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Activation of Protein Kinase C and Disruption of Endothelial Monolayer Integrity by Sodium Arsenite—Potential Mechanism in the Development of Atherosclerosis

Flavia E. Pereira, J Douglas Coffin, and Howard D. Beall

Department of Biomedical and Pharmaceutical Sciences and Center for Environmental Health Sciences, The University of Montana, Missoula, MT 59812

Abstract

Arsenic exposure has been shown to exacerbate atherosclerosis, beginning with activation of the endothelium that lines the vessel wall. Endothelial barrier integrity is maintained by proteins of the adherens junction (AJ) such as vascular endothelial cadherin (VE-cadherin) and β -catenin and their association with the actin cytoskeleton. In the present study, human aortic endothelial cells (HAECs) were exposed to 1, 5 and 10 μ M sodium arsenite [As(III)] for 1, 6, 12 and 24 h, and the effects on endothelial barrier integrity were determined. Immunofluorescence studies revealed formation of actin stress fibers and non-uniform VE-cadherin and β -catenin staining at cell-cell junctions that were concentration and time-dependent. Intercellular gaps were observed with a measured increase in endothelial permeability. In addition, concentration-dependent increases in tyrosine phosphorylation (PY) of β -catenin and activation of protein kinase C α (PKC α) were observed. Inhibition of PKC α restored VE-cadherin and β -catenin staining at cell-cell junctions and abolished the As(III) induced formation of actin stress fibers and intercellular gaps. Endothelial permeability and PY of β -catenin were also reduced to basal levels. These results demonstrate that As(III) induces activation of PKC α , which leads to increased PY of β -catenin downstream of PKC α activation. Phosphorylation of β -catenin plausibly severs the association of VE-cadherin and β -catenin, which along with formation of actin stress fibers, results in intercellular gap formation and increased endothelial permeability. To the best of our knowledge, this is the first report demonstrating that As(III) causes a loss of endothelial monolayer integrity, which potentially could contribute to the development of atherosclerosis.

Keywords

sodium arsenite; stress fibers; VE-cadherin; β -catenin; vascular permeability; PKC α

Author to whom all correspondence and reprint requests should be addressed: Howard D. Beall The University of Montana Department of Biomedical and Pharmaceutical Sciences 32 Campus Drive Missoula, MT 59812 Phone: (406) 243-5112 Fax: (406) 243-5228 Email: howard.beall@umontana.edu

E-mail address of all authors: Flavia E. Pereira: flavia.pereira@umontana.edu J Douglas. Coffin: douglas.coffin@umontana.edu Howard D. Beall: howard.beall@umontana.edu

Conflict of Interest Statement

There are no conflicts of interest.

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Introduction

Arsenic, an element present in the earth's crust, has been widely distributed in the environment by human activities such as mining, smelting, manufacturing and pesticide application. Epidemiological studies analyzing the correlation between cardiovascular diseases and arsenic exposure, have linked arsenic to atherosclerosis (Wang et al., 2002), ischemic heart disease (Tseng et al., 2003) and hypertension (Rahman et al., 1999). Drinking water is a significant source of arsenic exposure to humans. Using mouse models of atherosclerosis, we and others have demonstrated that chronic exposure to arsenic through drinking water exacerbates atherosclerotic plaque formation (Bunderson et al., 2004, Simeonova et al., 2003). Atherosclerosis can lead to serious clinical manifestations such as heart attack and stroke.

There are numerous regulatory points in the vascular system that may be disrupted and lead to atherogenesis. The integrity of the vascular endothelium is maintained by proteins of the adherens junctions (AJ), which includes the transmembrane protein VE-cadherin linked to the cytoplasmic protein β -catenin which in turn is linked to the actin cytoskeleton through α -catenin (Ivanov et al., 2001). This association is critical to maintaining cell-cell adhesion and structural integrity. The tyrosine phosphorylation (PY) of AJ proteins can alter their interaction with each other and the cytoskeleton. Endogenous mediators such as histamine have been shown to increase PY of both β -catenin and VE-cadherin, resulting in dissociation of VE-cadherin from the actin cytoskeleton with a subsequent increase in vascular permeability (Andriopoulou et al., 1999). The PY of β -catenin has been shown to cause a fivefold reduction in its affinity for E-cadherin (Lilien and Balsamo, 2005, Roura et al., 1999). Accordingly, vascular endothelial growth factor (VEGF) induced PY of VE-cadherin and β -catenin has been implicated in the loosening of cell-cell contacts and increased vascular permeability (Esser et al., 1998).

Bidirectional signaling between the cadherin-catenin complex and the actin cytoskeleton has been observed. Inhibition of VE-cadherin function induces stress fibers and conversely, stress fibers regulate VE-cadherin localization (Hordijk et al., 1999). Stress fibers consist of proteins such as myosin, tropomyosin and α -actinin intermittently associated with the actin filaments (Byers et al., 1984). Stress fibers are contractile elements and have been shown to develop *in vivo* during endothelial cell adaptation to unfavorable or pathological situations such as atherosclerosis and hypertension (van Nieuw Amerongen and van Hinsbergh, 2001, White et al., 1983, Wong et al., 1983). It has been shown that the apolipoprotein(a) component of lipoprotein(a), a risk factor for atherosclerotic disorders, induces stress fiber formation and VE-cadherin dispersion accompanied by cell retraction, intercellular gap formation and increased endothelial permeability (Pellegrino et al., 2004). Thus, development of stress fibers in endothelial cells adversely affects monolayer integrity. The effects of arsenic on the proteins involved in maintaining endothelial monolayer integrity remain unknown.

