Published in final edited form as:

Toxicol Appl Pharmacol. 2008 September 1; 231(2): 135–141. doi:10.1016/j.taap.2008.04.002.

Arsenic alters vascular smooth muscle cell focal adhesion complexes leading to activation of FAK – src mediated pathways

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Abstract

Chronic exposure to arsenic has been linked to tumorigenesis, cardiovascular disease, hypertension, atherosclerosis, and peripheral vascular disease; however, the molecular mechanisms underlying its pathological effects remain elusive. In this study, we investigated arsenic-induced alteration of focal adhesion protein complexes in normal, primary vascular smooth muscle cells. We demonstrate that exposure to environmentally relevant concentrations of arsenic (50 ppb As³⁺) can alter focal adhesion protein so-association leading to activation of downstream pathways. Co-associated proteins were identified and quantitated via co-immunoprecipitation, SDS-PAGE, and Western blot analysis followed by scanning densitometry. Activation of MAPK pathways in total cell lysates was evaluated using phosphor-specific antibodies. In our model, arsenic treatment caused a sustained increase in FAK-src association and activation, and induced the formation of unique signaling complexes (beginning after 3 hours As³⁺ exposure and continuing throughout the 12-hour time course studied). The effects of these alterations were manifested as chronic stimulation of downstream PAK, ERK and JNK pathways. Past studies have demonstrated that these pathways are involved in cellular survival, growth, proliferation, and migration in VSMCs.

Keywords

Arsenic; FAK; Focal Adhesion; Src; Vascular Smooth Muscle Cell

Introduction

Fundamental cellular processes such as proliferation and survival are intimately tied to a cell's ability to sense and appropriately respond to its surroundings. Aberrant cellular responses to extracellular stimuli can have serious consequences, including hypertrophy, hyperplasia, and transformation. Key mediators of cellular response to the extracellular matrix (ECM) are integrins, transmembrane receptors that respond to biochemical and

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biophysical cues from the ECM by initiating the appropriate intracellular signaling pathways (Aplin *et al.*, 1998; Howe *et al.*, 1998). Integrins function as a physical link between the ECM and the intracellular cytoskeleton at adhesion points. Upon stimulation by ECM proteins, such as fibronectin and collagen, or growth factors, they initiate signaling events leading to cytoskeletal reorganization and changes in shape, adhesion, and motility (Romer *et al.*, 2006). Although traditionally viewed as mediators of adhesion and cytoskeletal organization, it has become increasingly clear that integrins play a multifaceted role in cellular signaling by initiating and coordinating intracellular pathways involved in growth, survival, proliferation, and migration (Parsons, 2003).

Focal adhesion (FA) complexes facilitate coordination and cooperation among a wide range of receptors, including integrins, G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) (Aplin *et al.*, 1998; Howe *et al.*, 1998; Abram and Courtneidge, 2000; Parsons, 2003; Bromann *et al.*, 2004; Luttrell and Luttrell, 2004; Shupnik, 2004; Romer *et al.*, 2006). Integrins lack inherent catalytic activity; therefore, signal transduction is mediated through the assembly of FAs, large multi-protein complexes constructed from a diverse set of molecules including scaffolding proteins, GTPases, kinases, phosphatases, proteases and lipases (Brown and Turner, 2004; Romer *et al.*, 2006). In addition to their highly variable composition, FAs vary in both size and rate of assembly and disassembly, or turnover (Parsons *et al.*, 2000). Larger FAs have a slow turnover rate and are thought to effectively sequester signaling components, while smaller FAs undergo rapid turnover and redistribution of components. The dynamic and heterogeneous nature of these complexes allows for both temporal and spatial regulation of signaling events.

It is not well understood how cells regulate these interactions and the selective assembly of complexes to elicit the appropriate response to specific environmental cues; however, focal adhesion kinase (FAK) and src kinase are known to be key regulators of early FA assembly and subsequent signal transduction (Mitra and Schlaepfer, 2006). Integrin activation in response to extracellular stimuli leads to a rapid increase in FAK and src recruitment and corresponding increase in their catalytic activities (Cox *et al.*, 2006). In normal cells FAK and src activation occurs through numerous pathways and is a point of convergence for multiple overlapping pathways mediated by integrins, RTKs, and GPCRs. Additionally, FAK and src can stimulate downstream pathways through transactivation of various receptors, including the epidermal growth factor receptor (Simeonova *et al.*, 2002; Ishizawa and Parsons, 2004).

