

Cdc42 Regulates Arsenic-induced NADPH Oxidase Activation and Cell Migration through Actin Filament Reorganization*

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Although arsenic is a human carcinogen, the molecular mechanisms of its action remain to be understood. The present study reports that exposure to arsenic induced actin filament reorganization, resulting in lamellipodia and filopodia structures through the activation of Cdc42 in SVEC4-10 endothelial cells. It was also found that arsenic induced the formation of the superoxide anion (O_2^-) in SVEC4-10 cells. Immunoprecipitation and Western blotting analysis demonstrated that arsenic stimulation induced serine phosphorylation of p47^{phox}, a key component of NADPH oxidase, indicating that arsenic induces O_2^- formation through NADPH oxidase activation. Inhibition of arsenic-induced actin filament reorganization by either overexpression of a dominant negative Cdc42 or pretreatment of an actin filament stabilizing reagent, jasplakinolide, abrogated arsenic-induced NADPH oxidase activation, showing that the activation of NADPH oxidase was regulated by Cdc42-mediated actin filament reorganization. This study also showed that overexpression of a dominant negative Rac1 was sufficient to abolish arsenic-induced O_2^- production, implying that Rac1 activities are required for Cdc42-mediated NADPH oxidase activation in response to arsenic stimulation. Furthermore, arsenic stimulation induced cell migration, which can be inhibited by the inactivation of either Cdc42 or NADPH oxidase. Taken together, the results indicate that arsenic is able to activate NADPH oxidase through Cdc42-mediated actin filament reorganization, leading to the induction of an increase in cell migration in SVEC4-10 endothelial cells.

Arsenic has long been considered a toxic and carcinogenic metal. There is substantial evidence indicating that the exposure of humans to arsenic via contaminated air and drinking water causes cancer (1). Both epidemiologic and clinical studies indicated that arsenic exposure is linked to the high incidence of skin, bladder, liver, kidney, and lung cancers, as well as other human diseases (2). Arsenic has been shown to induce carcinogenesis *in vivo* by inducing a wide spectrum of changes in cell signal transduction, including the formation of reactive oxygen species (ROS),¹ an increase in tyrosine phosphoryla-

tion, the activation of mitogen-activated protein kinase and NF- κ B pathways, alterations in cell differential and proliferation, and the induction of apoptosis (3).

ROS is a collective term for the intermediates formed during oxidative metabolism, encompassing both oxygen radicals and non-radical reactive oxygen derivatives including superoxide anion (O_2^-), the hydroxyl radicals (\cdot OH), and hydrogen peroxide (H_2O_2). Mammalian cells have the antioxidant defense systems to remove low physiological concentrations of ROS. However, extremely high concentrations of ROS can cause DNA, protein, and lipid damage in cells. It has been proven that arsenic induces the formation of O_2^- in several different kinds of cells, including vascular endothelial cells (4), human vascular smooth muscle cells (5), acute promyelocytic leukemia cells (6), murine keratinocytes (7), and chronic lymphocytic leukemia cells (8). It has been proposed that ROS generated by arsenic-mediated reactions cause DNA damage, lipid peroxidation, and protein modification as well as alterations of antioxidant defenses, which are associated with the alterations of cell signal transduction (3). Although increasing evidence shows that ROS play an important role in arsenic-induced carcinogenesis, its mechanism of formation remains to be investigated. Recent study has demonstrated that arsenic is able to up-regulate NADPH oxidase, which is the main source of arsenic-induced ROS generation (9). The mechanism of arsenic-induced NADPH oxidase activation itself is not clear.

Actin is one of the most abundant proteins in eukaryotic cells. There are two different forms of actin, mono-actin (G-actin) and actin filaments (F-actin). Actin filaments are involved in a wide variety of cell functions, including cell motility, cell surface remodeling, cell shape changes during mitosis, cell contraction and separation during cytokinesis, cell-cell/cell-substrate interaction, cell signal transduction, angiogenesis, and endocytosis (10–12). Actin filaments function in cellular processes by undergoing dynamic structural reorganization or remodeling to form discrete structures at the cell periphery that mediate attachment to the substratum (13). These discrete structures are lamellipodia, filopodia, membrane ruffles, and stress fibers (13). The formation of these structures is regulated by both extracellular and intracellular signals and is essential for cell adhesion, migration, and signal transduction (11, 13).

Understanding arsenic-induced actin filament reorganization is important for elucidation of the overall mechanisms involved in arsenic-induced cellular injury. It is controversial

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¹ The abbreviations used are: ROS, reactive oxygen species; CRIB,

Cdc42/Rac interactive binding region; Cdc42DN, dominant negative form of Cdc42 cells; DPI, diphenyleneiodonium; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; TRITC, tetramethylrhodamine isothiocyanate; WASP, Wiskott-Aldrich syndrome protein.

as to whether the exposure of cells to arsenic will induce actin filament reorganization. Earlier reports indicated that arsenic exposure induces the dissociation of actin stress fibers and membrane perturbations in 3T3 cells (14, 15). There is also a report showing that arsenic is unable to induce actin filament reorganization even at such a high concentration that causes a marked retardation of cell growth in Chinese hamster V79 lung fibroblast cells (16). Moreover, the molecular mechanisms by which arsenic induces actin filament reorganization have not been elucidated, and little is known about the functional implications of arsenic-induced actin filament reorganization in cell signal transduction and cell metastasis.

In this report, we sought to examine whether arsenic exposure would induce actin filament reorganization in SVEC4-10 endothelial cells and determine the underlying mechanisms involved. The present study also attempts to understand the mechanisms of arsenic-induced NADPH oxidase activation and cell migration with an emphasis on the involvement of Cdc42 and actin filament reorganization. We demonstrated that arsenic stimulation induced actin filament reorganization to form lamellipodia and filopodia structures through the activation of Cdc42 in SVEC4-10 endothelial cells. We also found that arsenic stimulation produced superoxide anion (O_2^-) and activated NADPH oxidase, indicating that arsenic may induce O_2^- through the activation of NADPH oxidase. Furthermore, we showed that the activation of NADPH oxidase was regulated by Cdc42-mediated actin filament reorganization upon arsenic stimulation. We also demonstrated that Rac activities were required for Cdc42-mediated O_2^- production. Interestingly, the results obtained here also showed that the NADPH oxidase activity was involved in arsenic-stimulated cell migration via Cdc42-mediated actin filament reorganization in SVEC4-10 endothelial cells. This study defined a unique cell-signaling pathway that may be involved in arsenic-induced carcinogenesis. The results of this study also provided the first evidence showing that Cdc42-induced actin filament reorganization mediates the activation of NADPH oxidase.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, FITC-phalloidin, diphenyleneiodonium (DPI), TRITC-phalloidin, FITC-anti-mouse antibody, arsenite, nitro blue tetrazolium, *N,N*-dimethylformamide, superoxide dismutase, Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), and cytochalasin D were purchased from Sigma. Jasplakinolide was purchased from Calbiochem. Anti-phospho-serine antibody was purchased from Zymed Laboratories Inc.. The anti-p47^{phox} and anti-p67^{phox} antibodies were both purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Cdc42 antibody was purchased from BD Biosciences. The anti-c-Myc monoclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Dihydroethidium was purchased from Molecular Probes (Eugene, OR).