Several signaling intermediates have been implicated in the regulation of endothelial barrier function. In particular, the various protein kinase C (PKC) isotypes have a host of cellular substrates and are involved in numerous biological processes. PKCs are serine/threonine kinases and have been grouped into 3 subfamilies: classical, novel and atypical PKCs (Newton, 2001). The classical PKCs (cPKC), which consists of α , β and γ subtypes, are activated in a calcium-dependent manner and require diacylglycerol and phosphatidylserine. Amongst the cPKCs, endothelial cells express α and β isotypes (Mattila et al., 1994). The cPKCs have been implicated in the modulation of endothelial barrier function (Yuan, 2003). Activation of PKC α by thrombin has been shown to induce actin stress fibers and increase endothelial permeability (Sandoval et al., 2001). Conflicting data have been published regarding the effects of arsenic on the modulation of intracellular calcium levels and cPKCs; effects that are

dependent on the cell line studied and the duration of arsenic treatment (Chen et al., 2000, Liu and Huang, 1997).

Various mechanisms for arsenic-induced atherosclerosis have been proposed including activation of the endothelium that is characterized by up-regulation of adhesion molecules and inflammatory mediators (Simeonova and Luster, 2004). In particular, *in situ* arsenic exposure through intradermal injections on the dorsal surface of rats has been shown to induce vascular permeability (Chen et al., 2004). However, the mechanism of increased vascular permeability remains unknown. The endothelium maintains a semipermeable barrier and damage to the endothelial lining can result in increased permeability. This could lead to subsequent recruitment and migration of monocytes and oxidized low density lipoproteins (oxLDL) into the intima of the blood vessel, thereby promoting the development of atherosclerotic lesions. Hence, the objective of this study was to determine the effects of arsenic on endothelial barrier integrity and characterize the pathways for arsenic-induced increases in vascular permeability.

Materials and Methods

Cell culture

Human aortic endothelial cells (HAECs) were purchased from Cambrex Bio Science (Walkersville, MD) and were grown in the supplier's formulated medium: EBM-2 medium supplemented with EGM-2 SingleQuots (0.04% hydrocortisone, 0.4% hFGF-B, 2% FBS and 0.1% each of VEGF, R³-IGF-1, ascorbic acid, heparin, hEGF and GA-1000). The cells were incubated at 37°C under a humidified atmosphere containing 5% CO₂. Cells were used between passages 2 and 10.

Immunofluorescence

HAECs were grown on 18 mm round coverslips inserted into 12-well plates and coated with 0.1 mg/ml solution of Poly-D-Lysine hydrobromide (Sigma-Aldrich, Saint Louis, MO). HAECs were seeded at a density of 2×10^5 - 3×10^5 cells/well and were grown to confluence for 48-72 h. Cells were treated with As(III) (GFS Chemicals, Columbus, OH) and the cPKC inhibitor, Gö 6976 (Calbiochem, San Diego, CA), was added 1 h prior to and during the As (III) treatment wherever applicable. Cells were fixed with 1% paraformaldehyde/ phosphate-buffered saline (PBS) for 30 min at room temperature (RT), permeabilized using 0.1% saponin solution/ PBS for 10 min at RT and then blocked with 5% goat serum/PBS (Sigma-Aldrich, Saint Louis, MO) for 30 min at RT. Cells were then incubated with 2 µg/ml rabbit anti-VE-cadherin polyclonal antibody (Cayman Chemicals, Ann Arbor, MI), 2 µg/ml rabbit anti-β-catenin polyclonal antibody (Cayman Chemicals, Ann Arbor, MI) or 0.24 µg/ml rabbit antibody against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) in PBS for 2 h at RT or 24 h at 4°C followed by incubation with 10 µg/ml Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Inc. Eugene, OR) for 1 h at RT in the dark. For detection of stress fibers, HAECs were incubated with 16.5 nM Alexa Fluor 568-conjugated phalloidin (Molecular Probes, Inc. Eugene, OR) for 25 min at RT in the dark. Cells were counterstained with Hoechst stain for 5 min at RT, coverslipped with Fluorosave (EMD Biosciences, San Diego, CA) and stored in the dark until imaged. Slides were photographed with a Nikon Digital Eclipse DXM1200 camera attached to a Nikon E-800 microscope using a 60X oil immersion lens.

Permeability Assay

HAECs (1×10^5 - 1.5×10^5) were seeded on 0.4 µm pore polyester membrane transwell inserts (Corning Incorporated, Acton, MA) coated with 0.1 mg/ml solution of Poly-D-Lysine hydrobromide in 12 well plates. Cells were grown to confluence for 48-72 h. The volume of

medium in the Transwell was 500 μ l with 1.5 ml in the plate well at all times. The As(III) treatments were added to the Transwells and the cPKC inhibitor, Gö 6976, was added 1 h prior to and during the As(III) treatment wherever applicable. At the final 1 h of respective treatments, fluorescein isothiocyanate (FITC)-labeled dextran (average molecular weight 40,000, final concentration of 1 mg/ml; Sigma-Aldrich, Saint Louis, MO) was added to each Transwell including control wells. After completion of the treatment period, the fluorescence intensity of the medium in the plate well was analyzed using a SpectraMax GeminiXS fluorescence plate reader (Molecular Devices Corporation, Sunnyvale, CA). For each sample, 5 aliquots of 200 μ l each were analyzed using an excitation wavelength of 490 nm and emission wavelength of 520 nm.