Although FAK and src are critical to normal cellular responses and processes, aberrant expression or activity can contribute to cellular transformation and pathological processes associated with tumorigenesis, cardiovascular disease, chronic inflammatory disease, and mental retardation (Schlaepfer *et al.*, 2004; Mitra and Schlaepfer, 2006). Cellular transformation induced by constitutively active forms of src, or its viral homolog v-src, is associated with decreased cellular adhesion and acquisition of anchorage and growth factor independence. Src mediated transformation of epithelial cells results in a transition from the normal cuboidal morphology to one similar to fibroblasts, a process similar to the epitheloid to fibroblastoid transformation observed during tumorigenesis *in vivo*. Additionally, increased FAK and src activity was shown to promote E cadherin internalization and transcriptional up-regulation and expression of ECM proteases, both of which directly contribute to the acquisition of an invasive phenotype (Siesser and Hanks, 2006).

Because of their unique properties, vascular smooth muscle cells (VSMCs) have proven a useful model system for studying migration and tumorigenesis. As with other mesenchymal cells, VSMCs differentiate then exist in a G₀ growth-arrested state *in vivo*. However, growth factors or ECM components can stimulate their re-entry into the cell cycle, growth,

proliferation, and migration. Thus, *in vitro* VSMCs display many of the same characteristics associated with tumorigenesis and metastasis (Berk, 2001; Gerthoffer, 2007). Additionally, aberrant VSMC growth, migration and proliferation contribute to the development and progression of many cardiovascular diseases (Intengan and Schiffrin, 2001; Touyz *et al.*, 2001). Hypertension and atherosclerosis are associated with VSMC hyperplasia in large and intermediate arterioles, and eutrophic remodeling in small vessels. Moreover, migration and proliferation of medial VSMCs to form a neointima is an important pathological process in atherosclerosis associated intimal thickening. Experimental animal studies have reported significant increases in VSMC proliferation and the number of layers in the media of vessels from animals with chronic hypertension (Berk, 2001).

Arsenic is a known carcinogen and tumor promoter. Epidemiological studies link arsenic exposure with increased risks of cancer of the skin, kidney, lung, bladder and liver (Centeno *et al.*, 2002; Tapio and Grosche, 2006). Additionally, chronic ingestion of low-levels of arsenic has been associated with increased occurrence of diabetes, hypertension and cardiovascular disease (Simeonova and Luster, 2004; Yang, 2006; Wang *et al.*, 2007). The occurrence of blackfoot disease, an endemic peripheral vascular disease, has been specifically linked to long-term ingestion of contaminated drinking water (Yang, 2006). Various possible mechanisms of arsenic toxicity have been proposed including oxidative stress, altered protein expression, and altered DNA repair mechanisms (Goering *et al.*, 1999; Kitchin, 2001; Simeonova and Luster, 2002; Rossman *et al.*, 2004; Schoen *et al.*, 2004; Pysher *et al.*, 2007). Although the biological effects of arsenic exposure have been well documented, the molecular mechanisms underlying its effects remain elusive.

In this study, we investigated arsenic-induced alteration of FA protein co-association and related signaling pathways in normal VSMCs. We demonstrate that low, environmentally relevant concentrations of arsenic (50 ppb As³⁺) can induce sustained increases in FAK-src association and activation. Furthermore, we show concomitant chronic stimulation of downstream signaling pathways leading to PAK, ERK, and JNK activation. Past studies have demonstrated that these pathways are involved in cellular survival, growth, proliferation, and migration in VSMCs.

