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Arsenite induces p70S6K1 activation and HIF-1α expression in prostate cancer cells

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Abstract

Arsenite is ubiquitous in the environment, particularly in the form of contaminated water. Although this metal is a known human carcinogen, its exact mechanism of action remains unclear. P70S6K1 phosphorylates the ribosomal 40S protein leading to increased protein translation, and is an important regulator of cell growth and proliferation. Hypoxia inducible factor-1 (HIF-1) is a basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β. HIF-1 activates the transcription of a number of genes that mediate angiogenesis and tumor formation. In this study we demonstrated that arsenite treatment increased levels of p70S6K1 phosphorylation and p70S6K1 activity in a PI3K and mTOR sensitive manner. We have also shown that arsenite specifically induces HIF-1α, but not HIF-1β, protein levels in prostate cancer cells in a mTOR-dependent manner. (Mol Cell Biochem **255**: 19–23, 2004)

Key words: arsenite, HIF-1, p70S6K1, PI3K, mTOR

Introduction

Arsenite is widely present in the environment due to both natural sources and human pollution. This metal has previously been associated with many types of cancer, however, the exact mechanism of arsenite-induced carcinogenesis remains to be elucidated. Previous studies have indicated that the activation of certain signaling cascades by arsenite is necessary for arsenite-induced transformation [1]. Thus it appears that the interaction between arsenite and signaling molecules plays an important role in its effects on the cell.

p70S6K1 is a serine/threonine kinase that plays an important role a number of cellular processes [2–4]. This kinase phosphorylates the 40S ribosomal subunit leading to the increased translation of proteins [5]. The activation of p70S6K1 is complex and requires its phosphorylation at a number of different sites [6]. The exact role of phosphorylation at each of these sites is unclear, however it is known that the phosphorylation of p70S6K1 Thr-229, Thr-389 and Ser-371 is necessary for full activation of p70S6K1, and is inhibited by both rapamycin, a specific inhibitor of the mamallian target of rapamycin (mTOR/ FRAP), and wortmannin, a phosphatidylinositol-3 kinase (PI3K) inhibitor [5]. It has been shown previously that mTOR regulates p70S6K1 activity, although the exact manner in which this occurs is unclear [7]. Recent studies suggest that PI3K serves as an intermediate between cell surface receptors and p70S6K1 [5, 8] and that one downstream target of PI3K, PDK1, directly phosphorylates p70S6K1 [9, 10]. Previous data indicate that arsenite induces p70S6K1 activity in non-cancerous cells [11, 12], though the exact mechanism of this effect remains unclear. However, it has been suggested that this effect is dependent upon mTOR as well as other as yet unidentified signaling molecules [11].

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β [13, 14]. The activity of HIF-1 is controlled primarily at the levels of HIF-1α present in the cells

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[15]. HIF-1 induces the transcriptional activation of a number of genes including vascular endothelial growth factor (VEGF), heme oxygenase 1, inducible nitric oxide synthase, several glycolytic enzymes and p21 [16, 17]. It has also been shown that HIF-1 plays an important role in tumorigenicity and angiogenesis in nude mice [18–20]. HIF-1 can be induced by growth factors, oncogenes and a loss of function due to mutation of the von Hippel Lindau tumor suppressor gene [21]. Recent studies have indicated that HIF-1 activity is regulated by the PI3K and mTOR signaling pathways in response to vanadate treatment and growth factor stimulation [22–25].

The exact mechanism by which arsenite stimulation leads to increased levels of HIF-1α is not known, however p70S6K1, being downstream of both PI3K and mTOR, could play a role. Therefore in this study we want to further examine the effects of arsenite on HIF-1α and p70S6K1. Specifically, we would determine [1] whether arsenite treatment induces p70S6K1 phosphorylation and activation in a PI3K and mTOR dependent manner and [2] whether arsenite increases levels of HIF-1α via the mTOR signaling cascade.

Materials and methods

Cell culture and reagents

Arsenic (III) chloride ($AsCl_3$) (Sigma-Aldrich, St. Louis, MO, USA), rapamycin and LY294002 were initially dissolved in DMSO. DU145 prostate cancer cells were maintained in minimum essential medium with Earle's salts (MEM) (Invitrogen, San Diego, CA, USA), 10% fetal bovine serum (Intergen, Purchase, NY, USA), 3% chicken serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured at 37°C in a 5% CO_2 incubator until confluence and were detached using trypsin (0.025%)/EDTA solution for sub-culture.

