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Hydrogen peroxide mediates arsenite activation of p70^{s6k} and extracellular signal-regulated kinase

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Abstract

To define the mechanism of arsenite-induced tumor promotion, we examined the role of reactive oxygen species (ROS) in the signaling pathways of cells exposed to arsenite. Arsenite treatment resulted in the persistent activation of p70^{s6k} and extracellular signal-regulated kinase 1/2 (ERK1/2) which was accompanied by an increase in intracellular ROS production. The predominant produced appeared to be H_2O_2, because the arsenite-induced increase in dichlorofluorescein (DCF) fluorescence was completely abolished by pretreatment with catalase but not with heat-inactivated catalase. Elimination of H_2O_2 by catalase or *N*-acetyl-L-cysteine inhibited the arsenite-induced activation of p70^{s6k} and ERK1/2, indicating the possible role of H_2O_2 in the arsenite activation of the p70^{s6k} and the ERK1/2 signaling pathways. A specific inhibitor of p70^{s6k}, rapamycin, and calcium chelators significantly blocked the activation of p70^{s6k} induced by arsenite. While the phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and LY294002 completely abrogated arsenite activation of p70^{s6k}, ERK1/2 activation by arsenite was not affected by these inhibitors, indicating that H_2O_2 might act as an upstream molecule of PI3K as well as ERK1/2. Consistent with these results, none of the inhibitors impaired H_2O_2 production by arsenite. DNA binding activity of AP-1, downstream of ERK1/2, was also inhibited by catalase, *N*-acetyl-L-cysteine, and the MEK inhibitor PD98059, which significantly blocked arsenite activation of ERK1/2. Taken together, these studies provide insight into mechanisms of arsenite-induced tumor promotion and suggest that H_2O_2 plays a critical role in tumor promotion by arsenite through activation of the ERK1/2 and p70^{s6k} signaling pathways.

Keywords: Arsenite; H_2O_2; p70^{s6k}; Extracellular signal-regulated kinase 1/2

Introduction

Arsenic is ubiquitous in the environment, occurring mainly as arsenite and arsenate [1]. It is released into the environment during energy production from coal, oil shale, and geothermal sources. Epidemiological studies have shown that chronic exposure to arsenic can result in liver injury, peripheral neuropathy, and an increased incidence of cancers of the kidney, lung, skin, bladder, liver, and colon [2–6]. While a single exposure to arsenic appears to be nonmutagenic, arsenic potentiates the mutagenic effects of short-wavelength UV radiation [7], suggesting that arsenite acts as a tumor promoter in the carcinogenic process; however, the mechanism has not yet been defined.

Activation of tumor cell proliferation requires transcriptional upregulation of the immediate early genes, as well as an accelerated rate of translation of various genes, which are regulated by intracellular activation of several signaling

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protein kinase cascades [8, 9]. Among them, two protein kinase cascades, referred to as the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway and the $p70^{s6k}$ signaling pathway, have been demonstrated to be responsible for the upregulation of transcription [10] and translation in response to various growth factors [11], respectively. Ligand-mediated dimerization of growth factors triggers the activation of receptor tyrosine kinases, resulting in autophosphorylation of tyrosine residues [12,13], and these residues then serve as docking sites for the recruitment of downstream signaling mediators [14,15].

One of the signaling pathways bifurcating at the specific docking sites of receptor tyrosine kinase is the ERK1/2 signaling pathway consisting of Grb2/Sos, Ras, Raf-1, MEK1, and ERK1/2 [16]. Activated ERK1/2 is responsible for the phosphorylation of a variety of cellular proteins, including downstream kinases and transcription factors that are involved in the transcriptional regulation of the immediate early genes (e.g., *c-myc*, *c-jun*, and *c-fos*) [10]. The other signaling pathway is the $p70^{s6k}$ pathway, which bifurcates at a growth factor receptor docking site distinct from that of the Ras/ERK1/2 pathway [17]. Many studies, including point mutational analysis of platelet-derived growth factor (PDGF) receptor [18], and the effect of various mutants of phosphatidylinositol 3-kinase (PI3K) [19] and PKB [20,21], and specific inhibitors of PI3K and FK506-rapamycin-associated protein (FRAP) on $p70^{s6k}$ activity [18,22] have suggested that the $p70^{s6k}$ signaling pathway includes PI3K, PDK1, PKB, and FRAP as upstream mediators of $p70^{s6k}$ activity, although the function of PKB as a regulator of the $p70^{s6k}$ signaling pathway has been challenged [23, 24]. The major substrate of $p70^{s6k}$ appears to be the 40 S ribosomal protein S6 [25], whose multiple phosphorylation in the cytosol has been implicated in the selective translational upregulation of a family of mRNA transcripts that contain a polypyrimidine tract at their 5′ transcriptional start site [26, 27].

Dysregulation of the $p70^{s6k}$ pathway or ERK1/2 pathway through alterations in any one of several mediators involved in the cascade can lead to cellular transformation [28,29]. Indeed, many tumors exhibit elevated $p70^{s6k}$ [29] or ERK1/2 [30,31] activity. It is not surprising, therefore, that a common feature of known tumor promoters (e.g., phorbol ester) is their ability to perturb the $p70^{s6k}$ or ERK1/2 signaling pathway.

There is emerging evidence that reactive oxygen species (ROS) at low concentration may function as a signaling intermediator of cellular responses [32,33]. The generation of ROS by external stimuli is associated with various cellular processes, such as cell proliferation [34] and apoptosis [35]. ROS have been shown to stimulate components of signaling pathways implicated in growth factor and cytokine effects. The components include transcription factors, NF-κB [36], and AP-1 [37], as well as ERK1/2 [33,38,39] and $p70^{s6k}$ [34], which play an important role in cell proliferation and various intracellular signaling pathways. Arsenite has also been shown to cause a significant elevation of $p70^{s6k}$ or ERK1/2 activity in rat cardiomyocytes and PC12 cells [40,41]. Furthermore, the production of ROS in response to arsenite treatment has been observed in various cell lines [42,43], suggesting that arsenite may act early in the growth factor signaling pathway. Therefore, a better understanding of the signal transduction pathway leading to $p70^{s6k}$ and ERK1/2 activation by arsenite could provide important insight into its mechanism of tumor promotion. In this report, we focused our attention mainly on the early events in the signaling pathway leading to $p70^{s6k}$ and ERK1/2 activation, and provided evidence that arsenite treatment results in the production of hydrogen peroxide (H_2O_2), leading to the activation of $p70^{s6k}$ and ERK1/2. The ability of arsenite to usurp this growth regulatory pathway might likely contribute to its tumor promotion.