Methods

Reagents

Tissue culture media, fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen (Carlsbad, CA). Protein assay reagent was from Bio-Rad (Hercules, CA). SuperSignal West Dura enhanced chemiluminescence substrate was obtained from Pierce (Rockford, IL). Antibodies were obtained as follows; Akt, Rac1, FAK, p130cas, and PAK1 from Santa Cruz Biotechnology (Santa Cruz, CA), PDK1, JNK, pJNK, ERK, pERK, pP38, pPAK, and src from Cell Signaling Technology (Beverly, MA), paxillin from BD Biosciences (San Jose, CA).

Cell Culture

Primary rat VSMC were isolated from aortic explants from adult Sprague-Dawley rats and used between passages 4 and 12. Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cell monolayers were routinely grown on 10-cm tissue culture dishes in a humidified 37°C/5% CO₂ incubator. Arsenic treatment was accomplished through addition of arsenite (As³⁺, from a concentrated stock solution of NaAsO₂) to serum free media. VSMCs were made quiescent via serum starvation for 24–36 h, after which media was changed out with fresh

serum-free media containing 50 ppb (0.67 μ M) arsenic (or serum-free media without arsenic for control samples).

Immunoprecipitations and Western Blot Analysis

After treatment, cells were rinsed with ice-cold PBS (phosphate buffered saline, pH 7.4) and lysed. For immunoprecipitations cells were lysed for 10 min on ice in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 2 mM Na_3VO_4 , 1% NP-40, 20 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), after which the cells were collected into microcentrifuge tubes via gentle mechanical disruption and allowed to lyse for an additional hour at 4°C with tumbling. After lysis, cellular extracts were centrifuged at 4°C for 10 min, and protein concentration determined using bovine serum albumin as a standard. Cell lysates containing equivalent amounts of total protein (typically 150 μ g) were incubated with protein specific antibodies overnight at 4°C under constant rotation, and then an additional hour after the addition of protein G-agarose beads. The immunoprecipitates were collected by centrifugation (3000 $\times$ g for 5 s) and washed 3 times with lysis buffer, then resuspended in Laemmli buffer and heated (5 min at 90°C). Antibody titrations were performed to determine optimal concentrations and to ensure that antibody concentration was not limiting during immunoprecipitations. To collect total cellular protein, cells were lysed in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT). Protein content was determined as described, and samples containing equivalent amounts of total protein (20 μ g) were separated by SDS-PAGE, electrophoretically transferred to PVDF membranes, and analyzed by Western blot. Protein expression was visualized using enhanced chemiluminescence.

Statistical Analysis

Western blots shown are representative of results obtained; all experiments were repeated a minimum of 4 times. Bands were quantitated using Scion Image (Frederick, Maryland) and Image J software. Expression values (determined by band densities) are given in arbitrary units, where the sum of the values of all bands in a series is normalized to 100 and the relative expression level for each sample within that series reported as a percentage of that total. Error bars are representative of the standard deviations with N = 8 (figure 1, figure 3 & figure 4) or N = 4 (figure 5). For co-precipitations (where N = 8) values are based on 4 replicate immunoprecipitations of each protein; for example, the src-FAK values in figure 1B were calculated using 4 replicate src immunoprecipitations immunoblotted for co-associated FAK, and 4 replicate FAK immunoprecipitations immunoblotted for co-associated src. Significant differences ($p < 0.05$) were determined using one-way ANOVA (analysis of variance) and Bonferroni's multiple comparison tests at the 95% confidence interval.

Results

Basal State FA Protein Co-association in VSMCs

It is now well-known that proteins co-associate and form multi-protein complexes under both basal and stimulated conditions. In the basal state, pathway-related kinases, phosphatases, scaffolding proteins, substrates, and downstream effectors form pre-arranged signaling complexes, which are believed to facilitate a rapid response to stimuli and reduce non-specific crosstalk among overlapping pathways (Brown and Turner, 2004). The formation of FAs in particular is a complex and dynamic process during which multi-protein signaling complexes are assembled via ordered recruitment from over 50 different potential component proteins (Romer *et al.*, 2006). Although it is not well understood how this spatial and temporal assembly is regulated at the cellular level, initial tyrosine phosphorylation

events and signal propagation are known to be orchestrated by FAK and src kinases. Furthermore, early FAK-src mediated events often involve paxillin or p130cas, two key scaffolding proteins and direct FAK-src substrates (Brown and Turner, 2004; Wozniak *et al.*, 2004; Mitra and Schlaepfer, 2006).