Immnunoblot analysis

DU145 cells were grown to 90% confluence in 100 mm plates and cultured in the presence or absence of $AsCl_3$. When used, the PI3K inhibitor, LY292004 and the mTOR inhibitor, rapamycin, were added to DU145 cells 30 min prior to treatment with $AsCl_3$ as indicated. Cells were collected in phosphate-buffered saline (PBS), and lysed on ice for 20 min using RIPA buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS and 5 mM EDTA) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 0.5 mM dithiothreitol, 2 mM pepstatin A and 1 mM phenylmethylsulfonyl floride on ice for 20 min. The resulting solution was centrifuged at 4°C at 13,000 rpm for 15 min. The supernatant containing cellular extracts was collected and stored at –70°C until use. The protein concentration was measured using Bio-Rad® protein assay reagents (Richmond, CA, USA). SDS/polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve 40 µg aliquots of total cellular protein, which were then transferred to nitrocellulose. After transfer, membranes were blocked in 5% nonfat dry milk in 1 × TBS overnight at 4°C. Membranes were then probed using antibodies against p70S6K1 and phospho-p70S6K1 (S389) (Cell signaling, Beverly, MA, USA). Protein bands were probed with horseradish peroxidase-conjugated antibodies (NEN, Boston, MA, USA), and visualized through an enhanced chemiluminescence reagent (NEN, Boston, MA, USA).

p70S6K1 assay

The p70S6K1 assay was performed as we described previously [26]. Briefly, DU145 cells were treated with arsenite for 1.5 h. When used, inhibitors were added to the medium 30 min before arsenite treatment. The cells were then harvested in cold PBS and lysed in cold immunoprecipitation assay buffer for 30 min. The lysate was centrifuged at 4°C at 13,00 rpm for 15 min. The protein concentration in the lysate was measured as in the immunoblot analysis. Two hundred µg of protein was incubated with 1.5 µg anti-p70S6K1 antibodies (Santa-Cruz Biotechnology) at 4°C for 1 h, followed by incubation with 30 ml of protein A/G-agarose beads (50% slurry; Santa Cruz Biotechnology) for 1 h at 4°C. The agarose beads were washed twice in cold PBS, then once in assay dilution buffer (20 mM 4-morpholinepropanesulfonic acid (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM DTT). $p70^{s6k}$ activity in these samples was then measured using a S6 kinase assay kit (Upstate Biotechnology, NY, USA) per the manufacturer's instructions. Aliquots (20 µl) of supernatant was spotted in a P81 phosphocellulose filter and washed first in 0.75% phosphoric acid, then in acetone. The filters were counted in a Wallace 1410 liquid scintillation counter (Perkin-Elmer).

Results

Induction of p70S6K1 phosphorylation by arsenite in DU145 cells

To determine whether arsenite could induce p70S6K1 phosphorylation, we incubated DU145 prostate cancer cells with arsenite at various concentrations for 1.5 h. Levels of phospho-p70S6K1 were increased by arsenite in a dose-dependent manner (Fig. 1). The observed increase was concentration-dependent over a 0–10 µM range. Total levels of p70S6K1

were not affected by arsenite treatment (Fig. 1). To determine the effects of length of exposure to arsenite on p70S6K1 phosphorylation, DU145 prostate cancer cells were incubated with 50 mM arsenite for a number of different time periods and were analyzed for p70S6K1 phosphorylation. Levels of phosphorylated p70S6K1 were maximal at 1.5 h after the exposure, and were reduced to lower levels at subsequent time points (Fig. 2).