Materials and methods

Cell culture

JB6 cells (Balb-c mouse epidermal cells) were cultured in minimum essential medium (MEM) containing 8% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Cells were serum deprived in serum-free medium for 24 h prior to treatment with arsenite.

Preparation of cell extracts

Seventy-eight percent confluent cells were made quiescent by culturing them for 24 h in MEM without fetal bovine serum. Serum-deprived cells were treated at 37°C with arsenite for the indicated times. Alternatively, cells were pretreated for the indicated times at 37°C with serum-free MEM containing EGTA (5 mM), BAPTA-AM (20 μM), catalase (300 units/ml, *Aspergillus niger*), rapamycin (5 nM), wortmannin (500 nM), LY294002 (25 μM), or PD98059 (50 μM) before arsenite stimulation. After incubation, cells were rinsed twice with an ice-cold wash buffer solution containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride, and then extracted in the same buffer containing 0.1% Nonidet P-40. Cell extracts were collected with a plastic scraper, homogenized, and cleared by centrifugation at 4°C for 15 min at 12,000 rpm. Aliquots of the supernatant were frozen in liquid nitrogen and stored at −70°C.

Immunoblotting

Cell lysates were boiled in Laemmli sample buffer for 5 min. Cell lysates containing 50 μg of total protein were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on 15% slab gels for the determination of $p70^{s6k}$

and ERK1/2, and proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min in Tris-buffered saline containing 0.5% Tween 20 and 5% (w/v) dry skim milk powder, and incubated overnight with anti-$p70^{s6k}$ and anti-ERK1/2 antisera. The membranes were then washed with blocking solution and incubated for 90 min with alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody. Bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (Bio-Rad).

Immunoprecipitation and $p70^{s6k}$ activity assay

$p70^{s6k}$ was immunoprecipitated by incubating 20 μg (total 200 μl) of protein/assay of cell extract to an antibody directed to the C-terminal 18 residues of $p70^{s6k}$ for 2 h incubation at 4°C. Immunoprecipitation was facilitated by the addition of protein A–Sepharose (25 μl) for 30 min at 4°C on a shaking plate. The beads were then washed twice at 4°C with an extraction buffer containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, 0.1 mM PMSF, and 0.1% Nonidet P-40, and once with a dilution buffer containing 50 mM Mops (pH 7.0), 1 mM DTT, 5 mM $MgCl_2$, 10 mM *p*-nitrophenyl phosphate, and 0.1% Nonidet P-40. $p70^{s6k}$ activity was assayed using the S6 peptide as a substrate by incubating the immunoprecipitate for 30 min in 25 μl of reaction mixture containing 50 mM Mops (pH 7.0), 5 mM $MgCl_2$, 1 mM DTT, 10 mM *p*-nitrophenyl phosphate, 0.1% Nonidet P-40, 0.6 μM PKI, and 12 μM ATP (plus 0.75 μCi [γ-^{32}P]ATP). The reaction was terminated by the addition of 10 μl of 100 mM EDTA (pH 7.0). Following a brief centrifugation, the supernatant was spotted on P-81 paper. Unincorporated [γ-^{32}P]ATP was eliminated by three 10-min washes in 5% phosphoric acid, and phosphorylated S6 peptide bound to the paper was counted. Assays were carried out in duplicate. The results were calculated as units of S6 kinase activity per milligram of protein lysate and expressed as the fold increase over the control value. One unit of activity represents the transfer of 1 pmol of $^{32}P_i$ into S6 peptide per minute under the assay conditions.

Measurement of intracellular reactive oxygen species

Cells were grown on coverslips for 2 days and serum-free MEM for 1 day. The serum-deprived cells were stabilized in serum-free MEM without phenol red for at least 30 min and stimulated with arsenite for different times. Sometimes, cells were preincubated with various inhibitors of protein kinases or antioxidants for 30 min before treatment with arsenite. For the last 5 min of stimulation, 5 μM 2′, 7′-dichlorofluorescein (DCF) was added to monitor intracellular H_2O_2. Then, the cells were immediately observed with a laser scanning confocal microscope (Carl Zeiss LSM 410). The images of samples excited by a 488-nm argon laser were filtered through a long-pass 515-nm filter. Thirty cells were randomly selected from each experiment and the mean relative fluorescence intensity of each group of cells was then measured with the Karl Zeiss vision system (KS400, Version 3.0).

Electrophoretic mobility shift assay of activator protein 1 of AP-1

Oligonucleotide probe containing the activator protein 1 (AP-1) binding site TGACTCA (12-*o*-tetreadecanoylphorbol-13-acetate response element) was labeled with [α-^{32}P]dATP using Klenow fragment. The sequences of probe used in this work were 5′-TTG CGC ATG ACT CAC-3′ (sense) and 5′-GTG GGT GAG TCA TGC-3′ (antisense). Binding reactions were performed at room temperature for 30 min with 5 μg of nuclear protein in 20 μl of binding buffer (10 mM Hepes–KOH, pH 7.7, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 1 μg/ml leupeptin, pepstatin, and aprotinin) containing 1 μg of poly-[dI–dC · dI–dC] and 100,000 cpm ^{32}P-labeled probe. The specificity of the binding reaction was confirmed by competition assay with a 100-fold molar excess of unlabeled oligonucleotide probe. DNA–protein complex was separated from unbound probe on native 6% polyacrylamide gels. After electrophoresis, the gel was vacuum-dried and analyzed with the Fluorescent Image Analyzer FLA-2000 (Fujifilm, Japan).

Results

Activation of $p70^{s6k}$ and ERK1/2 in response to arsenite

We have reinvestigated previous reports that arsenite activates $p70^{s6k}$ [41] and ERK1/2 [40,44] to obtain kinetic information. Thus, mouse JB6 epidermal cells were treated with various concentrations of arsenite and activity of $p70^{s6k}$ and ERK1/2 was measured. As shown in Fig. 1, arsenite treatment led to dose-dependent phosphorylation and activation of $p70^{s6k}$, as measured by its slower migration on Western blots of one-dimensional SDS–PAGE and immune complex kinase assay. An increase in $p70^{s6k}$ activity was apparent above 10 μM arsenite and maximum $p70^{s6k}$ activity was observed at 100 μM (Fig. 1A). The increase in phosphorylation and $p70^{s6k}$ activity following arsenite treatment was apparent as early as 10 min, with maximum activity at 60 min, which was sustained longer than 90 min (Fig. 1B). Similarly, ERK1/2 phosphorylation was also dose-dependent and appeared from 10 min, with maximal phosphorylation at 60 min, which was prolonged up to 90 min (Figs. 2A, B). Therefore, the results presented in Figs. 1 and 2 not only confirm the earlier observations, but also provide additional information on the kinetics of $p70^{s6k}$ and ERK1/2 activation. The sustained activation of $p70^{s6k}$ and ERK1/2 with arsenite may be compared with the