To evaluate FA protein co-association in the basal state, VSMC were made quiescent via serum starvation for 24–36 h then harvested. The cell lysates were assayed for total protein content via the Bradford method, and the specified FA proteins, along with their co-associated proteins, were immunoprecipitated. Immunoprecipitations were performed using FAK, src, paxillin, and p130cas specific antibodies, and the co-precipitated proteins resolved by SDS-PAGE and transferred to PVDF membranes. After transfer, membranes were probed with an anti-phosphotyrosine antibody, then stripped and re-probed with appropriate antibodies to identify the co-precipitated proteins. As shown, FAK, src, and paxillin co-precipitated in unstimulated VSMCs, indicating that co-association of these proteins occurs in the basal state (Fig. 1A, lane a). Interestingly, although p130cas was also found to co-precipitate with paxillin (Fig. 2, lane a), it did not co-precipitate with either src or FAK (data not shown), indicating that while p130cas appears to associate with paxillin it is largely excluded from complexes containing src or FAK under these conditions.

Arsenic Alters FA Protein Association and Increases FAK-src Activation

To evaluate the effects of arsenic on FA protein co-association, VSMC were made quiescent via serum starvation for 24–36 h then treated with arsenic (as arsenite, As^{3+}) for the indicated times, with the VSMC remaining in the serum starved state throughout the treatment period. To simulate chronic, low-level rather than acute stimulation, VSMCs were treated for 3, 6, or 12 h with 50 ppb As^{3+} (0.67 μM), which is well below the concentrations known to cause oxidative stress or toxic effects. After treatment, cell lysates were harvested and immunoprecipitations performed as described previously. As shown, arsenic treatment significantly altered FAK, src, paxillin, and p130cas co-precipitation (Fig. 1A & 2, lanes b–d). Moreover, this effect persisted throughout the time course studied, indicating that arsenic induces a sustained alteration of FA protein complexes in this system. FAK and src are potent co-activators; therefore, increased FAK-src co-association equates to increased activation. Based on the extent of co-precipitation observed, arsenic treatment caused increased FAK-src co-association and activation, as well as increased association with their substrate paxillin (approximately 4-fold after 12 h) (Fig. 1B). Interestingly, a simultaneous decrease in both p130cas tyrosine phosphorylation and co-association with paxillin was observed (Fig. 2, lanes b–d). This finding seemed counterintuitive since, like paxillin, p130cas is a major FAK-src substrate; however, this effect might be the result of the involvement of other proteins not normally present in these complexes.

Arsenic Induces Akt Co-association with FA Proteins

In addition to the aforementioned alterations, arsenic induced FA protein co-association with additional proteins, including Akt (Fig. 3). In the basal state, Akt did not co-precipitate with src, FAK, or paxillin (Fig. 3A, lane a). However, in arsenic treated VSMCs co-precipitation of Akt, src, FAK, and paxillin was observed (Fig. 3A, lanes b–d). Furthermore, this behavior occurred throughout the time course studied, indicating that, in addition to altering the extent of FA protein co-association, arsenic induces a sustained alteration in the components of FA protein complexes. Several groups have reported src and Akt co-association (Chen *et al.*, 2001; Conus *et al.*, 2002; Jiang and Qiu, 2003). Unlike the current study, however, co-association with other FA proteins was not reported. Additionally, in this study no significant Akt activation was observed regardless of association with src (data not shown). Instead, Akt appeared to be involved via association mediated effects rather than its kinase activity.

Increased Activation of FAK-src Mediated Downstream Signaling Pathways

In general, increased FAK-src activity promotes motility, cell cycle progression, proliferation, growth and survival. The FAK-src substrate paxillin is a multifunctional scaffolding protein with numerous potential binding partners and, thus, capable of coordinating signaling events and transduction to numerous different downstream pathways (Brown and Turner, 2004). Therefore, it was important to identify the specific downstream pathways activated in response to altered FAK-src-paxillin activity in the present study.