Inhibition of arsenite-induced p70S6K1 phosphorylation by rapamycin and LY2942002

To examine the upstream signals involved in induction of p70S6K1 phosphorylation by arsenite, DU145 cells were pretreated with solvent, LY2922002 (a PI3 Kinase inhibitor) or rapamycin (an inhibitor of mTOR) 30 min prior to exposure to 50 μM arsenite for 1.5 h. Arsenite treatment markedly induced p70S6K1 phosphorylation, which was inhibited by rapamycin at all concentrations tested (Fig. 3). LY2942002 (20 μM) also completely inhibited arsenite-induced p70S6K1 phosphorylation (Fig. 3). Total levels of p70S6K1 were not affected by LY294002 or rapamycin. These data indicate that arsenite induces phosphorylation of p70S6K1 via PI3K and mTOR in DU145 prostate cancer cells.

Arsenite induces p70S6K1 activity in a LY294002- and rapamycin-dependent manner

To determine whether arsenite induces p70S6K1 activity through the PI3K and mTOR signaling pathways, we pretreated DU145 cells with solvent, rapamycin or LY292004 for 30 min, then exposed the cells to several concentrations of arsenite for 1.5 h. Consistent with the levels of p70S6K1 phosphorylation, arsenite treatment led to increased levels of

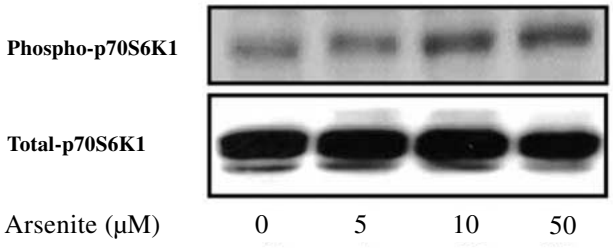

Fig. 1. Effects of arsenite concentration on p70S6K1 phosphorylation. DU145 cells were treated with 0, 5, 10 or 50 μM arsenite for 1.5 h. Total cellular protein extracts were prepared and 40 μg of protein was used for immunoblot analysis. Specific antibodies against phospho-p70S6K1 (S389) and total p70S6K1 were used to detect protein levels.

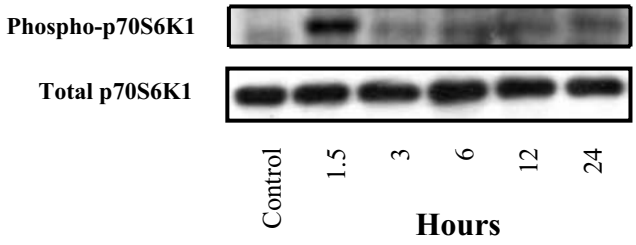

Fig. 2. Effects of treatment time with arsenite on p70S6K1 phosphorylation. DU145 cells were treated with arsenite (50 μM) for 0, 1.5, 3, 6, 12 and 24 h. Forty μg of total cellular protein extracts were analyzed by immunobloting using antibodies specific for phospho-p70S6K1 (S389) and total p70S6K1.

p70S6K1 activity, which was inhibited by the PI3K inhibitor, LY294002 and the mTOR inhibitor, rapamycin (Fig. 4).

HIF-1α expression was induced by arsenite and inhibited by rapamycin

Recent studies indicate that HIF-1α is induced by growth factors via mTOR signaling [23–25]. To investigate whether arsenite induces HIF-1α expression through a PI3K and mTOR signaling pathway, DU145 cells were pretreated with solvent or rapamycin 30 min prior to exposure to arsenite for 6 h. The cellular proteins were analyzed using antibodies against HIF-1α and HIF-1β proteins as described previously [22]. Arsenite treatment increased the levels of HIF-1α in the cells, which was inhibited by rapamycin (Fig. 5). In contrast, levels of HIF-1β were not affected by arsenite or rapamycin treatment. This result indicates that arsenite specifically increases levels of HIF-1α, but not HIF-1β in prostate cancer cells.

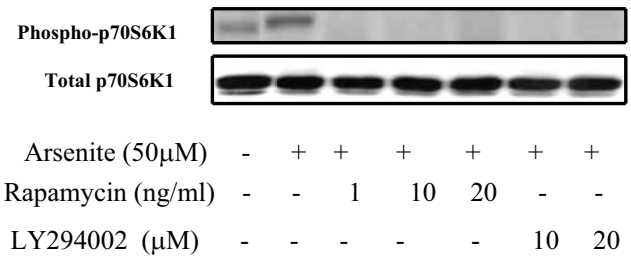

Fig. 3. Effects of LY294002 and rapamycin on arsenite-induced p70S6K1 phosphorylation. DU145 cells were pretreated with solvent, LY292004, or rapamycin 30 min prior to treatment with 50 μM arsenite for 1.5 h. Total cellular protein extracts were prepared, and 40 μg of protein extract was used for immunoblot analysis. Specific antibodies against phospho-p70S6K1 (S389) and total p70S6K1 were used for the assay.