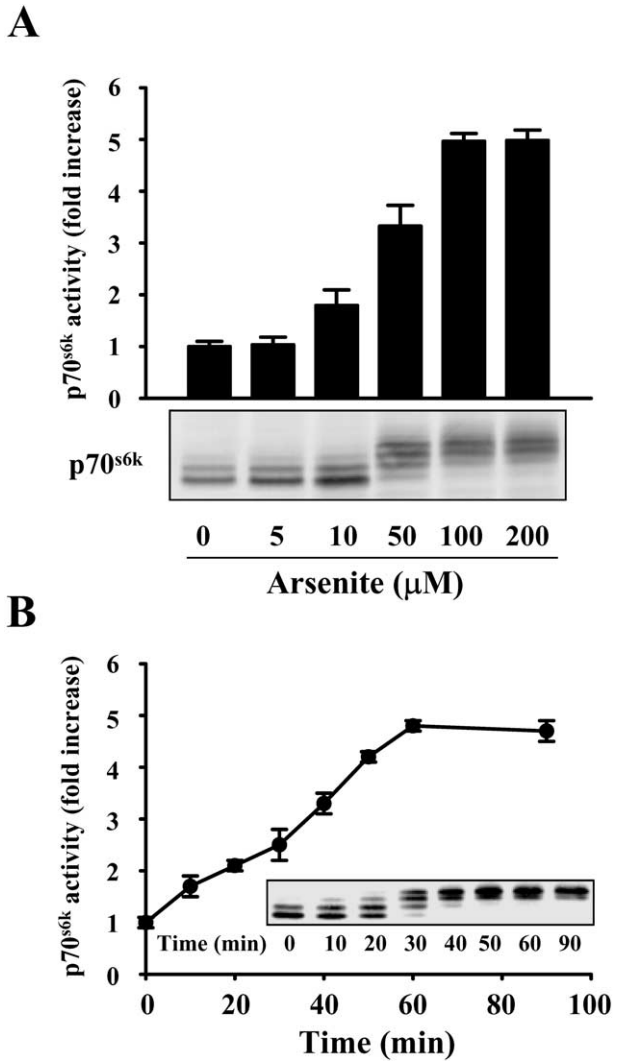

Fig. 1. $p70^{s6k}$ was activated by arsenite in a dose- and time-dependent manner. (A) Serum-deprived JB6 cells were stimulated with the indicated concentrations of arsenite for 60 min. Cell lysates were immunoprecipitated using a specific antibody for $p70^{s6k}$ and assayed for S6 kinase activity as described under Materials and methods. $p70^{s6k}$ activity was expressed as the fold increase over the control value. The results shown represent the means ± SEM of three independent experiments. The levels of $p70^{s6k}$ phosphorylation in the above-described cell lysates were monitored as slower migration of a family of bands on SDS–PAGE and immunoblot analysis using a specific antibody for $p70^{s6k}$. (B) After treatment with arsenite (100 μM) for the indicated times in serum-deprived JB6 cells, cell lysates were immunoprecipitated and assayed for S6 kinase activity as described under Materials and Methods. Inset: Levels of $p70^{s6k}$ phosphorylation in the cell lysates.

transient activation usually observed with growth factors such as EGF [45]. However, the magnitude of $p70^{s6k}$ activation by arsenite (about fivefold activation) is comparable to that seen with EGF [46].

Involvement of intracellular H_2O_2 in arsenite activation of $p70^{s6k}$ and ERK1/2

A recent study showed that H_2O_2 was able to mediate the activation of $p70^{s6k}$ and ERK1/2 by growth factor in JB6 cells [34]. As arsenite is known to produce ROS [42,43], we presumed that ROS might play a role as a mediator in the activation of $p70^{s6k}$ and ERK1/2 by arsenite. To determine the possibility, we first examined whether ROS were generated following treatment of JB6 cells with arsenite by measuring the intracellular generation of ROS with oxidation of the peroxide-sensitive fluorophore DCF. Microfluorometric studies with laser scanning confocal microscopy revealed that exposure of quiescent cells to arsenite resulted in a dose-dependent increase in DCF fluorescence (Figs. 3A, B). The increase in DCF fluorescence was apparent as early as 10 min after the arsenite treatment, and maximum production of ROS was observed at 60 min, and was sustained for up to 90 min with a slight decrease (Fig. 3C). The arsenite-stimulated increase in DCF fluorescence was completely abolished by preincubation with catalase (Figs. 4A, B) which is taken up by an unknown mechanism across the plasma membrane of vascular smooth muscle cells [33] as well as JB6 cells [34]. However, pretreatment of JB6 cells with heat-inactivated catalase did not affect arsenite-induced ROS production, indicating that the predominant ROS produced was H_2O_2 (Figs. 4A, B).

The dose response and time course of H_2O_2 production were well correlated with those of $p70^{s6k}$ and ERK1/2 activation; suggesting a possible role of H_2O_2 in the arsenite-induced activation of $p70^{s6k}$ and ERK1/2. To further confirm the role of H_2O_2 in arsenite-induced activation of $p70^{s6k}$ and ERK1/2, we attempted to reduce intracellular arsenite-induced H_2O_2 generation with antioxidants such as catalase and *N*-acetyl-L-cysteine (NAC), a thiol-containing compound, which nonenzymatically interacts and detoxifies reactive electrophiles and free radicals [46]. When JB6 cells were preincubated with catalase from *Aspergillus niger*

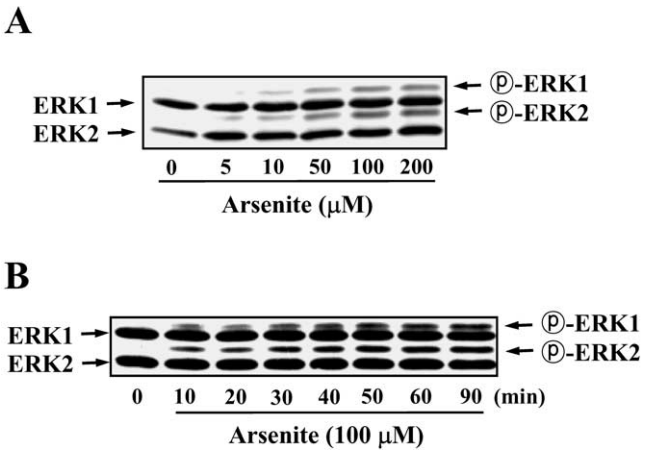

Fig. 2. ERK1/2 was activated by arsenite in a dose- and time-dependent manner. (A) Serum-deprived JB6 cells were stimulated with the indicated concentrations of arsenite for 60 min. (B) To examine the kinetics of arsenite activation of ERK1/2, serum-deprived JB6 cells were treated with arsenite (100 μM) for the indicated times. The activation of ERK1/2 in arsenite-stimulated cell lysates was monitored as slower migration of a family of bands on SDS–PAGE and immunoblot analysis using a specific antibody for ERK1/2. Results show a representative blot from three independent experiments.