Immunoprecipitation and Western blot analysis

The PY of VE-cadherin and β -catenin was analyzed by immunoprecipitation followed by Western blot. HAECs were seeded at a density of 7.5×10^5 cells/ 25 cm² cell culture flask and allowed to reach confluence for 48-72 h. Confluent cells were treated with As(III) and the cPKC inhibitor, Gö 6976 was added 1 h prior to and during the As(III) treatment wherever applicable. After completion of treatment, cells were rinsed with cold PBS containing phosphatase inhibitors (1 mM β -glycerophosphate, 1 mM Sodium pyrophosphate and 2.5 mM Sodium orthovanadate). The sodium orthovanadate was activated for maximal inhibition of protein tyrosine phosphatases (activation method obtained from the list of protocols provided by Upstate Cell Signaling Solutions). Cells were harvested using cold 1X RIPA buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 2 mM EGTA, 1% Igepal, 0.1% SDS and 0.5% deoxycholic acid) containing protease inhibitors (Complete protease inhibitor cocktail tablet; Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors. Cells were homogenized by sonication and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was sequentially incubated with 5 μ g/ml each of mouse anti-VE-cadherin monoclonal antibody followed by mouse anti- β -catenin monoclonal antibody (Abcam, Cambridge, MA) for 24 h at 4°C. Immune complexes were precipitated using immobilized protein G (Pierce, Rockford, IL). After incubation for 2 h at 4°C, the agarose bound immune complexes were washed three times with cold 1X RIPA buffer containing protease and phosphatase inhibitors and then heated at 70°C for 10 min in 1X solution of reducing agent and sample buffer (Invitrogen Life Technologies, Carlsbad, CA). The proteins were resolved using 4-12% NuPAGE Novex Bis-Tris gel (Invitrogen Life Technologies, Carlsbad, CA), transferred to a polyvinylidenedifluoride (PVDF) membrane, blocked with 5% BSA/ TBST (TBST: 1.57 gm/l Tris HCl (pH=8), 8.76 gm/l NaCl, 500 μ l/l Tween 20) for 1 h at RT and incubated with 2 μ g/ml mouse anti-phosphotyrosine monoclonal antibody (BD Transduction Laboratories, San Jose, CA) overnight at 4°C in 1% BSA/ TBST solution. This was followed by incubation of the membrane with 1 μ g/ml peroxidase labeled horse anti-mouse secondary antibody (Vector laboratories Inc., Burlingame, CA) for 1 h at RT in 1% BSA/ TBST solution. The protein bands were visualized by ECL Western blot detection system (Amersham, Buckinghamshire, England). To quantitate PY, the total protein content of immunoprecipitated VE-cadherin and β -catenin was determined as follows. After analysis of phosphotyrosine content, the membranes were stripped at 60°C for 1 h using stripping buffer (100 mM β -mercaptoethanol, 2% SDS and 62.5 mM Tris HCL, pH 6.8), blocked with 5% non-fat milk/ TBST for 1 h at RT and reprobbed for VE-cadherin and β -catenin protein using 1.25 μ g/ml rabbit anti-VE-cadherin and 1 μ g/ml rabbit anti- β -catenin polyclonal antibody (Cayman Chemicals, Ann Arbor, MI) overnight at 4°C in 0.4% non-fat milk/ TBST. This was followed by incubation with 5 μ g/ml peroxidase labeled goat anti-rabbit secondary antibody (Vector laboratories Inc., Burlingame, CA) for 1 h at RT in 0.4% non-fat milk/ TBST, and the protein bands were visualized as previously described.

To determine the protein content of VE-cadherin without immunoprecipitation, cells were rinsed with PBS and harvested with 1X RIPA buffer containing protease inhibitors. Cells were homogenized with 18 and 25 gauge needles and boiled for 10 min. Protein concentrations were determined by using the Bradford protein assay (Biorad, Hercules, CA). The proteins were resolved using 4-12% NuPAGE Novex Bis-Tris gel, transferred to PVDF membrane, blocked with 5% non-fat milk/ TBST for 2 h at RT and probed for VE-cadherin protein using 1.25 µg/ml rabbit anti-VE-cadherin polyclonal antibody for 2 h at RT or overnight at 4°C in 0.4% non-fat milk/ TBST. This was followed by incubation with 5 µg/ml peroxidase labeled goat anti-rabbit secondary antibody for 1h at RT in 0.4% non-fat milk/ TBST. The protein bands were visualized by ECL Western blot detection system. Protein loading was normalized using β-actin as loading control. β-actin protein was detected using 0.24 µg/ml mouse anti-β-actin monoclonal antibody (Abcam, Cambridge, MA) and 1 µg/ml peroxidase labeled horse anti-mouse as the secondary antibody.