To identify the downstream proteins involved, the applicable proteins were immunoprecipitated from cell lysates, and the co-precipitated proteins resolved by SDS-PAGE, then analyzed by Western blot. Based on the extent of co-precipitation and the interactions observed, PAK1, Rac1, 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt were determined to be significantly involved in downstream signal transduction (Fig. 4A). As shown, no co-precipitation was observed in the basal state VSMCs (Fig. 4A, lane a). However, in arsenic treated cells PAK1, Rac1, PDK1, Akt, and paxillin co-precipitation was observed throughout the time course studied. Arsenic altered PAK1 association in particular, inducing co-association with Rac1, PDK1, and Akt. Another significant effect noted was that arsenic induced co-association of src and PDK1 in this system.

PAK1 is a member of the p21 activated kinase (PAK) family of serine/threonine kinases. While generally known as downstream effectors of GTPases, including Rac1 and Cdc42, PAK activity can also be modulated by various other mechanisms including interactions with adaptor proteins, translocation, lipid binding, and phosphorylation (King *et al.*, 2000; Kumar and Vadlamudi, 2002; Weber *et al.*, 2004). Of interest in the current study is PAK1 activation via a src-PDK1-PAK1 pathway. Although PDK1 is primarily regulated via serine phosphorylation, src mediated activation via tyrosine phosphorylation (Tyr9 and Tyr373/376) has also been shown (Grillo *et al.*, 2000; Park *et al.*, 2001; Taniyama *et al.*, 2003; Weber *et al.*, 2004). In turn, PDK1 can activate PAK1 via phosphorylation of Thr423 and autophosphorylation of Ser199/204. To confirm PAK1 activation via the src-PDK1-PAK1 pathway in the present study, cell lysates containing total cellular protein were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with a p-Thr423 specific PAK antibody. As shown, no phosphorylation of PAK Thr423 was seen in the basal state (Fig. 5A, lane a), however phosphorylation was evident after 3 h treatment and was further increased after 6 and 12 h of treatment with 50 ppb arsenic (Fig. 5A, lanes b–d).

Both PAK and FAK-Src mediated signaling pathways have been shown to activate the three major MAPK pathways, ERK, JNK, and p38 (Eblen *et al.*, 2002; Bokoch, 2003; Weber *et al.*, 2004; Beeser *et al.*, 2005). To determine which of these pathways were activated in the the present study, cell lysates containing total cellular protein were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with phosphospecific antibodies against ERK1/2, JNK, and p38 (Fig. 5A). Arsenic treatment caused increased activation of both ERK and JNK; however, no increase in p38 activity was noted (Fig 5A, compare lanes b–d with a). As shown, increased ERK activation was noted after 3 h and continued to increase throughout the time course; however, significant JNK activation was only noted after 6 h of arsenic treatment and was further increased after 12 h (increasing approximately 5-fold between 6 and 12 h of treatment, Fig. 5B).

Discussion

In this study, we report the alteration of FA protein co-associations and related signaling pathways in VSMCs exposed to low, environmentally relevant concentrations of arsenic (50 ppb As³⁺). FAK-src association and activity were significantly increased after as little as 3

h of arsenic exposure, and continued to be elevated throughout the 12-hour time course studied. Furthermore, we show concomitant activation of downstream pathways leading to chronic stimulation of PAK1, ERK, and JNK pathways.

Because of their role in mediating signaling pathways that regulate critical cellular processes, FAK and src activity must be tightly regulated. Src is normally maintained in an inactive conformation through phosphorylation of its c-terminal regulatory Tyr530 and intramolecular interactions involving its SH2 and SH3 binding domains (Bjorge *et al.*, 2000; Boggon and Eck, 2004). Activation can occur through dephosphorylation of Tyr530 or protein-protein interaction with its SH2 or SH3 domain. Ligation of the SH2 or SH3 binding domain displaces the inhibitory intramolecular interactions, thereby destabilizing the inactive conformation and allowing autophosphorylation of Tyr416 in the kinase-activating loop. One mechanism of FAK and src activation is co-activation via their co-association. ECM binding or adhesion can stimulate integrin and GPCR mediated autophosphorylation of FAK at Tyr397, which creates a high-affinity binding site for the src SH2 domain (Schlaepfer *et al.*, 2004; Cox *et al.*, 2006). FAK-src association results in activation of src and subsequent phosphorylation of FAK on multiple tyrosine residues including Tyr576/577, which increases kinase activity, and Tyr407, 861, and 925, which serve as additional docking sites for SH2 containing binding partners (Mitra and Schlaepfer, 2006). In addition to this mechanism of co-association, active src can both activate FAK and create its own binding site via phosphorylation. Although both FAK and src are capable of independently initiating signaling cascades through a vast array of scaffolding proteins and downstream effectors, they often remain associated and work cooperatively to mediate signal transduction and activate downstream pathways, including the ERK, JNK, and p38 MAPK pathways (Mitra and Schlaepfer, 2006).