Cell Culture and Plasmid Construction—SVEC4-10 cells, an immortal mouse endothelial cell line, were purchased from the American Type Culture Collection (Manassas, VA) and routinely passaged in Dulbecco's modified Eagle medium with 10% fetal calf serum. Both a dominant active form of Cdc42 (pHEFdpCdc42V12) and a dominant negative form of Cdc42 (pHEFdnCdc42N14) were gifts from Dr. Alan Hall (University College London, UK) and Dr. Wang Lu-Hai (Mount Sinai School of Medicine, New York NY). pGST-PAK-CRIB is a gift from Dr. Pontous Aspenstrom (Ludwig Institute for Cancer Research, Uppsala, Sweden). Both pHEFdnCdc42N14 and pHEFdpCdc42V12 were transfected into SVEC4-10 cells with Lipofectin (Invitrogen), and the transfected cells were selected by G-418 to make stable transfected cell lines.

Immunofluorescence Assay—SVEC4-10 cells were grown on coverslips. After treatment, cells were fixed and permeabilized as described previously (17), followed by labeling with FITC-phalloidin for 30 min and mounting to the slides with Fluoromount (Fisher, Pittsburgh, PA). A Zeiss LSM 510 microscope was used to obtain images. Scale bars were generated and inserted by LSM software.

Wound Healing Assay—The wound healing assays were performed according to the methods described previously (10, 12). SVEC4-10 cells were grown on coverslips to 100% confluent monolayers and then

scratched to form a 100- μ m "wound" using sterile pipette tips. The cells were then cultured with the different treatments in serum-free media for 8–12 h, fixed on coverslips with formalin, and stained with TRITC-phalloidin. A Zeiss LSM 510 microscope was used to obtain images. Scale bars were generated and inserted by LSM software.

Cdc42 Activation Assay—Cdc42 activation assays were performed according to the method described by Edlund *et al.* (18). Briefly, the cells were washed with $1\times$ phosphate-buffered saline supplemented with 1 mM $MgCl_2$. After washing, the cells were lysed immediately with lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μ g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged at 14,000 rpm for 15 min. The supernatants were added with GST-WASP-CRIB in GST beads to pull-down active Cdc42 proteins, followed by incubation at 4 °C for 20 min. After incubation, the beads were washed two times with cold wash buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μ g/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). The Cdc42 protein was eluted with sample buffer and subjected to electrophoresis on a 15% SDS-polyacrylamide gel. The Western blot analysis was performed using anti-Cdc42 polyclonal antibody. Protein bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences).

Superoxide Anion (O_2^-) Assay in Adherent Cells—SVEC4-10 cells were serum-starved overnight and then stimulated with 10 μ M arsenic for different periods of time. During the stimulation periods, dihydroethidium was added at a final concentration of 5 μ M for 30 min. After incubation, the cells were washed two times with phosphate-buffered saline and mounted on coverslips. A Zeiss LSM 510 microscope was used to obtain images. Scale bars were generated and inserted by LSM software.

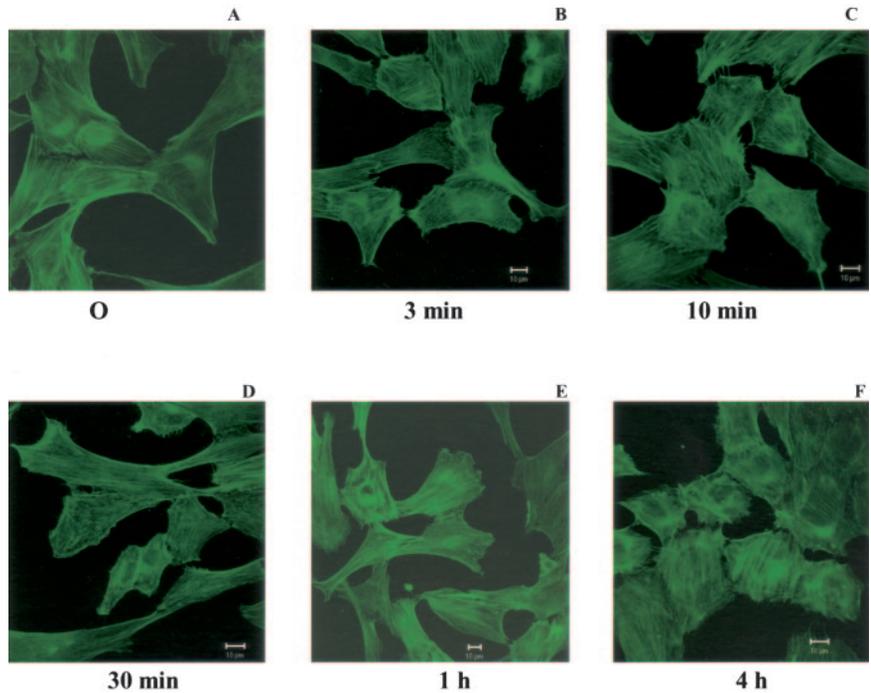
p67^{phox} Translocation Assay—The p67^{phox} protein translocation assay was performed according to the methods described by Zang *et al.* (19) and Zhao *et al.* (20). Briefly, the cells were sonicated for 4 s and then centrifuged at 100,000 $\times g$ for 1 h to pellet membrane fraction in the homogenization buffer (20 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, and protease inhibitors). After centrifugation, the membrane fraction (pellet) was resuspended in the homogenization buffer with 1% Triton X-100. The suspended mixture was sonicated for 4 s and then centrifuged at 15,000 $\times g$ for 15 min. The membrane proteins were in the supernatants that were analyzed by Western blot assays.

Measurement of NADPH Oxidase Activities—Measurement of the activity of NADPH oxidase was performed according to the method described by Dib *et al.* (21). Briefly, the cells were incubated with 0.2% nitro blue tetrazolium during the last 10 min of the incubation. After the treatment, the adherent cells were scraped off and pelleted. The pellets were then resolved in *N,N*-dimethylformamide, and the supernatants were used in measuring the activities of NADPH oxidase with a spectrophotometer (515 nm).

