory phosphoprotein 4E-BP1 binds to and inhibits translation initiation factor eIF4E. Its phosphorylation is believed to be mediated via a pathway closely related to that activating p70 S6k [6-10]. To test whether the arsenite-induced activation of the p70 S6k pathway led to the expected downstream consequences, we studied the phosphorylation of 4E-BP1 as assessed by its migration on SDS-PAGE where more highly phosphorylated species of the protein migrate more slowly [7,8,50]. Arsenite-treatment of cells resulted in this characteristic upward band shift (Fig. 4A) such that the fastest migrating band disappeared and subsequently the uppermost one became the predominant species. This effect was completely blocked by rapamycin (Fig. 4A). Arsenite treatment also brought about the dissociation of 4E-BP1 from eIF4E which normally accompanies its phosphorylation (Fig. 4B).

## CONCLUSIONS

In this paper, we demonstrate that p70 S6k is activated by arsenite in adult cardiomyocytes. This is the

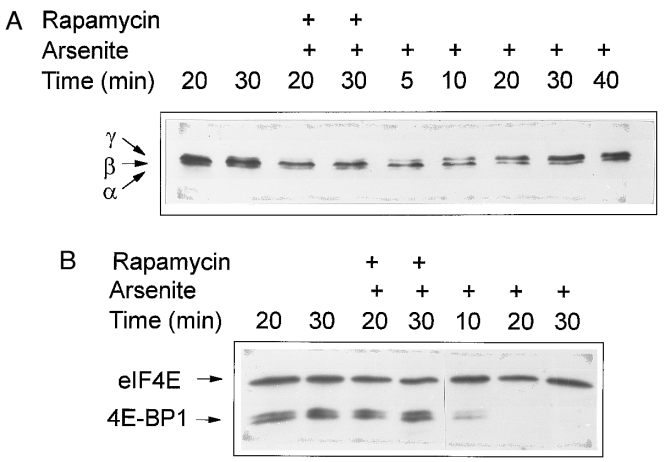



**FIG. 4.** Effects of arsenite on the phosphorylation state of 4E-BP1 and its association with eIF4E. Panel A: Isolated cardiomyocytes were preincubated with or without rapamycin (100nM for 20 min, as indicated). Cells were then further incubated with or without arsenite (1mM) for the times shown (min). Cells were extracted and 4E-BP1 was immunoprecipitated and analysed by SDS–PAGE and western blotting with anti-4E-BP1 antiserum. The figure shows a western blot. Labelled arrows indicate the positions of migration of the three species of 4E-BP1 (which differ in their states of phosphorylation) and can be separated on SDS–PAGE (termed $\alpha$, $\beta$ and $\gamma$). Panel B: As panel A (but note times) but eIF4E and 4E-BP1 were isolated by affinity chromatography on m7GTP-Sepharose and then analysed by SDS–PAGE and western blotting.

first demonstration that arsenite activates p70 S6k in any cell type. This finding is surprising, given that activation of p70 S6k is normally associated with treatment of cells by mitogens or analbolic stimuli. Arsenite has been previously shown to activate two other kinase cascades, those regulating the MAP kinase homologues p38 MAP kinase [51] and the SAP kinase family [52]. Two other agents which could be regarded as inducers of stress responses have been previously reported to activate p70 S6k: these are the protein synthesis inhibitors cycloheximide and anisomycin [9,53]. However, since arsenite does not inhibit protein synthesis in cardiac myocytes (data not shown), blockage of translation cannot be a common pathway leading to p70 S6k activation. Since the p70 S6k pathway and the two stress-activated kinase cascades mentioned above all have the potential to regulate transcription, their activation under stress conditions might serve to activate genes required to mediate (or, on the other hand, to counter) the effects of these stresses on the cell.

Recent work has indicated that the Rho family GTPases, Cdc42 and Rac1, are involved in the activation of both p38 MAP kinase and the SAP kinases [42,54,55]. Still more recently, Chou and Blenis [56] have provided evidence that these GTP-binding proteins can also mediate the activation of p70 S6k. Since, in general, arsenite can activate all three pathways, it may therefore be that these small G-proteins play a common role in their activation. In heart cells, we found that the activation of p70 S6k by arsenite was partially sensitive to inhibitors of PI 3-kinase, suggesting that, as in response to other stimuli, arsenite activation of p70 S6k may involve this enzyme (reviewed in [1]). However, the observation that such inhibition was only partial, even at sub-maximally effective concentrations of arsenite, suggests that other (parallel) steps are also involved. The ability of SB203580 also partially to block p70 S6k activation is of particular interest since it suggests that p38 MAP kinase, which is potently activated by arsenite, may also play a role in stimulating p70 S6k under these conditions, although this effect seems not to be additive with the input which requires PI 3-kinase.

The ability of arsenite to activate p70 S6k is not a general consequence of stress-treatment of heart cells since, as reported here, neither heat shock nor hydrogen peroxide treatment (both of which can activate other stress-activated kinases) resulted in the stimulation of p70 S6k. Very recently, while this paper was in preparation, Krause *et al.* [57] have reported that another stress stimulus (incubation in hypotonic medium) activates p70 S6k in hepatocytes and that this was blocked by wortmannin and rapamycin.

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