Past studies have reported co-association of src and Akt via interaction between the src SH3 binding domain and a conserved c-terminal proline rich motif in Akt (Pro424 – Pro427, mouse Akt1 numbering) (Jiang and Qiu, 2003). Activated src was shown to subsequently phosphorylate Akt on several tyrosine residues; however, the exact residues and effect of this phosphorylation remain debated (Chen *et al.*, 2001; Conus *et al.*, 2002; Jiang and Qiu, 2003). Based on this known Akt-src co-association and the other co-associated FA proteins observed in this study, we hypothesize that arsenic treatment induces co-association of Akt and src and, consequently, Akt co-association with FAK and paxillin is mediated by src. An intriguing observation is the correlation between increased Akt-src association, and the increases in FAK, src, and paxillin co-association (Fig. 1 & Fig. 3, lanes b–d). These results suggest a mechanism by which Akt association enhances src activity, which then increases src-FAK association and, consequently, their co-association with the downstream effector and scaffolding protein paxillin (Brown and Turner, 2004). Src is known to exist in various states of activation, with dual occupation of both its SH2 and SH3 domains reportedly increasing its activity compared to occupation of only one of its binding domains. This agrees with the model our data suggest; where FAK is bound via the src SH2 domain and Akt via the src SH3 domain in arsenic treated VSMCs. Paxillin co-association would then be mediated through FAK.

FAK-src activation and subsequent phosphorylation of FA components is a major regulator of FA turnover, a critical factor in cellular modulation of migration, growth, survival, and gene expression. In general, FA regulation occurs as a dynamic balance between a Rho dominant phenotype, characterized by large FAs with slow turnover, and a Rac dominant phenotype, characterized by small, dynamic FAs with rapid turnover. Movement towards the Rac phenotype favors decreased ECM and cell-cell adhesion, and increased motility and invasiveness, while the Rho phenotype favors increased ECM and cell-cell adhesion,

decreased motility, and growth arrest. In cancer cells especially, rapid FA turnover equates to enhanced motility, invasiveness, and metastasis.

FAK and src modulate FA behavior both directly and through indirect mechanisms via activation of downstream effectors such as PAK1 (Webb *et al.*, 2004; Romer *et al.*, 2006). Src phosphorylation of FAK promotes rapid cycling of FAK in and out of FAs, increased FA turnover, and disassembly of large FAs in favor of a Rac phenotype (Webb *et al.*, 2004). PAK is involved in signaling events regulating morphogenesis, motility, survival, mitosis, and angiogenesis (Bokoch, 2003). Moreover, PAK activity is believed to play a role in tumorigenesis, and a correlation between PAK1 activity and cancer invasiveness has been reported (Kumar and Vadlamudi, 2002; Bokoch, 2003). PAK is a downstream effector of Rac, and can regulate cytoskeletal dynamics and FA turnover in both Rac dependent and independent manner. An interesting observation in the current study is that Rac – PAK activity increases rapidly then declines slightly between the 6 and 12-hour time points, while PDK1 – PAK association increases slowly at first then appears to rapidly surpass Rac – PAK activity after the 6-hour time point. The lag in PDK1-PAK association also appears to match well with the trend in PDK1-src association (Fig. 4B). Based on these observations it is possible that, once activated via src, PDK1 competes with Rac for association with PAK1, thus causing the observed decrease in Rac-PAK association.