RESULTS

Arsenic Stimulation Induces Actin Filament Reorganization in SVEC4-10 Cells—Actin filaments play major roles in cell signal transduction and carcinogenesis. They have been indicated as being the targets for injury by the epigenetic carcinogenic metal compounds in the environment (14). Arsenic is a toxic heavy metal that can induce stress-related cell signal transduction. We sought to clarify whether arsenic stimulation would induce actin filament reorganization. SVEC4-10 cells, an immortalized endothelial cell line, were serum-starved overnight and stimulated with 10 μ M arsenic for different periods of time ranging from 3 min to 4 h. After the stimulation, the cells were immediately fixed, permeabilized, and stained with FITC-phalloidin, followed by morphological analysis of the cells using confocal microscopy. The unstimulated SVEC4-10 cells stretched out evenly, and no protrusions were found at the leading edge of the cells. The actin filaments were well organized, and few cell motile structures and rosette-like dots were found at the cell leading edge and cell body, respectively, indicating that the unstimulated SVEC4-10 cells do not undergo a significant reorganization of actin filaments (Fig. 1A). Upon stimulation with 10 μ M arsenic for 3 min, the cell morphology was changed, and the actin filaments were remodeled (Fig. 1B). The cells began to round up. There were some actin protrusion

FIG. 1. Arsenic stimulation induces actin filament reorganization in SVEC4-10 cells. SVEC4-10 cells were grown on coverslips and serum-starved overnight. The cells were stimulated with 10 μM arsenic for different periods of time as indicated (A–F). After stimulation, the cells were fixed, permeabilized, and stained with FITC-phalloidin for actin filaments. Confocal microscopy analysis was performed to analyze the integrity of actin filaments.



structures at the leading edge of the cells. These protrusion structures are lamellipodia and filopodia. Some of the actin stress fibers were dissociated to form rosette-like structures around the cell body. These morphological changes indicate that the actin filaments were reorganized upon arsenic stimulation within 3 min in SVEC4-10 endothelial cells. After the stimulation with arsenic for 10–30 min, a substantial number of lamellipodia and filopodia were found at the leading edge of the cells, and actin stress fibers were further dissociated, indicating that the actin filaments underwent a more profound reorganization (Fig. 1, C and D). Arsenic-induced actin filament reorganization lasted for >4 h (Fig. 1, E and F). Dose-dependent experiments demonstrated that the lowest concentration of arsenic that could induce the reorganization of actin filaments within 30 min of stimulation was 10 μM (data not shown). Our results demonstrated that arsenic stimulation was sufficient to induce actin filament reorganization to form lamellipodia and filopodia structures at the leading edge of the cells and rosette-like structures in the cell body in SVEC4-10 endothelial cells, indicating that actin cytoskeleton architecture is a molecular target of arsenic-induced stress injury in endothelial cells.

Arsenic Stimulation Induces Actin Filament Reorganization through the Activation of Cdc42—The reorganization of actin filaments is tightly regulated during cell signal transduction. To investigate whether arsenic stimulation would activate the small Rho GTPases Rac and Cdc42, the main actin filament regulatory proteins (13), SVEC4-10 cells were stimulated with 10 μM arsenic for various periods of time ranging from 3 min to 4 h, and the cell lysates were subject to glutathione *S*-transferase fusion protein pull-down assays using GST-PAK-CRIB and GST-WASP-CRIB, respectively. Our results demonstrated that arsenic stimulation induced the activation of Cdc42 within 3 min, and the activation lasted \sim 1 h (Fig. 2A). No Rac activation was found (data not shown).

We then examined whether the activation of Cdc42 regulated arsenic-induced actin filament reorganization. A dominant negative form of Cdc42 was stably expressed into SVEC4-10 cells (SVEC-Cdc42DN) to inhibit the activity of Cdc42. Both SVEC4-10 cells and SVEC-Cdc42DN were stimulated with 10 μM arsenic for 30 min, and immunofluorescence assays were

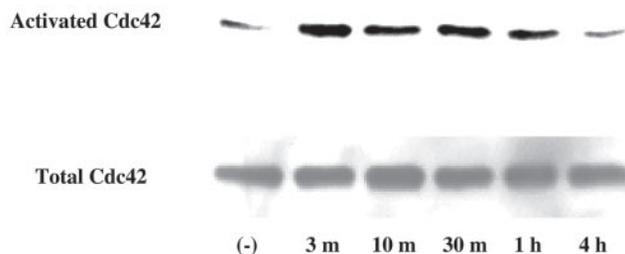
performed to analyze the integrity of actin filaments. The results showed that the inhibition of Cdc42 activity abrogated arsenic-induced actin filament reorganization (Fig. 2B). Taken together, the results demonstrated that arsenic-induced actin filament reorganization was regulated by the activation of Cdc42 in SVEC4-10 cells.

Arsenic Stimulation Induces NADPH Oxidase Activation—Using confocal microscopy, we sought to determine whether stimulation with arsenic would induce the production of O_2^- in SVEC4-10 endothelial cells. SVEC4-10 cells were stimulated with 10 μM arsenic for different periods of time and then examined for the production of O_2^- by staining the cells with 5 μM dihydroethidium during the last 30 min of stimulation. Dihydroethidium stains cells a blue color, which can be detected by confocal microscopy. When degraded by O_2^- , the dye changes color from blue to red. The *red color* was shown on the *left side* of each panel in Fig. 3, whereas the *blue color* was shown on the *right side* of each panel. Our results showed that the unstimulated SVEC4-10 cells exhibited the blue color and little or no red color (Fig. 3A, *section 1*), demonstrating that the unstimulated cells do not generate substantial amounts of the O_2^- radical. Upon arsenic stimulation for 3 min, the blue color disappeared, and the cells were stained to a red color (Fig. 3A, *sections 2–5*), demonstrating the production of the O_2^- radical. The arsenic-induced red color peaked at 30 min and lasted at least 4 h after stimulation. Our results indicate that arsenic stimulation induces the production of O_2^- in SVEC4-10 endothelial cells.

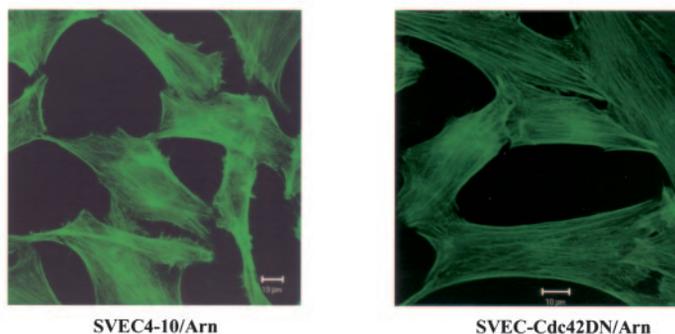
NADPH oxidase is one of major enzymes that are involved in O_2^- production (22). It was demonstrated that arsenic stimulation activated NADPH oxidase in endothelial cells (23). We sought to determine whether arsenic-induced O_2^- production is through the activation of NADPH oxidase in SVEC4-10 cells. Serine phosphorylation of p47^{phox} is an essential step in the activation of NADPH oxidase, and the induction of p47^{phox} phosphorylation in serines is a strong indication of NADPH oxidase activation (22). We performed both immunoprecipitation and Western blotting assays using anti-p47^{phox} and anti-phosphoserine antibodies to determine whether arsenic would stimulate serine phosphorylation of p47^{phox} in SVEC4-10 cells. The results showed that arsenic stimulation induced an increase in serine phosphorylation

A

FIG. 2. Arsenic stimulation (Arn) induces actin filament reorganization through the activation of Cdc42 in SVEC4-10 cells. A, arsenic stimulation induces the activation of Cdc42. SVEC4-10 cells were serum-starved overnight and then stimulated with 10 μM arsenic for different periods of time as indicated. The cells were lysed after the stimulation, and the lysates were subjected to GST-WASP-CRIB pull-down assays to analyze the activities of Cdc42. The reactions of the pull-down assays were resolved by electrophoresis on a 15% SDS-polyacrylamide gel, and an anti-Cdc42 antibody was used to detect Cdc42. B, arsenic stimulation induces actin filament reorganization through the activation of Cdc42. Both SVEC4-10 cells and SVEC-Cdc42DN cells were grown on coverslips and serum-starved overnight. The cells were stimulated with 10 μM arsenic for 30 min. After the stimulation, the cells were fixed, permeabilized, and stained with FITC-phalloidin for actin filaments. Confocal microscopy analysis was performed to analyze the integrity of actin filaments.