To determine the activation of PKCα, HAECs were seeded at a density of 7.5×10^5 cells in 25 or 75 cm² cell culture flasks and after 3-5 days, confluent cells were treated with As(III). Cells were rinsed with cold PBS, harvested using homogenizing buffer (50 mM Tris HCl, pH 7.5, 1 mM EDTA and protease inhibitors) and sonicated. To separate the membrane and cytosolic fractions, the lysates were centrifuged at 100,000× g for 1 h at 4°C. The supernatant was collected as the cytosolic fraction. The pellet was dissolved in 1X RIPA buffer containing protease inhibitors, sonicated and centrifuged again at 100,000× g for 1 h at 4°C. The supernatant was the membrane fraction. Protein concentrations were determined by using the Bradford protein assay. The proteins were resolved using 4-12% NuPAGE Novex Bis-Tris gels. To detect good quality protein bands, different amounts of cytosolic and membrane fraction proteins were loaded; 10-20 µg protein for the cytosolic fraction and 40-55 µg protein for the membrane fraction. After separation, the proteins were transferred to PVDF membranes, blocked with 5% non-fat milk/ TBST for 1 h at RT and probed for PKCα using 0.4 µg/ml mouse anti-PKCα monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C in 0.4% non-fat milk/ TBST. This was followed by incubation with 1 µg/ml horse anti-mouse secondary antibody conjugated with horseradish peroxidase for 1 h at RT in 0.4% non-fat milk/ TBST. Protein bands were visualized by ECL Western blot detection system. Na⁺/K⁺ ATPase was used as loading control for the membrane fraction using 0.4 µg/ml mouse anti-Na⁺/K⁺ ATPase monoclonal antibody (Upstate Cell Signaling Solutions, Lake placid, NY) and GAPDH was used as loading control for the cytosolic fraction using 1 µg/ml mouse anti-GAPDH monoclonal primary antibody (Chemicon International, Inc., Temecula, CA). In both cases, 1 µg/ml horse anti-mouse secondary antibody was used.

Statistical analysis

Results are presented as means ± SE. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls multiple comparison test. Differences were considered significant at $p < 0.05$.

Results

Dose response of HAECs to cPKC inhibitor Gö 6976

Indolocarbazole Gö 6976 has been shown to be a potent and selective inhibitor of cPKCs via interference with binding of ATP to the kinase (Martiny-Baron et al., 1993). Preliminary experiments at 5 min, 15 min, 30 min, 1 h, 1.5 h, 2 h and 6 h revealed that 10 µM As(III) induces intercellular gaps beginning at 1 h of treatment (data not shown) that were identified by immunofluorescence staining of VE-cadherin. To determine the effective concentration of Gö 6976 to be used in our experiments, cells were exposed to 10 µM As(III) alone or to 10

nM, 50 nM, 100 nM, 250 nM, 500 nM and 1 μ M Gö 6976 in the presence of 10 μ M As(III) as depicted in Fig. 1. The duration of As(III) treatment was 1 h and the respective Gö 6976 treatments were applied 1 h prior to and during As(III) treatment. As analyzed by immunofluorescence staining of VE-cadherin, 250 nM-1 μ M concentrations of Gö 6976 (Fig. 1 F-H) were able to inhibit the As(III) induced gap formation. Therefore, in all the further experiments, 250 nM Gö 6976 was used to inhibit the activity of cPKCs. Also, the effect of Gö 6976 on cell viability was assessed using the MTT assay (Bunderson et al., 2002) and no loss of viability was observed at concentrations 10 nM-500 nM up to 24 h (data not shown).

Effect of As(III) exposure on VE-cadherin localization and intercellular gap formation

Based on results from preliminary experiments, the concentration and time-dependent effects of As(III) on VE-cadherin localization and intercellular gap formation were analyzed by immunofluorescence staining of VE-cadherin. Cells were treated with 1, 5 and 10 μ M As(III) for 1, 6, 12 and 24 h. At the 1 h time point (Fig. 2), the frequency of gap formation observed with 10 μ M As(III) treatment (Fig. 2D) was much greater than 5 μ M As(III) (Fig. 2C) and only a few gaps were observed with 1 μ M As(III) treatment (Fig. 2B) as compared to the untreated cells (Fig. 2A). Cells appeared to be pulling apart from the adjacent cells. Some areas of undamaged monolayer could be observed. Also, non-uniform staining of VE-cadherin was observed where the gaps appeared. To corroborate the fact that the observed effects were not due to cells undergoing apoptosis, immunofluorescence staining for cleaved caspase-3 was performed. Figures 2E and F demonstrate little cleaved caspase-3 staining for either untreated or 10 μ M As(III) treated cells, respectively. Also, MTT assay showed no loss of cell viability at any of the As(III) concentrations used up to 48 h (data not shown). The cPKC inhibitor, Gö 6976, inhibited gap formation at all concentrations of As(III) treatment and uniform distribution of VE-cadherin could be observed at cell-cell junctions. The images of 250 nM Gö 6976 only treatment (Fig. 2G) and 10 μ M As(III) + 250 nM Gö 6976 treatment (Fig. 2H) are shown.

At the 6 h time point (Fig. 3), a similar concentration-dependent effect of As(III) was observed but the size and frequency of gaps was greater than that observed at the 1 h time point. Exposure to 1 μ M (Fig. 3B) and 5 μ M As(III) (Fig. 3C) revealed non-uniform staining of VE-cadherin where the gaps appeared whereas treatment with 10 μ M As(III) (Fig. 3D) resulted in loss of VE-cadherin staining at the cell-cell junctions. Immunofluorescence staining of cleaved caspase-3 in untreated (Fig. 3E) and 10 μ M As(III) treated cells (Fig. 3F) revealed no differences in apoptosis. Also, Gö 6976 inhibited gap formation at all concentrations of As(III) treatment and restored VE-cadherin staining at the cell-cell junctions. The images of 250 nM Gö 6976 only (Fig. 3G) and 10 μ M As(III) + 250 nM Gö 6976 treatment (Fig. 3H) are shown. At the 12 and 24 h time points, concentration-dependent effects of As(III) treatment were similar to those seen at earlier time points (data not shown). However, the frequency and size of the gaps observed were maximal at the 6 h time point.