The discovery of arsenic-induced MAPK pathway activation in the present study may have considerable biological implications. The ERK and JNK pathways are critical regulators of VSMC growth, migration, and proliferation (Nelson *et al.*, 1998; Huang *et al.*, 2004). Moreover, ERK and JNK mediate transcription of matrix altering proteins, such as matrix metalloproteases (MMPs) and plasminogen activator inhibitor, which are implicated in many pathologic vascular processes (Chen and Feener, 2004). In healthy arteries, VSMCs remain differentiated and quiescent, existing primarily within the medial layer. However, VSMC hyperplasia, hypertrophy, and invasion of the subendothelial space occur in pathological vascular conditions such as hypertension and atherosclerosis. Past studies have reported increased VSMC growth and ERK activation in spontaneously hypertensive rats both *in vivo* and *in vitro*. Additionally, VSMCs from arteries of hypertensive human patients showed augmented and sustained AngII stimulated activation of ERK and corresponding increases in gene transcription, *c-fos* overexpression, enhanced AP1 activity, and augmented growth (Touyz *et al.*, 2001). This correlation between hypertension and prolonged ERK activation is particularly interesting in light of the sustained ERK activity observed in the present study.

Some insight into the mechanism of arsenic mediated signaling events can be gained through evaluation of similarities to known agonist mediated pathways in VSMCs. Angiotensin II, a well-known promoter of VSMC hyperplasia and hypertrophy, mediates signaling through alterations in intracellular Ca²⁺, ROS, and other signaling pathways (Touyz and Schiffrin, 2000; Gregg *et al.*, 2003; Clempus and Griendling, 2006; Hunyady and Catt, 2006). AngII stimulation can induce transactivation of the EGFR and PDGFR via ROS sensitive pathways, and the IGF-1R through a ROS independent pathway. Additionally, AngII stimulation induces activation of src, PDK1, PAK1, Rac, ERK, JNK, and p38 MAPK in VSMCs; In comparison to the present study, however, AngII stimulation of MAPK pathways is rapid and transient, with phosphorylation returning to basal levels within an hour. Additionally, while AngII is known to activate p38 MAPK in VSMCs, arsenic treatment did not activate p38 in the present study. Furthermore, although arsenic appeared to activate many of the same proteins as AngII, the exact pathways followed and downstream pathways activated differed. The ERK activation in the present study does bear some resemblance to stimulation by growth factors such as PDGF, which induce a biphasic MAPK response with an early peak in ERK activity followed by persistent activation at

approximately 45% of this peak level of activation (Nelson *et al.*, 1998). Therefore, although arsenic treatment appeared to promote some similar effects in the present study, the protein co-associations observed in the arsenic treated VSMC appear to be unique and have not been reported in studies involving growth factor or agonist stimulated pathways.

In summary, arsenic treatment induced alterations in the co-association and activity of FA proteins in primary VSMCs. These alterations resulted in a sustained increase in FAK-src association and activation, as well as formation of unique signaling complexes. The effects of these alterations were manifested as chronic stimulation of PAK1 and downstream ERK and JNK MAPK pathways (Fig. 6). Past studies have demonstrated associations between chronic stimulation of these pathways and vascular disease. Additionally these pathways are known mediators of VSMC growth, proliferation, and migration. Thus, the data presented here suggest a potential role for arsenic, via activation of these same pathways, as a mediator of VSMC behavior. Future studies will focus on identifying the effects of these arsenic-induced changes on protein transcription and expression, as well as overall phenotypic alteration.

Acknowledgments

This work was supported, in part, by ES04940, ES12007, AG19710 (R.R.V.). M.D.P. was supported by a postdoctoral fellowship from the Interdisciplinary Physiological Sciences Program at the University of Arizona (National Institutes of Health Training Grant HL07249). We thank Suzanne Regan for assistance with VSMCs.

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Fig. 1.