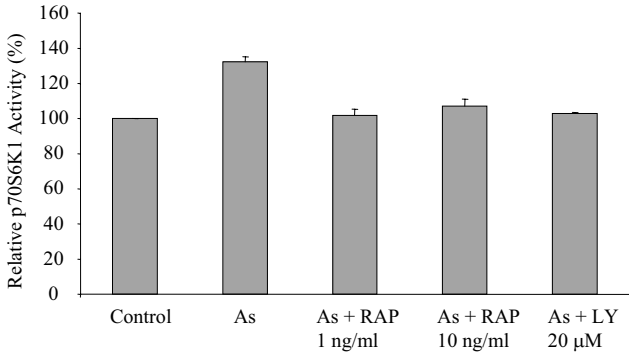

Fig. 4. Effects of LY294002 and rapamycin on arsenite-induced p70S6K1 activity. DU145 cells were treated as described in Fig. 3. p70S6K1 activity was assayed using a S6 kinase assay kit per the manufacturer's instructions. The data are values from replicated experiments, which are normalized to the values obtained in the untreated cell control.

Discussion

In this study, we show that arsenite induces p70S6K1 in a PI3K and mTOR dependent manner in DU145 prostate carcinoma cells. Arsenite is a known human carcinogen, having been associated with a number of different types of human cancer [27]. The exact mechanism by which arsenite leads to tumor formation remains unknown [27], although some evidence indicates that much of the tumorigenic potential of arsenite is due to its ability to activate certain signal transduction cascades, leading to altered gene expression [28]. This would be in line with our findings of activated downstream targets of the PI3K and mTOR signaling pathways in response to arsenite treatment.

We also demonstrated that arsenite specifically increased levels of HIF-1α, which is the regulatory subunit of the transcriptional factor HIF-1 [15]. This result is consistent with recent observations that HIF-1α is induced by arsenite [29]. Furthermore, results indicate that mTOR signaling was involved in the induction of HIF-1α by arsenite.

p70S6K1 is involved in many cellular functions, including cell proliferation and tumor formation [30, 31], while HIF-1α has been implicated in both tumor formation and angiogenesis [18–20]. This study demonstrates that arsenite treatment induced p70S6K1 activation and increased HIF-1α levels in human prostate cancer cells, suggesting that both of these signaling molecules play a role in arsenite-induced carcinogenesis. The next goal of this research is to determine the specific roles of p70S6K1 and HIF-1α in arsenite-induced tumor formation, either by using knockout cell lines for these signaling molecules or by using dominant negative constructs of HIF-1α and p70S6K1.

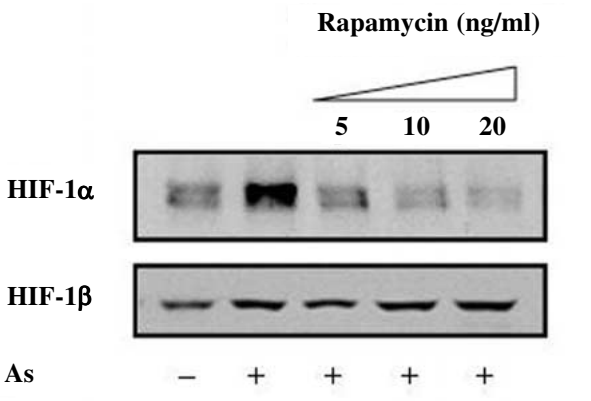

Fig. 5. Effects of arsenite treatment on HIF-1α levels. DU145 prostate carcinoma cells were treated with arsenite (50 μM) for 6 h. Cells in the inhibitor group were pre-treated with rapamycin for 30 min prior to arsenite treatment. Total cellular protein extracts were prepared and 40 μg of protein was used for immunoblot analysis. Antibodies against HIF-1α and HIF-1β proteins were used for the immunoblotting assay [22].

Acknowledgement

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