Effect of As(III) treatment on VE-cadherin protein content

The immunofluorescence results in Fig. 1-3 showed that VE-cadherin distribution was altered by As(III) exposure. Therefore, the effects of 1, 5, and 10 μ M As(III) treatments on the protein levels of VE-cadherin at the 24 h time point were determined. Western blotting analysis revealed a concentration-dependent effect of As(III) on VE-cadherin protein levels in the HAECs (Fig. 4A and B). Approximately 8, 15 and 30% reductions in VE-cadherin protein were observed with 1, 5 and 10 μ M As(III) treatment, respectively, compared to the control (untreated) HAECs (Fig. 4B).

Effect of As(III) exposure on β -catenin localization

The cytoplasmic domain of VE-cadherin is linked with β -catenin, so it was of interest to determine whether As(III) affects β -catenin distribution. Exposure to As(III) for 6 h revealed the most damaging effects on the endothelial monolayer. Hence, the effect of As(III) treatment on β -catenin localization in HAECs was analyzed by immunofluorescence staining at the 6 h time point (Fig. 5). As previously observed with VE-cadherin localization, β -catenin staining showed a corresponding concentration-dependent effect with As(III) treatment. The frequency of gap formation with 10 μ M As(III) (Fig. 5D) was greater than 5 μ M As(III) (Fig. 5C) and only a few gaps were observed with 1 μ M As(III) treatment (Fig. 5B). Non-uniform distribution or loss of β -catenin staining was evident wherever gaps could be observed. Also, 250 nM Gö 6976 treatment inhibited the gap formation induced by 10 μ M As(III) (Fig. 5E) and restored the β -catenin staining at cell-cell junctions.

Effect of As(III) exposure on PY of VE-cadherin and β -catenin

The association of VE-cadherin with β -catenin is critical for the stability of AJ, and PY of either one or both the proteins can alter endothelial integrity. As(III)-induced disruption of the endothelial monolayer was evident starting at the 1 h time point. Therefore, the effect of As(III) exposure on PY of VE-cadherin and β -catenin was analyzed at 1 h by using immunoprecipitation followed by Western blotting. The phosphorylation of β -catenin was determined by probing the membrane with anti-phosphotyrosine monoclonal antibody following electrophoresis (Fig. 6A). The same membrane was stripped and total β -catenin protein content was analyzed (Fig. 6B). The PY levels were determined by normalizing with the total β -catenin protein content (Fig. 6C). A concentration-dependent effect of As(III) exposure on PY of β -catenin was observed. Treatment of cells with 1 μ M As(III) showed no change in PY as compared to the control (untreated), whereas an increase in PY was observed with 5 μ M As(III), and 10 μ M As(III) treatment resulted in a significant increase in PY of β -catenin. The increase in PY induced by 10 μ M As(III) was completely inhibited by 250 nM Gö 6976 (Fig. 6C).

No PY of VE-cadherin was observed (data not shown). Even a basal level of PY could not be detected. Increasing the concentrations of phosphatase inhibitors in the lysis buffer to confirm that VE-cadherin was not dephosphorylated during harvesting or immunoprecipitation did not yield any positive results. Analysis of the total VE-cadherin protein content established the presence of VE-cadherin and confirmed the adequacy of immunoprecipitation and Western blotting (data not shown).

Effect of As(III) exposure on the formation of stress fibers

The cadherin/ β -catenin complex is associated with the actin cytoskeleton, and this association is very important for maintaining junctional stability and structural integrity of the endothelial cell monolayer. As previously mentioned, reorganization of actin microfilaments into stress fibers can adversely affect the monolayer integrity. Hence, the effects of 1, 5 and 10 μ M As(III) on reorganization of actin microfilaments were analyzed at 1, 6, 12 and 24 h by immunofluorescence staining using fluorophore-conjugated phalloidin. A concentration and time-dependent effect of As(III) treatment was observed. At the 1 h time point (Fig. 7), As(III) induced a concentration-dependent increase in stress fibers with the maximum stress fibers being observed with 10 μ M As(III) treatment (Fig. 7D). Treatment with Gö 6976 abolished the stress fiber formation at all concentrations of As(III) treatment. The images of 250 nM Gö 6976 only treatment (Fig. 7E) and 10 μ M As(III) + 250 nM Gö 6976 treatment (Fig. 7F) are shown.

At the 6 h time point (Fig. 8), the frequency and density of stress fibers induced by As(III) was much higher than that observed at the 1 h time point. Also, a concentration-dependent increase in stress fibers was observed with the maximum stress fibers being induced with 10 μ M As(III) treatment (Fig. 8D). Treatment with Gö 6976 reduced the stress fibers at all concentrations of As(III) treatment. The images of 250 nM Gö 6976 only treatment (Fig. 8E) and 10 μ M As(III) + 250 nM Gö 6976 treatment (Fig. 8F) are shown. At the 12 and 24 h time points, similar concentration-dependent effects of As(III) treatment were observed (data not shown). Stress fiber formation, like the intercellular gap formation, was maximal at the 6 h time point.