B



of p47^{phox} within 3 min, and the peak of the serine phosphorylation occurred \sim 30 min after the stimulation. The phosphorylation lasted $>$ 4 h (Fig. 3B). The data presented here demonstrated that arsenic stimulation induced the activation of NADPH oxidase in SVEC4-10 endothelial cells. Moreover, the profile of arsenic-induced serine phosphorylation of p47^{phox} is similar to that of arsenic-induced O₂⁻ production, indicating that NADPH oxidase is a major enzyme that produces O₂⁻ upon arsenic stimulation in SVEC4-10 endothelial cells. To further prove arsenic-induced activation of NADPH oxidase, we performed a p67^{phox} translocation assay. The results showed that p67^{phox} was translocated to the cell membrane fraction upon arsenic stimulation (Fig. 3C), which is one of hallmarks of NADPH oxidase activation (22). Taken together, the results demonstrated that arsenic induced the activation of NADPH oxidase in SVEC4-10 cells.

Arsenic Stimulation Induces NADPH Oxidase Activities through the Activation of Cdc42 and the Reorganization of Actin Filaments—The major focus in this section is to investigate how arsenic stimulation induces the activation of NADPH oxidase in SVEC4-10 cells. In our previous report, we showed that the activation of Cdc42 mediated ethanol-induced ROS production in SVEC4-10 cells (10). The present study investigates whether the induction of NADPH oxidase activity was mediated by Cdc42 activation upon arsenic stimulation in the same cells. Both SVEC4-10 cells and SVEC-Cdc42DN cells were stimulated with 10 μM arsenic for 30 min, and then the production of superoxide-induced formazan was measured to determine the activity of NADPH oxidase (21). Our results showed that arsenic stimulation induced an increase in superoxide-induced formazan produc-

tion in SVEC4-10 cells that was abolished by the disruption of Cdc42 activity, indicating that the activation of Cdc42 mediates arsenic-induced O₂⁻ production in SVEC4-10 cells (Fig. 4A). We also pretreated the cells with superoxide dismutase, a superoxide-specific scavenger, to examine whether superoxide dismutase would abrogate the superoxide-induced formazan formation. The results showed that the pretreatment of superoxide dismutase inhibited arsenic-induced formation of formazan (Fig. 4A), proving that Cdc42 specifically mediates arsenic-induced O₂⁻ production. Immunoprecipitation analysis demonstrated that the disruption of Cdc42 abrogated arsenic-induced serine phosphorylation of p47^{phox} in SVEC4-10 cells (Fig. 4B), indicating that Cdc42 regulates NADPH oxidase activation in response to arsenic stimulation. We further examined whether Cdc42 regulated the activation of NADPH oxidase through the reorganization of actin filaments. SVEC4-10 cells were pretreated with the actin filament inhibitors jasplakinolide or cytochalasin D, respectively, and were then stimulated with arsenic for 30 min. Jasplakinolide is an inhibitor of actin filament reorganization that stabilizes actin filaments, and cytochalasin D is an actin filament inhibitor that disrupts the structures of actin filaments (24). They induce the opposite morphological changes in actin filaments. Cytochalasin D, to a lesser extent, mimics the effects of Cdc42 on actin filaments in terms of disruption of actin filament structure. Our immunoprecipitation and Western blotting analysis showed that pretreatment with jasplakinolide abolished arsenic-induced NADPH oxidase activation, whereas pretreatment with cytochalasin D only partially inhibited arsenic-induced NADPH oxidase activation (Fig 4B), indicating that Cdc42-me-

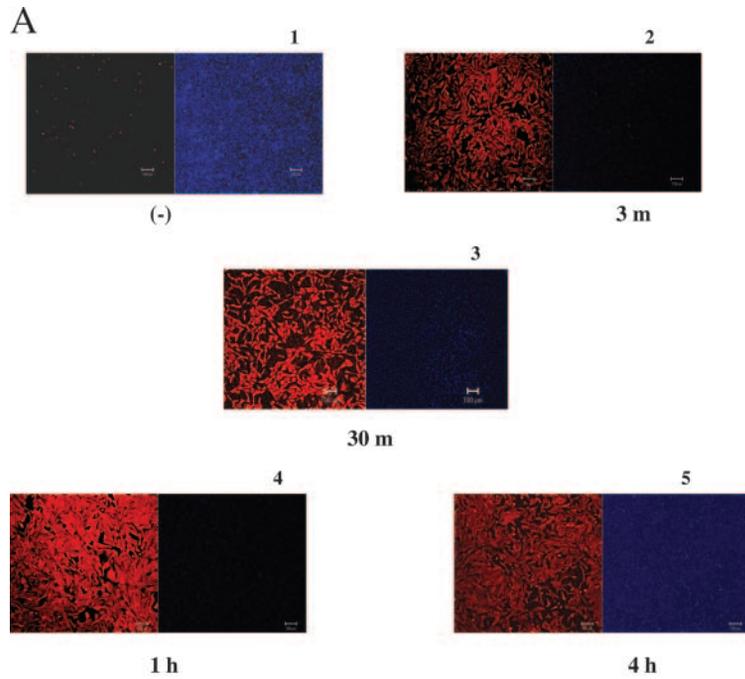
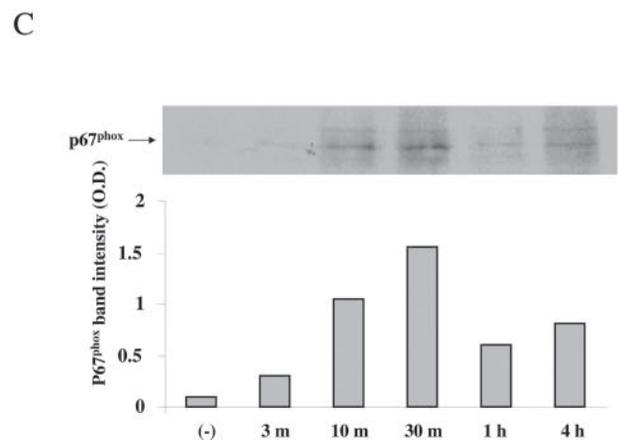
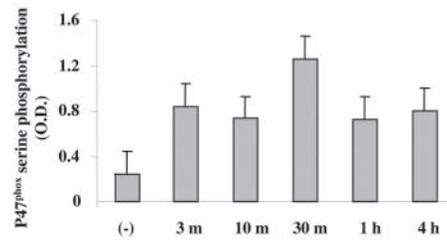
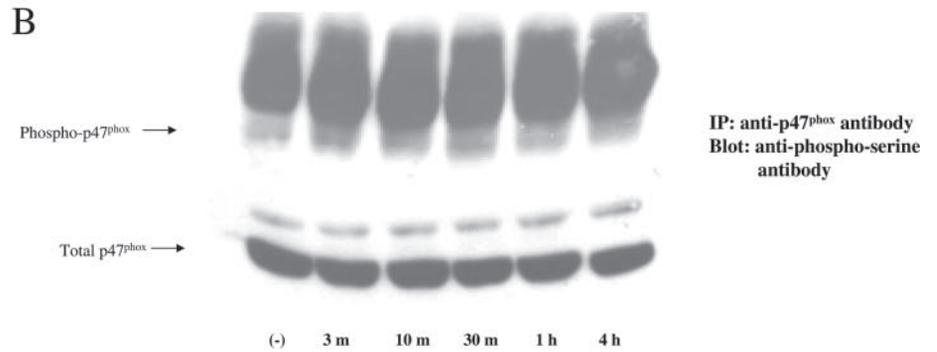
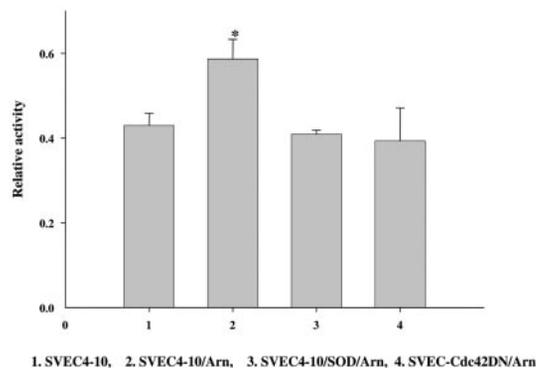