Arsenic alters FAK, src, and paxillin co-association in VSMCs. Cell lysates from serum-starved, quiescent VSMCs treated with 50 ppb arsenic (As^{3+}) for 0, 3, 6, or 12 h were immunoprecipitated (IPd) with anti-FAK, anti-src, and anti-paxillin antibodies. The IPd proteins were separated by SDS-PAGE, and co-precipitated proteins identified by Western blot (IB). All experiments were repeated a minimum of 4 times, and the blots shown (A) are representative of the results obtained. (B) Relative FAK, src, and paxillin co-association based on protein co-immunoprecipitation. Western blot protein bands were quantitated by densitometry as described in the Experimental Procedures. FAK-src co-association was calculated from src blots of FAK IPs and FAK blots of src IPs. Src-paxillin and FAK-

paxillin co-associations were calculated from paxillin blots of src or FAK IPs and src or FAK blots of paxillin IPs. Expression, or relative co-association (y-axis) is in arbitrary units normalized to total 100 within each series. Significant differences determined by one-way ANOVA and Bonferroni's multiple comparison tests at the 95% confidence interval, values presented as mean $\pm$ SEM, $p < 0.05$, $N=8$ for each series. Protein molecular weights shown on the left.

Fig. 2.

Arsenic treatment causes decreased p130cas phosphorylation and association with paxillin. Cell lysates from serum-starved, quiescent VSMCs treated with 50 ppb arsenic (As^{3+}) for 0, 3, 6, or 12 h were immunoprecipitated (IPd) with anti-p130cas, and anti-paxillin antibodies. The IPd proteins were separated by SDS-PAGE, then co-precipitated proteins identified and tyrosine phosphorylation determined by Western blot (IB). All experiments were repeated a minimum of 4 times, and the blots shown are representative of the results obtained. Protein molecular weights shown on the left.

Fig. 3.

Arsenic induces Akt co-association with FA proteins in VSMCs. Cell lysates from serum-starved, quiescent VSMCs treated with 50 ppb arsenic (As^{3+}) for 0, 3, 6, or 12 h were immunoprecipitated (IPd) with anti-FAK, anti-src, anti-paxillin, and anti-Akt antibodies. The IPd proteins were separated by SDS-PAGE, and co-precipitated proteins identified by Western blot (IB). All experiments were repeated a minimum of 4 times, and the blots shown (A) are representative of the results obtained. (B) Relative FAK, src, paxillin, and Akt co-association based on protein co-immunoprecipitation as described in Fig. 1. Protein molecular weights shown on the left.

Fig. 4.

Cell lysates from serum-starved, quiescent VSMCs treated with 50 ppb arsenic (As^{3+}) for 0, 3, 6, or 12 h were immunoprecipitated (IPd) with anti-PAK, anti-src, anti-PDK1, anti-Rac, and anti-Akt antibodies. The IPd proteins were separated by SDS-PAGE, and co-precipitated proteins identified by Western blot (IB). All experiments were repeated a minimum of 4 times, and the blots shown (A) are representative of the results obtained. (B) Relative src, PDK1, PAK, Rac, and Akt co-association based on protein co-immunoprecipitation as described in Fig. 1. Protein molecular weights shown on the left.

Fig. 5.

Arsenic induces activation of PAK1, ERK, and JNK pathways in VSMCs. Serum-starved, quiescent VSMCs were treated with 50 ppb arsenic (As^{3+}) for 0, 3, 6, or 12 h. Cell lysates containing total cellular protein (20 μg) were analyzed by SDS-PAGE and Western blot with antibodies recognizing phospho-ERK1/2, total ERK1/2, phospho-JNK1/2, total JNK1/2, phospho-p38, phospho-(Thr423)PAK, and total PAK. Lane e shows the positive control for phospho-p38. All experiments were repeated a minimum of 4 times, and the blots shown (A) are representative of the results obtained. (B) Relative PAK1, ERK, and JNK activation based on phosphorylation. Protein bands were quantitated by densitometry. Expression, or relative activation (y-axis) is in arbitrary units normalized to total 100 within each series,

significant differences determined by one-way ANOVA and Bonferroni's multiple comparison test's and values presented as mean $\pm$ SEM, $p < 0.05$, $N=4$ for each series. Molecular weights indicated on the left based on protein standards.

Fig. 6.
Overview of the observed arsenic-induced effects and general pathway proposed for our VSMC model.