Effect of As(III) on the activation of cPKCs

The translocation of PKC from cytosol to the membrane is an important step in its activation process (Newton, 2001). Therefore, the effect of As(III) on the activation of cPKCs was analyzed by separation of the cytosolic and membrane fractions and determining the protein content in each fraction by Western blotting technique. Preliminary experiments showed that 10 μ M As(III) treatment induced translocation of PKC α from the cytosolic fraction to the membrane fraction at 1 h, but not at earlier time points (data not shown). Also, As(III) induced damage to the endothelial monolayer was observed starting at 1 h. Hence, cells were exposed to 1, 5 and 10 μ M As(III) and cPKC activation was analyzed at the 1 h time point (Fig. 9). Treatment with 5 and 10 μ M As(III) induced a significant increase in PKC α activation as determined by quantifying the protein content in the membrane fraction (Fig. 9A and B). Conversely, a decrease was observed with 1 μ M As(III) treatment. In the cytosolic fraction (Fig. 9C and D), a decreasing trend in the PKC α content was observed with increasing As(III) concentration indicative of PKC α translocation from the cytosol to the membrane. An exact proportionate change in the cytosolic fraction was not evident compared to the membrane fraction, but overall, protein levels were much greater in the cytosolic fraction than the membrane fraction. Phorbol esters are very potent activators of PKCs (Newton, 2001) and hence phorbol 12-myristate 13-acetate (PMA) was used as a positive control. Treatment with 1 μ M PMA for 1 h induced a robust activation of PKC α (data not shown).

Effect of As(III) on endothelial permeability

Exposure to As(III) caused detrimental effects on the endothelial monolayer and induced reorganization of actin microfilaments into stress fibers. Therefore, we hypothesized that As(III) would also increase permeability. The effect of 1, 5 and 10 μ M As(III) treatment on endothelial permeability was analyzed at the 6 h time point (Fig. 10). Confluent monolayers of HAECs grown on Transwell inserts were treated with the various As(III) concentrations and the permeability of the monolayer for FITC-dextran was determined by measuring the fluorescence intensity of the medium in the plate well. A 5% increase in permeability was observed with 1 and 5 μ M As(III) treatment, whereas 10 μ M As(III) induced approximately a 19% increase. Treatment with 250 nM Gö 6976 inhibited the increase in permeability induced by 10 μ M As(III).

Discussion

The results from this study show that PKC α is a key mediator in a proposed mechanism for As(III)-induced atherosclerosis. The data showed that PKC α plays a crucial role in localization of the AJ proteins, VE-cadherin and β -catenin, and in regulating endothelial gap formation, stress fiber formation and vascular permeability. As(III) treatment induced intercellular gaps thereby disrupting the endothelial monolayer as evidenced by loss or non-uniform staining of VE-cadherin and β -catenin where the gaps appeared. Reduced VE-cadherin protein content was also detected; a finding that may in part be responsible for the observed intercellular gaps. An increased plasma concentration of VE-cadherin has been associated with coronary

atherosclerosis (Soeki et al., 2004), and *in vitro* studies have shown that hydrogen peroxide induces internalization of VE-cadherin and gap formation (Kevil et al., 1998). Hence, the decreased VE-cadherin may be due to internalization and degradation or release into the cell culture medium. β -catenin was found to be phosphorylated with 5 and 10 μ M As(III) treatments, but no PY of VE-cadherin was detected. There are several non-receptor tyrosine kinases such as Fyn, Fer, cMet, Abl and Src that have been shown to associate with and modulate the phosphorylation state of β -catenin and its association with cadherin (Lilien and Balsamo, 2005) during various developmental and physiological processes. In particular, phosphorylation of β -catenin at tyrosine 654 by Src has been shown to cause a fivefold reduction in its affinity for E-cadherin (Roura et al., 1999), which belongs to the same class of type I classical cadherins as VE-cadherin (Ivanov et al., 2001). The phosphorylation and dephosphorylation state of AJ proteins plays a critical role in the formation and/or maintenance of stable cell-cell adhesions (Lilien and Balsamo, 2005, Schnittler, 1998). Therefore, PY of β -catenin could potentially reduce its affinity for association with VE-cadherin and cause dissociation or loosening of the AJ.

Arsenic has been shown to increase calcium-dependent PKC activity in Chinese hamster ovary cells at high doses after 4 h treatment (Liu and Huang, 1997). Similarly, transient activation of PKC α was observed with 20 μ M arsenic treatment in a mouse epidermal cell line (Chen et al., 2000). Our results showed that 5 and 10 μ M As(III) treatment induced significant activation of PKC α at the 1 h time point. The downstream effects of As(III)-induced PKC α activation were confirmed by using the cPKC inhibitor, Gö 6976. Inhibition of PKC α prevented gap formation and restored VE-cadherin and β -catenin at the cell-cell junctions. PY of β -catenin induced by As(III) was also reduced to basal levels. The effects of activation of PKC α on endothelial integrity have been well documented (reviewed in Yuan, 2003). Activation of PKC α by thrombin has been shown to modulate endothelial integrity by induction of stress fibers and increased endothelial permeability (Sandoval et al., 2001). Also, PY of β -catenin has been observed that is PKC dependent (Cohen et al., 1999).