FIG. 3. Arsenic stimulation activates NADPH oxidase in SVEC4-10 cells. *A*, arsenic stimulation induces the production of O_2^- . SVEC4-10 cells were grown on coverslips and serum-starved overnight. The cells were stimulated with $10 \mu\text{M}$ arsenic for different periods of time as indicated (sections 1–5). Dihydroethidium ($5 \mu\text{M}$) dye was added into the cell culture during the last 30 min of the stimulation. After staining, the cells were fixed and analyzed by confocal microscopy. *B*, arsenic stimulation induces the serine phosphorylation of p47^{phox} in SVEC4-10 cells. SVEC4-10 cells were serum-starved overnight and stimulated with $10 \mu\text{M}$ arsenic for different periods of time as indicated. After stimulation, the cells were lysed and the lysates were immunoprecipitated (IP) with an anti-p47^{phox} antibody. The immunoprecipitation reactions were resolved by electrophoresis on a 10% SDS-polyacrylamide gel, followed by Western blotting analysis using an anti-phosphoserine antibody. The densitometries of p47^{phox} phosphorylation were scanned using EGII software, and the results were plotted by SigmaPlot. *C*, arsenic stimulation induces p67^{phox} translocation. SVEC4-10 cells were serum-starved overnight and stimulated with $10 \mu\text{M}$ arsenic for different periods of time as indicated. After stimulation, the cells underwent the protein translocation assay. The cell membrane fractions were resolved by electrophoresis on an 8% SDS-polyacrylamide gel followed by the Western blotting analysis using an anti-p67^{phox} antibody. The densitometries of p67^{phox} were scanned using EGII software, and the results were plotted by SigmaPlot.



A



B

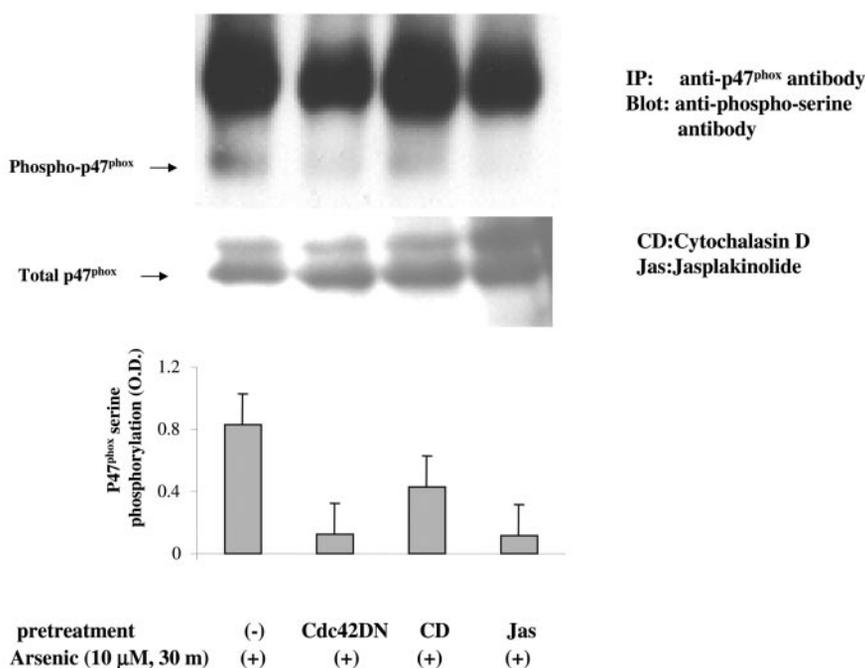


FIG. 4. Arsenic-induced activation (*Arn*) of NADPH oxidase is regulated by Cdc42-mediated actin filament reorganization. A, arsenic stimulation activates NADPH oxidase through Cdc42. Both SVEC 4-10 cells and SVEC-Cdc42DN cells were preincubated with 0.1% nitro blue tetrazolium and then treated with 10 μM arsenic for 30 min as indicated. Both SVEC4-10 cells alone and SVEC4-10 cells with superoxide dismutase (*SOD*) pretreatment (500 units/ml/h) were set as the negative controls. After the treatments, the cells were lysed, and the supernatants of the lysates were dissolved in *N,N*-dimethylformamide. The amount of formazan in the supernatants was detected by a spectrophotometer at 515 nm, which represents the amount of superoxide formation. The data are from three independent experiments. An *asterisk* indicates a significant difference from controls ($p < 0.01$, $n = 3$). B, arsenic-induced increase in serine phosphorylation of p47^{phox} is mediated by Cdc42 and actin filament reorganization. SVEC4-10 cells were pretreated with 1 μM cytochalasin D or 100 nM jasplakinolide for 1 h, respectively, as indicated. After the pretreatments, the cells were stimulated with 10 μM arsenic for 30 min. After the stimulation, the cells were lysed, and the lysates were immunoprecipitated (*IP*) with an anti-p47^{phox} antibody. The immunoprecipitation reactions were resolved by electrophoresis on a 10% SDS-polyacrylamide gel, followed by the Western blotting analysis using an anti-phospho-serine antibody. The densitometries of p47^{phox} phosphorylation were scanned using EGII software, and the results were plotted by SigmaPlot. C, arsenic stimulation induces superoxide production through Cdc42 and actin filament reorganization. The cells were grown on coverslips and serum-starved overnight. The cells were pretreated with either 1 μM cytochalasin D (*CD*) or 100 nM jasplakinolide (*Jas*) for 1 h as indicated and then stimulated with 10 μM arsenic for 30 min. Dihydroethidium (5 μM) dye was added into the cell culture during the last 30 min of the stimulation. After the staining, the cells were fixed and analyzed by confocal microscopy. D, Rac activities are required for arsenic-induced O_2^- production. *Section 1*, SVEC4-10 cells were grown on coverslips and transiently transfected with a dominant negative mutant of Rac1, pRK5mycRacN17. After incubation for 48 h, the cells were fixed and stained with both an anti-Myc antibody and a FITC-anti-mouse antibody, followed by confocal microscopy analysis. The *left side* shows pRK5mycRacN17-transfected cells. The *right side* shows all the cells visualized under a phase-contrast channel. *Section 2*, the pRK5mycRacN17-transfected cell lysates were resolved by electrophoresis on a 12% SDS-polyacrylamide gel followed by the Western blotting with an anti-Myc antibody. *Section 3*, both SVEC-RacN17 cells and SVEC4-10 cells were stimulated with 10 μM arsenic for 30 min. After the stimulation, the cells were lysed and the lysates were immunoprecipitated with an anti-p47^{phox} antibody. The immunoprecipitation reactions were resolved by electrophoresis on a 10% SDS-polyacrylamide gel followed by Western blotting analysis using an anti-phosphoserine antibody. *Section 4*, both SVEC4-10 cells (*left*) and SVEC-RacN17 cells (*right*) were grown on coverslips and serum-starved overnight. After serum starvation, the cells were stimulated with 10 μM arsenic for 30 min. Dihydroethidium (5 μM) dye was added into the cell culture during the stimulation. After the staining, the cells were fixed and analyzed by confocal microscopy.