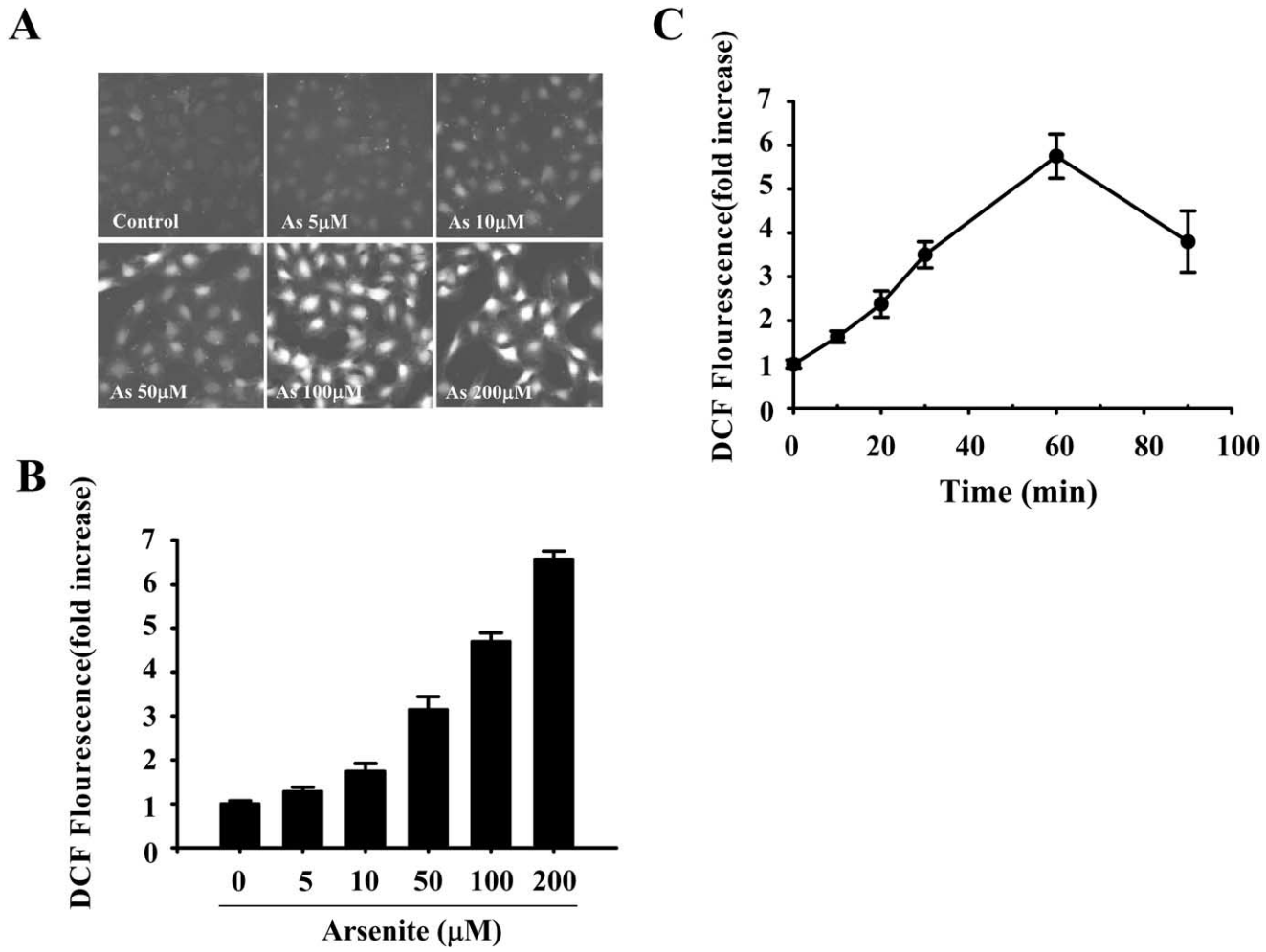

Fig. 3. H_2O_2 was produced by arsenite in a dose- and time-dependent manner. JB6 cells were grown on coverslips for 2 days and serum-free MEM for 1 day. The serum-deprived cells were stabilized in serum-free MEM without phenol red for at least 30 min. (A) After treatment with the indicated concentrations of arsenite (As) for 60 min, cells were incubated in the presence of 5 μM DCF for the last 5 min to monitor intracellular ROS. DCF fluorescence was measured with a confocal scanning laser microscope. Results are representative photographs from three independent experiments. (B) Thirty cells were randomly selected from each of the experiments in (A), and fluorescence intensity was then measured with the Karl Zeiss vision system (KS400, Version 3.0) and averaged. Fluorescence intensity of each group is expressed as the fold increase over the control value. Data shown represent means ± SEM of the fluorescence intensities of each group. (C) After treatment with arsenite (100 μM) for the indicated time, cells were incubated in the presence of 5 μM DCF for the last 5 min to monitor intracellular ROS. The fluorescence intensity is the mean ± SEM of three independent experiments.

(300 units/ml) or NAC (30 mM) for 30 min, $p70^{s6k}$ phosphorylation and activity (Fig. 4C), as well as ERK1/2 phosphorylation (Fig. 4D), decreased: activation of $p70^{s6k}$ and ERK1/2 was almost completely inhibited to near-basal levels. These results suggest that the sustained production of H_2O_2 by arsenite could be responsible for the persistent activation of $p70^{s6k}$ and ERK1/2, thereby leading to tumor promotion. Consistent with these results, ERK1/2 activation by arsenite has been demonstrated to be required for the arsenite-induced cell transformation [47].