Formation of stress fibers induced by As(III) was also abolished or reduced with Gö 6976 treatment. Studies have demonstrated the relation between PKC α activation, stress fiber formation and cell contraction. It has been found that PKC α induces the activation of myosin by serine/threonine phosphorylation of myosin light chain which results in actin-myosin interaction and subsequent stress fiber formation and cell contraction (Tiruppathi et al., 2003). Therefore, PKC inhibition plausibly inhibited the contractile force exerted by the stress fibers. Confluent endothelial cells consist of a band of actin filaments concentrated along the cell borders known as a dense peripheral band (DPB) that is also present *in vivo* (Gabbiani et al., 1983, Schnittler, 1998). The cadherin-catenin complex associates with the actin filaments of DPB, which is important for junctional stability. Our results showed that actin filaments were present in untreated cells along the cell borders and were mostly absent in central regions of the cell. Treatment with As(III) caused the loss of DPB and an increase in stress fibers traversing the cells. The effect of As(III) was observed to be concentration and time-dependent with the maximum stress fibers being formed with 10 μ M As(III) at the 6 h time point. Actin reorganization associated with the disruption of endothelial barrier is characterized by increased stress fiber density and reduction or loss of DPB (Lum and Roebuck, 2001). Stress fibers have the molecular machinery to generate contractile force and have been associated with gap formation and increased endothelial permeability (Pellegrino et al., 2004, Yuan, 2003).

Vascular permeability changes are associated with loss of endothelial integrity. Chen et al., (2004) and Tsai et al., (2005) showed that As(III) alters endothelial permeability in rodents. Using FITC-dextran, we found a 5% increase in endothelial permeability with 1 and 5 μ M As

(III) and approximately a 19% increase with 10 μ M As(III) treatment that was inhibited by Gö 6976 treatment. These changes were not as great as expected based on the gap formation observed and actin stress fiber formation. However, Chen et al., (2004) and Tsai et al., (2005) have clearly demonstrated that even a small increase in permeability could be pathologically important. Taken together, these results suggest that As(III) induces PY of β -catenin, which modulates AJ assembly resulting in non-uniform distribution or loss of VE-cadherin as well as β -catenin at the cell-cell junctions and promotes the formation of stress fibers which culminates in intercellular gap formation and increased permeability.

While the results presented here show PKC α dependency for the effects of As(III) on endothelial cell-cell adhesion, monolayer integrity and vascular permeability, they do not suggest that As (III) effects on endothelium are restricted to the PKC α pathway. Other signal transduction pathways intersect with PKC α signaling. The PKC enzymes belong to the family of serine/threonine kinases, and it has been suggested that crosstalk exists between the tyrosine and serine/threonine kinase signaling cascades (Bauman and Scott, 2002, Chang et al., 2001). Involvement of tyrosine kinases introduces the possibility that vascular endothelial growth factor (VEGF), a key modulator of AJ stability that signals through a tyrosine kinase pathway, may be involved. VEGF has been implicated in arsenic-related toxicity through activation of PKC δ (Soucy et al., 2004). In addition, the VEGF receptor tyrosine kinases have been shown to signal with PKC α activation and downstream AKT signaling (Gliki et al., 2002). Moreover, VEGF signals through a Ras-MEK-ERK pathway independent of PKC α suggesting that not all As(III)-related effects may be PKC α dependent. Stress kinases such as p38 and c-Jun amino-terminal kinase (JNK) could also potentially be involved in the observed effects of As(III). Barchowsky et al., (1999) showed that high levels of As(III) can induce cellular stress and activate stress kinases including p38. On the other hand, lower concentrations have been shown to induce DNA synthesis and reactive oxygen species (Barchowsky et al., 1996) without activating stress kinases (Barchowsky et al., 1999). The role of these alternative pathways in endothelial arsenic toxicity is the subject for future investigations.

In summary, we have determined a potential mechanism for arsenic-induced atherosclerosis. This study demonstrates that As(III)-induced activation of PKC α causes PY of β -catenin and formation of stress fibers. PY of β -catenin may cause weakening of the AJ, and this effect, in association with the contractile force generated by stress fibers, results in gap formation and increased endothelial permeability. Proteins at the AJ are largely responsible for maintaining the barrier and normally allow passage of selected molecules. Injury to the endothelium can promote accumulation of oxidized low density lipoproteins (oxLDLs) and monocytes/macrophages in the intima of blood vessels (Lusis, 2000), ultimately leading to formation of foam cells. The intercellular gaps and increased permeability caused by arsenic could potentially accelerate/enhance influx of oxLDLs and monocytes/macrophages across the endothelium and contribute significantly to the progression of atherosclerosis.

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FIG 1.

Dose response of cPKC inhibitor Gö 6976. Confluent HAEC monolayers were either untreated (A) or treated with 10 μ M As(III) (B) for 1 h and the intercellular gaps (indicated with arrows) were visualized by immunofluorescence staining of VE-cadherin (green). The nuclei were stained with Hoechst (blue). Pretreating the cells with increasing concentration of Gö 6976 for 1 h prior to and during 10 μ M As(III) treatment inhibited the gap formation in a concentration-dependent manner: 10 μ M As(III) + 10 nM Gö 6976 (C), 10 μ M As(III) + 50 nM Gö 6976 (D), 10 μ M As(III) + 100 nM Gö 6976 (E), 10 μ M As(III) + 250 nM Gö 6976 (F), 10 μ M As(III) + 500 nM Gö 6976 (G) and 10 μ M As(III) + 1 μ M Gö 6976 (H). Magnification=60X. The images are representative of 3 independent experiments performed.