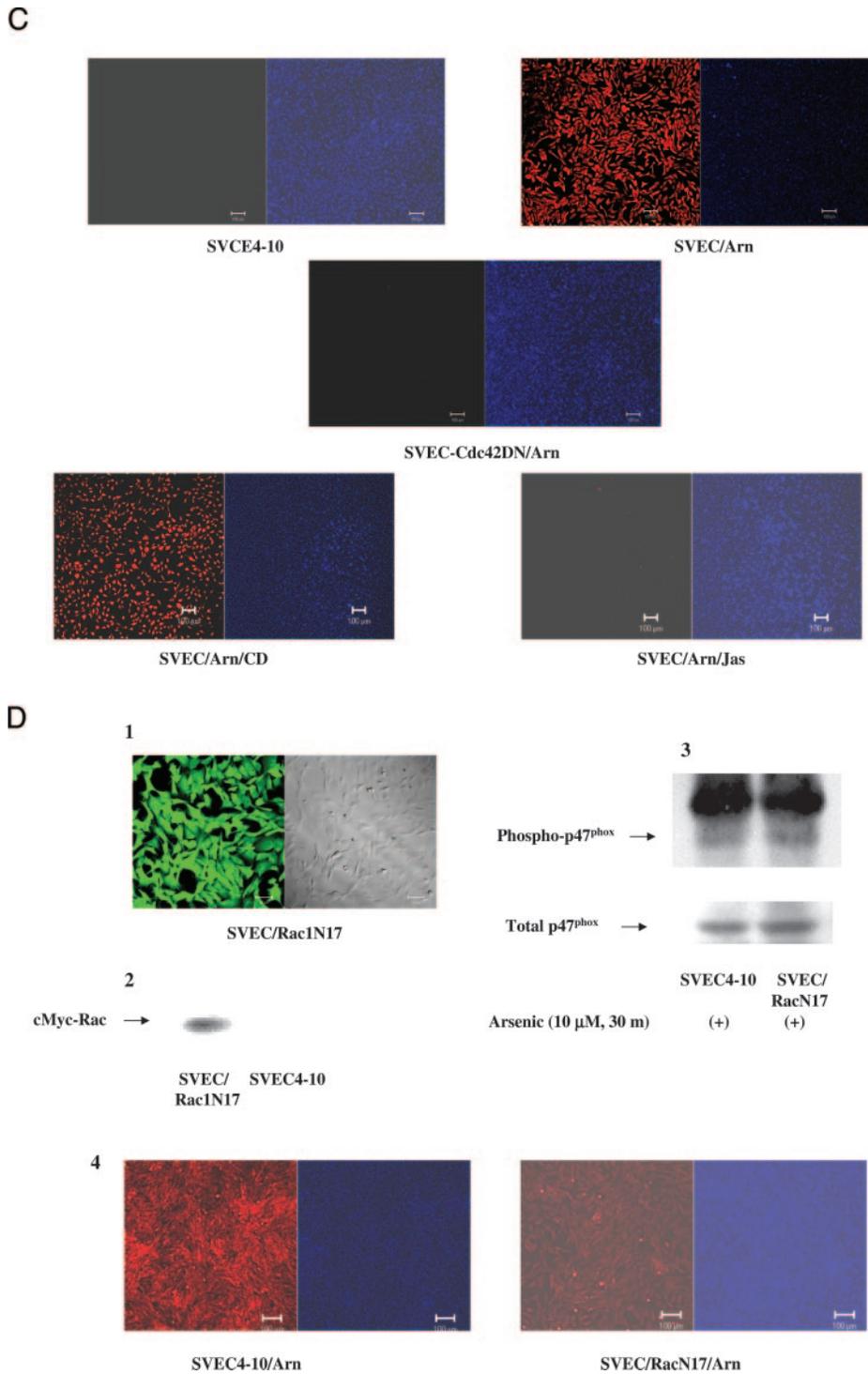


FIG. 4—continued

diated actin filament reorganization might regulate NADPH oxidase activation in response to arsenic stimulation. To further prove the effects of actin filament reorganization on NADPH oxidase activation, the pretreated SVEC4-10 cells were stimulated with arsenic followed by staining with dihydroethidium dye. SVEC-Cdc42DN cells were set as a negative control. The results demonstrated that arsenic-induced O_2^- production was abolished by either the disruption of Cdc42 activity or the inhibition of actin filament reorganization with jasplakinolide pretreatment (Fig. 4C). The pretreatment of cytochalasin D had a small effect on arsenic-induced O_2^- production (Fig. 4C).

Because Rac1 is both a downstream protein of Cdc42 and a key component of NADPH oxidase, we performed Rac activa-

tion assays to determine whether Rac1 was activated upon arsenic stimulation in SVEC4-10 cells. Our results demonstrated that no Rac activation was observed (data not shown). We then performed experiments to examine whether Rac1 activities were required for Cdc42-modulated NADPH oxidase activation in response to arsenic stimulation. A dominant negative Rac, pRK5mycRacN17, was transiently overexpressed into SVEC4-10 cells followed by stimulation with 10 μ M arsenic for 30 min. After stimulation, the cell lysates were subjected to immunoprecipitation assays to examine the serine phosphorylation level of p47^{phox}. We also detected the effects of RacN17 overexpression on arsenic-induced O_2^- production. Our results showed that >90% of the cells expressed RacN17 after 48 h of