Effect of calcium chelators on arsenite-induced $p70^{s6k}$ activity

Earlier studies showed that incubation of various cells with compounds that increase intracellular calcium concentration (ionophore A23187, thapsigargin) stimulates $p70^{s6k}$ [48], and that PDGF- or H_2O_2-induced $p70^{s6k}$ activation is ablated by Ca^{2+} chelation [24,34], indicating a possible role of Ca^{2+} in regulation of $p70^{s6k}$. However, the requirement of Ca^{2+} for ERK1/2 activation has been shown to vary with individual cell types and stimuli [34,49,50]. We therefore examined the possible involvement of Ca^{2+} in arsenite activation of $p70^{s6k}$ and ERK1/2. When JB6 cells were preincubated with either the extracellular calcium chelator EGTA (5 mM) or the cell-permeant calcium chelator BAPTA-AM (20 μM), the activation and phosphorylation of $p70^{s6k}$ by arsenite were completely inhibited (Fig. 5A). In comparison, neither EGTA nor BAPTA-AM significantly inhibited arsenite-induced phosphorylation of ERK1/2 (Fig. 5B). These results indicate that the arsenite-stimulated

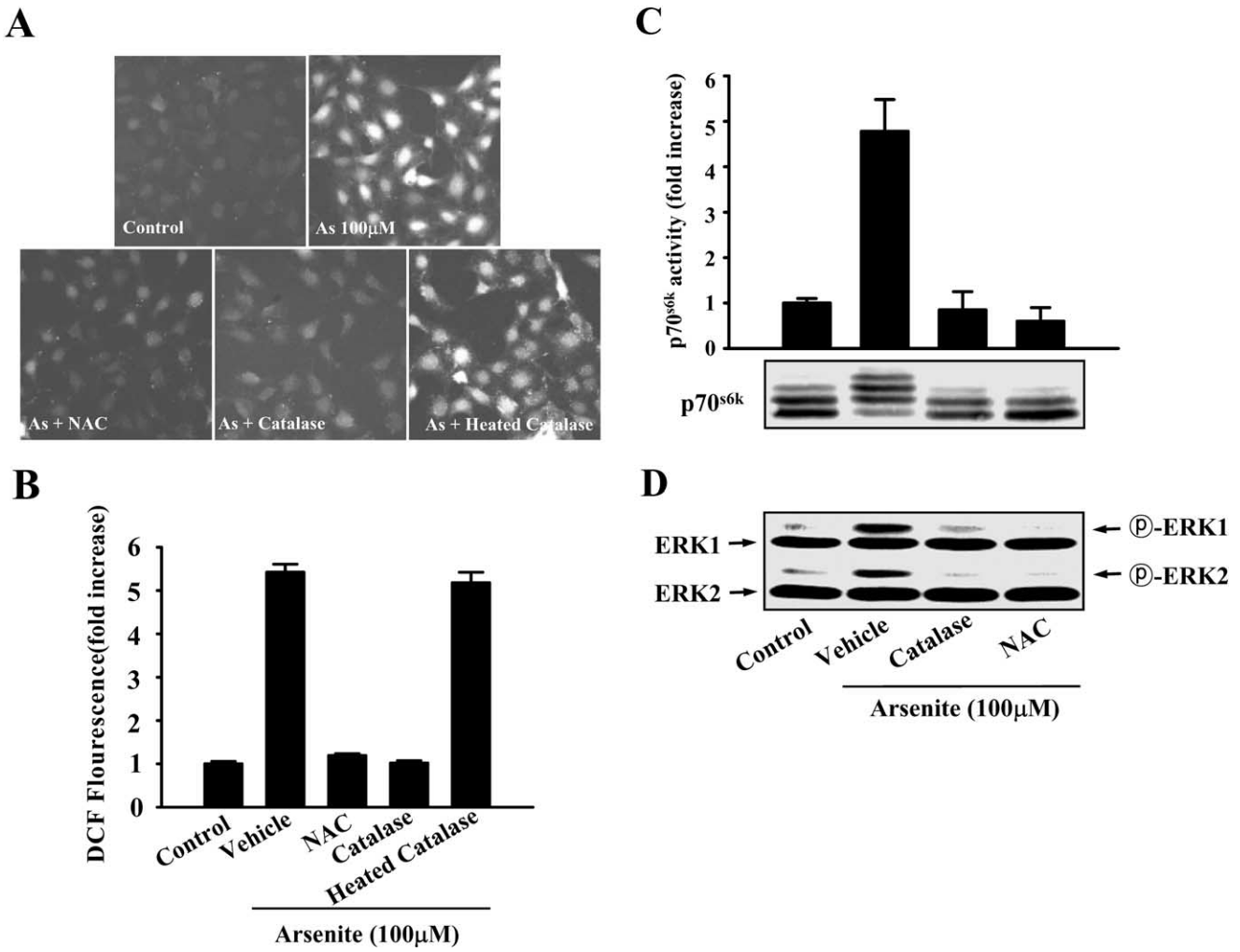

Fig. 4. H_2O_2 was an intracellular mediator in arsenite activation of $p70^{s6k}$ and ERK1/2. (A) Cells were pretreated with 30 mM NAC, catalase (300 units/ml, from *Aspergillus niger*), or heat-inactivated catalase for 30 min before stimulation with 100 μM arsenite. Results are representative photographs from three independent experiments. (B) Fluorescence intensity was measured and is expressed as the fold increase as described for Fig. 3. (C) Serum-deprived JB6 cells were stimulated with arsenite (100 μM) for 60 min. Alternatively, samples were preincubated with 30 mM NAC or 300 units/ml catalase for 30 min. Activity and phosphorylation of $p70^{s6k}$ were measured as described earlier. The results shown are means ± SEM of three independent determinations. (D) Phosphorylation of ERK1/2 was analyzed as described in Fig. 2. NAC, *N*-acetyl-L-cysteine.

$p70^{s6k}$ signaling pathway is intracellular calcium-dependent, whereas the arsenite activation of ERK1/2 is not, in JB6 cells. The requirement for Ca^{2+} appeared to be downstream of H_2O_2, because pretreatment of JB6 cells with EGTA and BAPTA-AM did not cause any alteration of H_2O_2 production by arsenite (Figs. 7A, B). Consistent with these results, H_2O_2 stimulation of Ca^{2+} release has also been observed in Rat-2 fibroblasts [51].

Differential involvement of PI3K in arsenite-activated $p70^{s6k}$ and ERK1/2 signaling pathways

In rat cardiomyocytes, it has been observed that arsenite activation of $p70^{s6k}$ is inhibited by inhibitors of PI3K, indicating the possible involvement of PI3K in the activation of $p70^{s6k}$ by arsenite [41]. Although it has been suggested that PI3K acts as an upstream molecule of H_2O_2 produced by PDGF [52], this has been disputed, because exposure of cardiomyocytes to H_2O_2 markedly increased PI3K activity and tyrosine phosphorylation of the p85 regulatory subunit [53]. Thus, we further characterized the role of PI3K as well as FRAP in arsenite-induced signaling pathways, using specific inhibitors for PI3K and FRAP. Pretreatment of JB6 cells with wortmannin (500 nM) or LY294002 (25 μM) completely inhibited arsenite activation and phosphorylation of $p70^{S6k}$ to almost basal levels (Fig. 6A), indicating the possible role of PI3K as a mediator of arsenite activation of $p70^{s6k}$. In constrast, the phosphorylation of ERK1/2 by arsenite was not affected by PI3K inhibitors (Fig. 6B), indicating that PI3K was not required for activation of the ERK1/2 signaling pathway by arsenite. Neither wortmannin nor LY294002 suppressed H_2O_2 production by arsenite

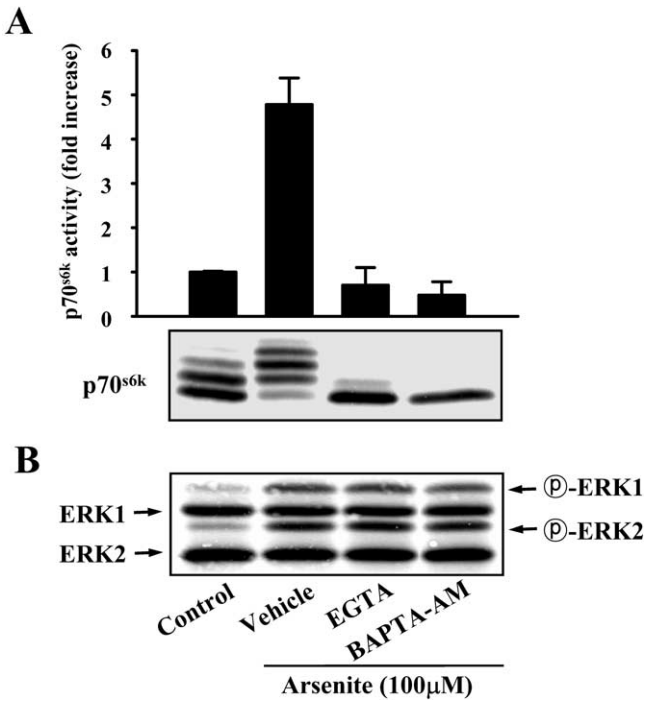

Fig. 5. Arsenite activation of 70^{s6k} was mediated by the intracellular Ca^{2+} signaling pathway. Serum-deprived JB6 cells were stimulated with 100 μM arsenite for 60 min. Alternatively, samples were preincubated with 5 mM EGTA or 20 μM BAPTA-AM for 30 min. (A) The activity and phosphorylation of $p70^{s6k}$ were assayed as described in Fig. 1. The results shown represent the means ± SEM of four independent experiments. (B) Phosphorylation of ERK1/2 was determined as described in Fig. 2.

(Figs. 7A, B). These observations further support the notion that PI3K acted downstream rather than upstream of H_2O_2 produced by arsenite, because arsenite activation of ERK1/2 would be inhibited by the decrease in H_2O_2 by PI3K inhibitors, if PI3K acted as an upstream molecule of H_2O_2. The FRAP-specific inhibitor rapamycin at 5 nM significantly inhibited arsenite-induced $p70^{s6k}$ activity, indicating the involvement of FRAP in arsenite activation of $p70^{s6k}$. Pretreatment of JB6 cells with the MEK-specific inhibitor PD98059 (50 μM) significantly inhibited ERK1/2 activation, but had no effect on $p70^{s6k}$ activity at the same concentration (Figs. 6A, B). Therefore, arsenite stimulation of $p70^{s6k}$ appears to be mediated through PI3K and FRAP.

Requirement of H_2O_2 and ERK1/2 for induction of activator protein 1 activity by arsenite

It is known that arsenite stimulates transcription factor AP-1 activity through the mitogen-activated kinase cascade in several cell systems [47,54,55]. AP-1 mediates many biological effects of tumor promoters and is an important regulator of cell growth. We therefore investigated the possible role of H_2O_2 and ERK1/2 in stimulation of AP-1 activity by arsenite, and measured the change in AP-1 binding affinity following treatment with 100 μM arsenite by electrophoretic mobility shift assay (EMSA). AP-1 DNA binding activity increased in a time-dependent manner, being apparent as early as 10 min after arsenite treatment, with maximum activation occurring at 90 min, which was sustained for up to 120 min (Fig. 8A). Arsenite activation of AP-1 was ablated by pretreatment with the MEK inhibitor PD98059, but not by specific inhibitors of the $p70^{s6k}$ signaling pathway—wortmannin, LY294002, and rapamycin—indicating the role of the ERK1/2 pathway as a mediator of AP-1 activation by arsenite (Fig. 8B). Furthermore, AP-1 activation by arsenite was completely inhibited by reducing H_2O_2 production through pretreatment of the cells with antioxidants such as catalase and NAC (Fig. 8C). The specificity of AP-1 DNA binding was confirmed by a competition assay with a 100-fold molar excess of unlabeled oligonucleotide probe (data not shown). Taken together, the results suggest that arsenite activation of AP-1 is mediated by H_2O_2-induced ERK 1/2 activation.

Discussion

Recently, ROS such as H_2O_2 and superoxide have gained acceptance as modulators of receptor-mediated signal transduction in a variety of cell types [56]. We have recently

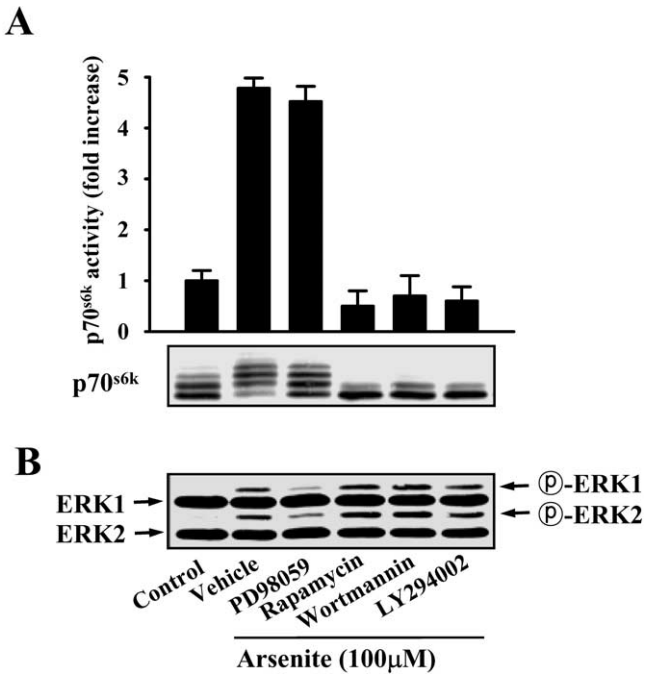

Fig. 6. The P13K signaling pathway was differentially involved in arsenite activation of $p70^{s6k}$ and ERK1/2. Serum-deprived JB6 cells were stimulated with 100 μM arsenite for 60 min. Alternatively, the cells were preincubated with 50 μM PD98059, 5 nM rapamycin, 500 nM wortmannin, or 25 μM LY294002 for 30 min. (A) Activity and phosphorylation of $p70^{s6k}$, and (B) phosphorylation of ERK1/2 were assayed as described earlier. The results shown are the means ± SEM and typical representative blot of three independent experiments.