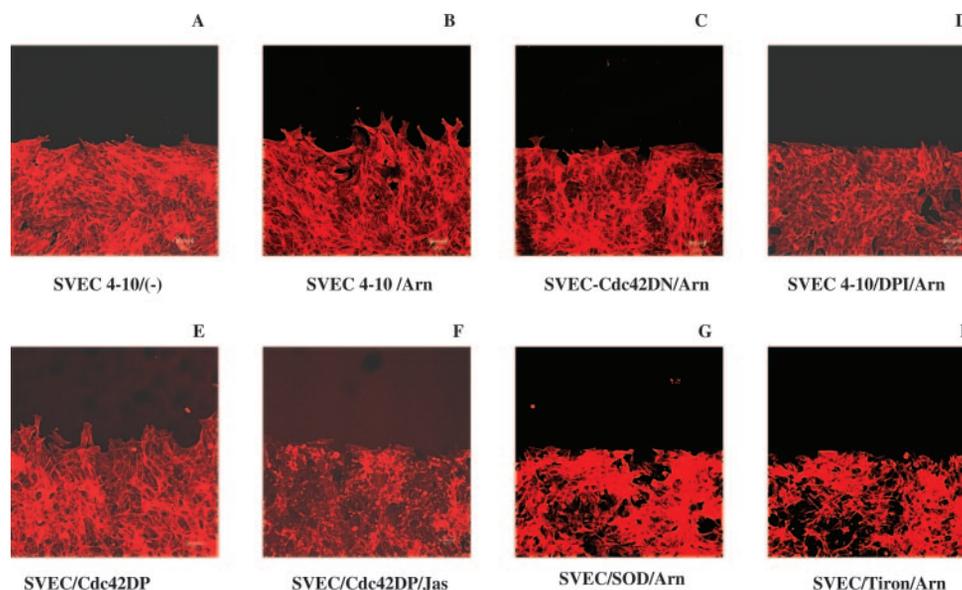


FIG. 5. **Arsenic stimulation (Arn) induces an increase in cell migration.** A and B, the cells were grown on coverslips to 100% confluent monolayers. Sterile pipette tips were used to scratch the confluent monolayer cells to form a 100- μm wound, and then the cells were grown for 8 h with (B) or without (A) 10 μM arsenic stimulation as indicated. C, SVEC4-10 cells were stably transfected with a dominant negative form of Cdc42. D, SVEC4-10 cells were pretreated with 10 μM DPI for 1 h. E, SVEC4-10 cells were stably transfected with a dominant active form of Cdc42 (Cdc42DP). F, SVEC-Cdc42DP cells were pretreated with 100 nM jasplakinolide (Jas) for 1 h. G, SVEC4-10 cells were pretreated with superoxide dismutase (SOD; 500 units/ml) for 1 h. H, SVEC4-10 cells were pretreated with 5 mM Tiron for 1 h.

transfection (Fig. 4D, section 1). The overexpression of RacN17 had no effects on arsenic-induced p47^{phox} serine phosphorylation, indicating that Cdc42-regulated p47^{phox} activation upon arsenic stimulation is independent of Rac activities (Fig. 4D, section 2). However, the overexpression of RacN17 was sufficient to abolish arsenic-induced O₂⁻ production, implying that the integrity of Rac activities was required for Cdc42-mediated NADPH oxidase activation to produce O₂⁻ upon arsenic stimulation (Fig. 4D, section 3). Taken together, our results indicate that the activation of Cdc42 regulates NADPH oxidase activities via the reorganization of actin filaments in response to arsenic stimulation in SVEC4-10 endothelial cells, which requires Rac activities.

Arsenic Stimulation Induces Cell Migration—Both Cdc42 and actin filaments are the key signal proteins that are involved in cell migration (11, 25). Arsenic-induced changes in actin filaments and Cdc42 indicate that arsenic may be able to enhance cell migration in SVEC4-10 cells. We determined whether arsenic would have the capability to induce cell migration and then investigated whether the increase in cell migration would be regulated by arsenic-induced changes in Cdc42, actin filaments, and NADPH oxidase. Cell wound healing assays were performed. SVEC4-10 cells were grown to 100% confluent monolayers on coverslips, starved for serum, and then scratched to form a 100- μm wound with the sterile pipette tips. After the cells were wounded, they were cultured for 8 h with or without 10 μM arsenic stimulation to examine the difference of cell motility at the edge of the wound. SVEC-Cdc42DN cells were used as a negative control to determine the importance of Cdc42 in arsenic-induced cell migration. We also investigated the requirements for NADPH oxidase activation in arsenic-induced cell migration via DPI pretreatment of the cells. DPI is an effective inhibitor of NADPH oxidase (26). Our results showed that the edge of the wound was smooth and that the cells at the edge were unable to move toward the wound without arsenic stimulation after 8 h of the incubation (Fig. 5A), indicating the cells are static in terms of migration. However, upon arsenic stimulation the edge of the wound became protrusive, and many cells migrated out of the boundary of the wound to heal the wound (Fig. 5B), showing that arsenic stim-

ulation induces an increase in cell migration. Our results also showed that either the disruption of Cdc42 activity by Cdc42DN or the inhibition of NADPH oxidase with DPI abolished arsenic-induced cell migration (Fig. 5, C and D). Furthermore, overexpression of a dominant active form of Cdc42 (SVEC-Cdc42DP) was sufficient to induce an increase in cell migration, which is totally abolished upon treatment with jasplakinolide (Fig. 5, E and F). Finally, our results demonstrated that the pretreatment with either 500 units/ml superoxide dismutase or 5 mM Tiron to inhibit the formation of O₂⁻ was sufficient to abrogate arsenic-induced cell migration (Fig. 5, G and H), indicating that arsenic-induced cell migration is a linear consequence of Cdc42 activity and O₂⁻ production. Taken together, the results show that arsenic stimulation induced an increase in cell migration, which was regulated by Cdc42 and NADPH oxidase.

DISCUSSION

The concentration of arsenic (10 μM) used here is relatively high compared with the highest concentration of arsenic (2 μM) that humans can tolerate without major side effects (2). However, in most *in vitro* experiments used to study arsenic-induced cell signal transduction the concentration ranges from 0.1 to 500 μM , depending on the duration of the treatment (2). In general, a high concentration of arsenic for *in vitro* experiments is needed to induce changes in cell signal transduction in a relatively short exposure time.

Arsenic is a carcinogen. It is associated with a high incidence of several types of human cancers (2). However, the molecular mechanisms by which it induces cancer development have not been well elucidated. In this study, we sought to investigate arsenic-induced NADPH oxidase activation and cell migration with emphasis on the role of Cdc42 and actin filament reorganization. The reorganization of actin filaments is a hallmark of cell transformation and the main driving force for cell migration, which is directly involved in angiogenesis, cancer invasion, and metastasis. The processes of actin filament reorganization are essential in cell extension, attachment, contraction, and release during cell movement (27). Our results showed that arsenic stimulation induced the formation of the cellular pro-

trusion structures lamellipodia and filopodia at the leading edges of cells and rosette-like dot structures in the cell body, which demonstrated that arsenic stimulation was sufficient to induce the reorganization of actin filaments in SVEC4-10 cells. The formation of lamellipodia and filopodia involves dynamic processes of actin filament polymerization and depolymerization and actin filament cross-linking (17, 28). These cell motile structures are the key features of transformed cells (29, 30). The reorganization of actin filaments is tightly regulated by small Rho GTPases, mainly Cdc42 and Rac (13). The results obtained from the present study demonstrated that arsenic-induced actin filament reorganization was regulated by the activation of Cdc42. In fibroblast cells, the activation of Cdc42 and Rac is in a hierarchical cascade wherein Cdc42 activates Rac (13). The increase in the formation of lamellipodia indicates that arsenic stimulation may activate Rac in SVEC4-10 cells. We performed Rac activation assays and found that no Rac activation was induced upon stimulation with arsenic (data not shown). It is not unprecedented that Cdc42 remodels actin filaments to form both lamellipodia and filopodia structures without the activation of Rac. Both our previous study in SVEC4-10 cells and those from other groups demonstrated that Cdc42 was able to induce the formation of lamellipodia independent of the activation of Rac (10, 18, 31).