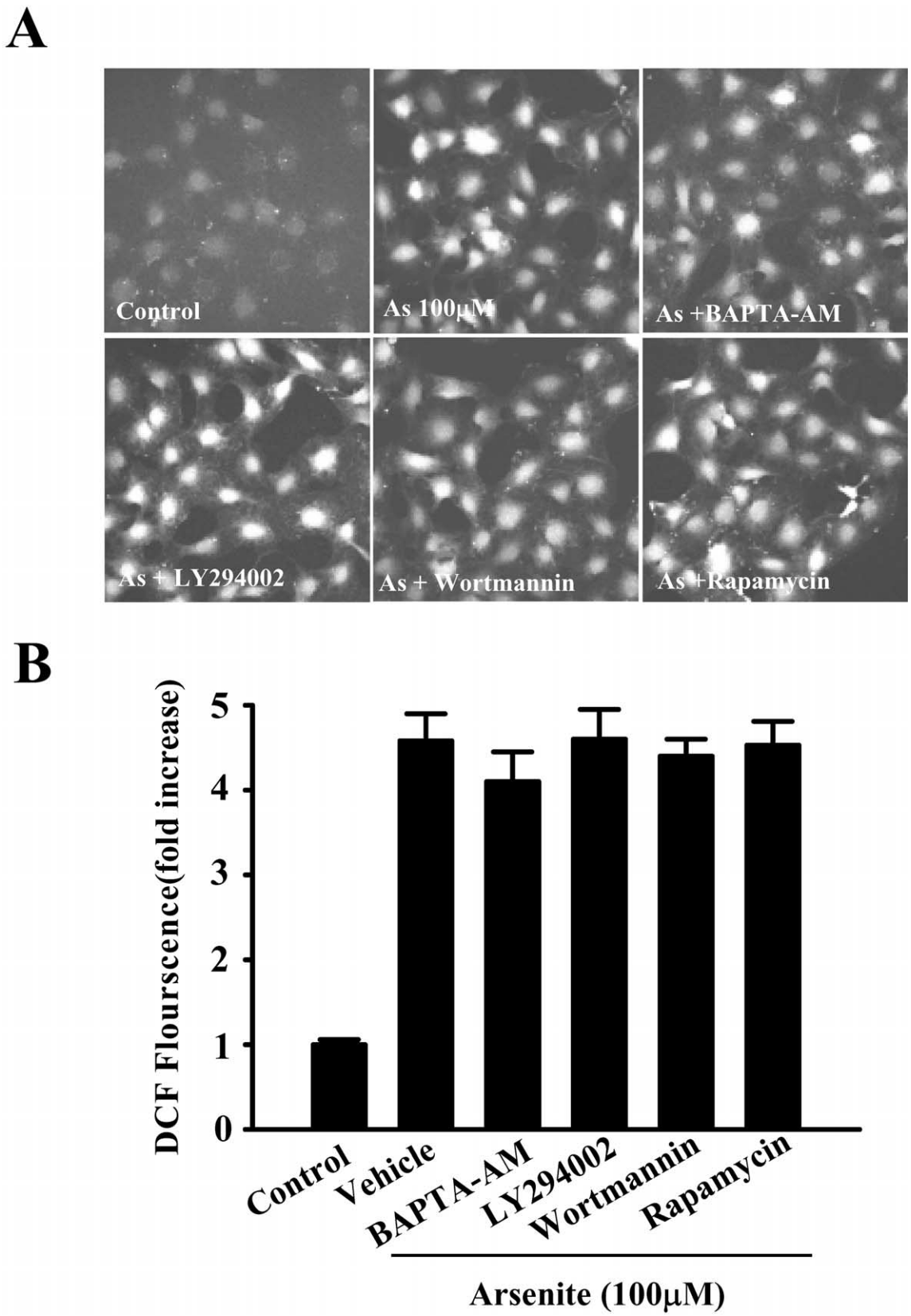

Fig. 7. Ca^{2+} chelator, PI3K inhibitors, and rapamycin did not inhibit H_2O_2 production by arsenite. Serum-deprived JB6 cells were stimulated with 100 μM arsenite for 60 min. Alternatively, the cells were preincubated with 20 μM BAPTA-AM, 25 μM LY294002, 500 nM wortmannin, or 5 nM rapamycin for 30 min. (A) DCF fluorescence was measured with a confocal scanning laser microscope. Results are representative photographs from three independent experiments. (B) ROS generation was measured by DCF fluorescence as described in Fig. 3.

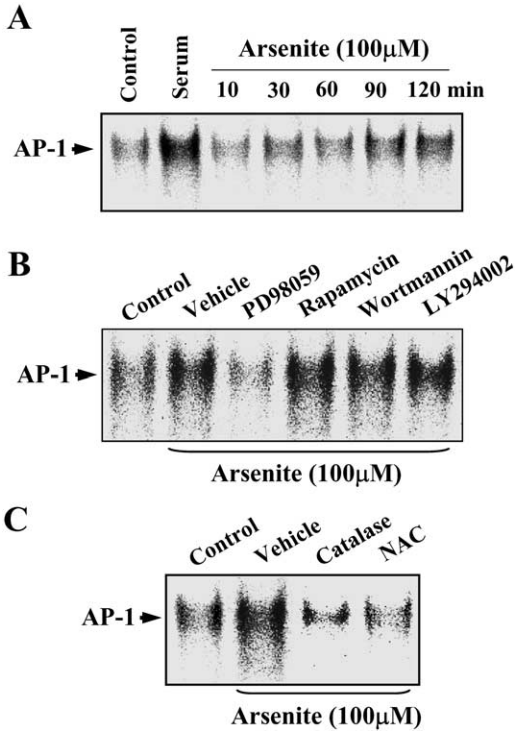

Fig. 8. AP-1 activation was mediated by the arsenite-activated H_2O_2–ERK1/2 signaling pathway. (A) Serum-deprived JB6 cells were stimulated with 100 μM arsenite for the indicated times. Nuclear extracts were prepared, and DNA binding activity of AP-1 was determined by EMSA as described under Materials and Methods. The positive control sample was stimulated with 10% serum for 20 min. (B) To determine the signaling pathway involved in arsenite activation of AP-1, serum-deprived JB6 cells were pretreated with 50 μM PD98059, 5 nM rapamycin, 500 nM wortmannin, or 25 μM LY294002 for 30 min. After stimulation with 100 μM arsenite for 90 min, nuclear extracts were prepared and DNA binding activity of AP-1 was determined by EMSA. (C) To elucidate the involvement of H_2O_2 in arsenite activation of AP-1, serum-deprived JB6 cells were pretreated with NAC (30 mM) or catalase (300 units/ml) for 30 min. After stimulation with 100 μM arsenite for 90 min, DNA binding activity of AP-1 was determined by EMSA.