NADPH oxidase is one of the key enzymes that are involved in the generation of O_2^- in a variety of cells. It has been demonstrated that NADPH oxidase is a major source of O_2^- production in endothelial cells and that the production of O_2^- plays a major role in vascular physiology and pathology (22, 32). NADPH oxidase is composed of several components, including $p40^{phox}$, $p67^{phox}$, $p22^{phox}$, $gp91^{phox}$, and Rac (22). Its activity is low in static endothelial cells (32). Upon stimulation, it can be activated in several minutes to several hours (32). One of the key steps of NADPH oxidase activation is the serine phosphorylation of $p47^{phox}$. It was demonstrated that the kinetics of $p47^{phox}$ serine phosphorylation paralleled that of NADPH oxidase activation (33). $p47^{phox}$ is an essential part of NADPH oxidase in endothelial cells as well as in phagocytes. It was found that $p47^{phox-/-}$ endothelial cells were unable to sufficiently produce ROS and transmit signaling upon different stimuli. Overexpression of $p47^{phox}$ into these $p47$ knock-out cells rescued the response of agonist-induced O_2^- generation (32, 34). The results obtained from the present study have demonstrated that arsenic stimulation of SVEC4-10 cells generates O_2^- both extracellularly and intracellularly. The increase in serine phosphorylation of $p47^{phox}$ indicates that arsenic is able to activate NADPH oxidase and that this activation is responsible for the arsenic-induced generation of O_2^- .

It has been demonstrated that NADPH oxidase activity is modulated by several enzymes in endothelial cells, including protein kinase C, phospholipase D, phospholipase A_2 , phosphatidylinositol 3-kinase, Src, and Rac (32). The present study provided the evidence that NADPH oxidase was regulated by Cdc42. Interestingly, Rac, which is a downstream of Cdc42 and is a key component of NADPH oxidase, is not activated in response to arsenic stimulation, which matches our previous observation that Cdc42 is able to induce ROS production independent of Rac activation upon ethanol stimulation in the same cell system (10). However, our results demonstrated that the integrity of Rac activities was essential for arsenic-induced O_2^- production, indicating that Cdc42-mediated O_2^- production is Rac-dependent. It was reported that the guanine nucleotide exchange factor Trio can activate the phagocyte NADPH oxidase in the absence of a GDP-to-GTP exchange on Rac in an *in vitro* cell-free NADPH oxidase assay (35). It has been suggested that a conformational

change in Rac-GDP upon its interaction with a guanine nucleotide exchange factor is sufficient for mediating NADPH oxidase activation (35). Overexpression of a dominant negative Cdc42 abrogated arsenic-induced actin filament reorganization and NADPH oxidase activation, indicating that Cdc42 modulates both actin filament reorganization and NADPH oxidase activation upon stimulation with arsenic. The present study attempted to correlate these two cellular responses. Although both cytochalasin D and jasplakinolide are actin filament modifiers, they induce the opposite morphological changes in actin filaments through different mechanisms. Cytochalasin D blocks actin polymerization to disrupt actin filament structures, whereas jasplakinolide induces actin polymerization to inhibit actin filament reorganization (24). Using these two actin filament modifiers, the present study shows that Cdc42 regulates NADPH oxidase activity through actin filament reorganization in response to arsenic stimulation. Actin filament reorganization has been found to activate several cell signal proteins, including Erk, JUN, p38, Rho kinase, and Src (36–40). The molecular mechanisms of the activation have not been elucidated. Actin filaments, as a main scaffold for cells, not only provide the mechanical supports to cells but also compartmentalize cellular proteins to localize cellular signal transduction. The reorganization of actin filaments upon stimulation may disrupt the actin filament-organized cell compartments and change the cellular localization of signal proteins, leading to changes in cell signal transduction. $p47^{phox}$ was found to directly associate with actin filaments in both endothelial cells and neutrophils (41, 42). Most of $p47^{phox}$ protein and basal NADPH activities are associated with actin filaments in unstimulated endothelial cells (43). It is possible that the actin filaments may provide a structural basis to stabilize NADPH oxidase in static cells. Upon stimulation with arsenic, the reorganized actin filaments may change the localization of $p47^{phox}$ and enable other cell signal proteins to phosphorylate $p47^{phox}$. Indeed, it was found that angiotensin II-induced activation of endothelial cell NADPH oxidase involved the subcellular redistribution of $p47^{phox}$ (41). It would be interesting to find out which signal protein(s) are involved in the serine phosphorylation of $p47^{phox}$ following Cdc42 activation and actin filament reorganization in response to arsenic stimulation.

Arsenic-induced formation of lamellipodia and filopodia indicates that arsenic may have an ability to increase cell migration, which has been demonstrated in the present study. This is the first evidence showing that arsenic induces cell migration in endothelial cells. The results obtained further demonstrate that arsenic induces cell migration through Cdc42-mediated NADPH oxidase. NADPH oxidase activity was found to mediate endothelial cell migration upon stimulation with growth factors (44). NADPH oxidase-produced ROS play a major role in various cell signal transduction pathways, including cell migration and angiogenesis. Cdc42 has also been demonstrated to be directly involved in cell migration (25). The present study also indicates that NADPH oxidase-produced O_2^- is a mediator for Cdc42-induced cell migration. The increase in cell migration upon arsenic stimulation suggests that arsenic may promote cancer development through the enhancement of cell migration, opening a new door for the investigation of the overall mechanisms of arsenic-induced carcinogenesis.

Taken together, this study demonstrated that arsenic stimulation is sufficient to do the following: (a) induce the reorganization of actin filaments; (b) induce the activation of Cdc42; (c) activate NADPH oxidase and generate O_2^- ; and (d) induce an increase in cell migration. Our study has also concluded that Cdc42 is able to regulate NADPH oxidase activity through actin filament reorganization, leading to an increase in cell migration in endothelial cells. Our study defines a unique

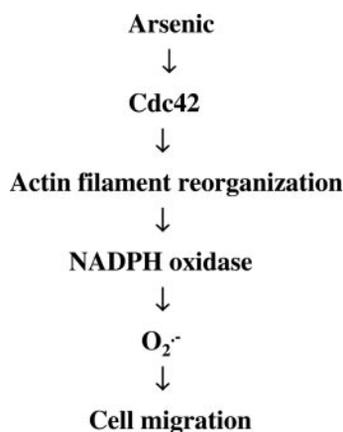


FIG. 6. Schematic representation of signal transduction from arsenic stimulation to cell migration.

signal transduction pathway that indicates Cdc42 mediates cell migration through actin filament reorganization and NADPH oxidase activation upon arsenic stimulation in endothelial cells (Fig. 6).

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Cdc42 Regulates Arsenic-induced NADPH Oxidase Activation and Cell Migration through Actin Filament Reorganization

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