demonstrated that the generation of H_2O_2 is required for PDGF- and EGF-induced activation of $p70^{s6k}$ and ERK1/2 [34]. In addition, arsenite has been shown to produce ROS as well as to activate $p70^{s6k}$ and ERK1/2 [40–43]. However, none of the previous studies linked the activation of $p70^{s6k}$ and ERK1/2 to ROS production in arsenite-stimulated cells. In the present study, we provide direct evidence for this linkage. Arsenite was found to induce activation of $p70^{s6k}$ and ERK1/2. However, this response differed from the ligand-induced response. While endogenous stimuli such as EGF have been demonstrated to cause transient activation of $p70^{s6k}$ and ERK1/2 [45], arsenite induced sustained activation of these kinases (Fig. 1, 2). Consistent with our observation, persistent activation (for up to 45 min) of ERK1/2 by arsenite has been shown in UROtsa cells, an SV40 immortalized human urothelium cell line [57]. The prolonged activation of these kinases by arsenite could be important in progression of the cell cycle, thereby leading to continued cell proliferation. These findings are in good agreement with other observations that $p70^{s6k}$ activity remains maximally activated for up to 14 h after serum induction, a time just before the cells first enter S phase [58]. Furthermore, microinjection of quiescent rat embryo fibroblasts with polyclonal antibodies against $p70^{s6k}$ during the first 10 h after serum induction totally abrogated entry of the cells into S phase, indicating that $p70^{s6k}$ function was essential throughout G_1 phase. In support of the above observation, $p70^{s6k}$ was also found to be constitutively activated in small cell lung cancer cells [29]. Pretreatment of the cells with rapamycin, a specific inhibitor of $p70^{s6k}$, inhibited growth of the cells at concentrations similar to those required for inducing inactivation of $p70^{s6k}$ [29]. Thus, the prolonged activation of $p70^{s6k}$ by arsenite would play an important role in promoting the growth of cancer cells.

In addition to the ability to activate $p70^{s6k}$ and ERK1/2, arsenite has been implicated in production of ROS [42,43]. In fact, arsenite induced time- and dose-dependent production of ROS. The kinetic pattern of ROS production by arsenite was very similar to that of activation of $p70^{s6k}$ and ERK1/2, indicating a possible link between ROS production and activation of $p70^{s6k}$ and ERK1/2: Arsenite activation of $p70^{s6k}$ and ERK1/2 was completely inhibited by antioxidants. Furthermore, the major ROS produced by arsenite appeared to be H_2O_2, because arsenite-induced production of ROS was significantly abolished by pretreatment with catalase but not with heat-inactivated catalase. Mutational analysis of PDGFβR has demonstrated that PI3K is required for PDGF-induced production of H_2O_2, indicating the role of PI3K as an upstream molecule of H_2O_2 [52]. However, contrary to the above, we earlier demonstrated that H_2O_2 acted as an upstream molecule of PI3K, FRAP, and Ca^{2+} in the $p70^{s6k}$ signaling pathway [34]. Consistent with this observation, specific inhibitors for PI3K, FRAP, or Ca^{2+} chelators abrogated arsenite activation of $p70^{s6k}$ without any alteration of H_2O_2 production, thus indicating PI3K, FRAP, and Ca^{2+} as downstream effectors of H_2O_2 in arsenite signaling leading to $p70^{s6k}$ activation. If PI3K acted as an upstream molecule of H_2O_2, inhibition of PI3K by specific inhibitors would induce inactivation of $p70^{s6k}$ as well as ERK1/2, because no H_2O_2 would be produced. However, inhibition of PI3K failed to inactivate ERK1/2, confirming PI3K as a downstream effector of H_2O_2, This possibility is further strengthened by an earlier observation that exposure of cardiomyocytes to H_2O_2 led to sustained activation of PI3K as well as tyrosine phosphorylation of the p85 regulatory subunit [53].

Several lines of evidence have demonstrated that arsenite may act early in the growth factor signaling pathway. In particular, arsenite activated ERK1/2 in rat PC12 cells through Ras-dependent signaling [40], and stimulated c-Src activity and tyrosine phosphorylation of several proteins including the proto-oncoprotein Shc and EGF receptor [40, 57]. These effects of arsenite were antagonized by anitioxidant, indicating that effects of arsenite on early growth

factor signaling might be mediated by a ROS which appeared to be H_2O_2, because production of ROS by arsenite was completely abolished by catalase treatment (Fig. 4A). Furthermore, H_2O_2 has also been shown to cause phosphorylation of growth factor receptors [53]. In support of the above observation, activation of $p70^{s6k}$ and ERK1/2 by arsenite was significantly inhibited by treatment with the protein tyrosine kinase inhibitor genistein (unpublished data).

Epidemiological studies have revealed that many skin cancers occur among people medically or occupationally exposed to arsenite and that high exposure to arsenite through drinking water is associated with increased risk of skin, bladder, kidney, lung, and colon cancers [6,59]. Consistent with these observations, chronic exposure of cultured cells to low concentrations of arsenic leads to transformation [60]. Furthermore, mice exposed to 0.01% arsenite in drinking water develop hyperplasia of the bladder urothelium within 4 weeks of exposure [61]. One possible explanation of its tumor-promoting properties in humans, animals, and cultured cells could be activation of early events in the growth factor signaling pathway by sustained production of H_2O_2 by arsenite. H_2O_2 produced by arsenite might activate growth factor receptor by increasing its tyrosine phosphorylation, thereby leading to stimulation of the $p70^{s6k}$ and ERK1/2 pathways, which are critical signaling events for cell proliferation [10,11], strongly indicating H_2O_2 to be a pivotal mediator of the tumor-promoting activity of arsenite. Finally, our findings presented herein provide one of the mechanisms for the tumor-promoting properties of arsenite and suggest H_2O_2 as a potential target for therapeutic strategies aimed at preventing or inhibiting arsenite-induced tumor growth.

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