FIG 2.

Effect on VE-cadherin localization and intercellular gap formation with As(III) exposure for 1 h. Confluent HAEC monolayers were either untreated (A) or treated with 1 μ M As(III) (B), 5 μ M As(III) (C) and 10 μ M As(III) (D) for 1 h and the intercellular gaps (indicated with arrows) induced with As(III) treatment were visualized by immunofluorescence staining of VE-cadherin (green). The nuclei were stained with Hoechst (blue). Cleaved caspase-3 staining (green) for untreated (E) and 10 μ M As(III) (F) treated cells is indicated by arrowheads. Pretreatment with 250 nM Gö 6976 for 1 h prior to and during 10 μ M As(III) treatment (H) inhibited the As(III) induced gaps whereas 250 nM Gö 6976 only treatment (G) did not have

any effect on the endothelial monolayer. Magnification=60X. The images are representative of 4 independent experiments performed.

FIG 3.

Effect on VE-cadherin localization and intercellular gap formation with As(III) exposure for 6 h. Confluent HAEC monolayers were either untreated (A) or treated with 1 μ M As(III) (B), 5 μ M As(III) (C) and 10 μ M As(III) (D) for 6 h and the intercellular gaps (indicated with arrows) induced with As(III) treatment were visualized by immunofluorescence staining of VE-cadherin (green). The nuclei were stained with Hoechst (blue). Cleaved caspase-3 staining (green) for untreated (E) and 10 μ M As(III) (F) treated cells is indicated by arrowheads. Pretreatment with 250 nM Gö 6976 for 1 h prior to and during 10 μ M As(III) treatment (H) inhibited the As(III) induced gaps whereas 250 nM Gö 6976 only treatment (G) did not have

any effect on the endothelial monolayer. Magnification=60X. The images are representative of 4 independent experiments performed.

FIG 4.

Effect of As(III) treatment on VE-cadherin protein levels. Confluent HAECs were treated with 1, 5 and 10 μM As(III) for 24 h and VE-cadherin protein content was analyzed by Western blotting technique. The VE-cadherin protein content is shown in the (A) upper panel and the lower panel represents the β -actin loading control. The images in (A) are representative of 7 experiments performed. The percent change in VE-cadherin protein relative to the control (untreated) is represented in (B). The control (100%) is indicated by the dotted line in (B). Error bars represent mean $\pm$ SE for $n=7$.

FIG 5. Effect of As(III) on β -catenin localization. Confluent HAECs were either untreated (A) or treated with 1 μ M As(III) (B), 5 μ M As(III) (C) and 10 μ M As(III) (D) for 6 h and β -catenin localization was visualized by immunofluorescence staining (green). Non-uniform staining or loss of β -catenin was observed wherever gaps occurred (indicated by arrows). Pretreatment with 250 nM Gö 6976 for 1 h prior to and during 10 μ M As(III) treatment (E) restored the β -catenin localization at cell-cell junctions. The nuclei were stained with Hoechst (blue). Magnification=60X. The images are representative of 3 independent experiments performed.

FIG 6.

Effect of As(III) on PY of β -catenin. Confluent HAECs were treated with 1, 5, and 10 μM As (III) for 1 h or pretreated with 250 nM Gö 6976 for 1 h prior to and during 10 μM As(III) treatment. The β -catenin was immunoprecipitated, and Western blotting technique was used to analyze the phosphorylation of tyrosine residues (A). The membrane was stripped and reprobed to determine the total β -catenin protein content (B). The images A and B are representative of 4-6 independent experiments performed. The percent change in PY with the various treatments relative to the control (untreated) is represented in (C). The control (100%) is indicated by the dotted line. Error bars represent mean $\pm$ SE for $n=4-6$. * and # indicates significantly different from control and 10 μM As(III), respectively, with $p < 0.01$.

FIG 7.

Effect of 1 h As(III) exposure on the formation of stress fibers. Confluent HAEC monolayers were either untreated (A) or treated with As(III); 1 μ M (B), 5 μ M (C) and 10 μ M (D) for 1 h, and the stress fibers induced with As(III) treatment were visualized by immunofluorescence staining with Alexa Fluor 568 conjugated phalloidin (red). Pretreatment with 250 nM Gö 6976 for 1 h prior to and during 10 μ M As(III) treatment (F) abolished the As(III) induced stress fibers whereas 250 nM Gö 6976 only treatment (E) did not have an effect on stress fiber formation. Magnification=60X. The images are representative of 4 independent experiments performed.

FIG 8.

Effect of 6 h As(III) exposure on the formation of stress fibers. Confluent HAEC monolayers were either untreated (A) or treated with As(III); 1 μ M (B), 5 μ M (C) and 10 μ M (D) for 6 h and the stress fibers induced with As(III) treatment were visualized by immunofluorescence staining with Alexa Fluor 568 conjugated phalloidin (red). Pretreatment with 250 nM Gö 6976 for 1 h prior to and during 10 μ M As(III) treatment (F) reduced the As(III) induced stress fibers whereas 250 nM Gö 6976 only treatment (E) did not have an effect on stress fiber formation. Magnification=60X. The images are representative of 4 independent experiments performed.

FIG 9.

Effect of As(III) on the activation of PKC α . Confluent HAECs were treated with 1, 5, and 10 μM As(III) for 1 h. The lysates were subjected to ultracentrifugation to separate the membrane and cytosolic fractions and the PKC α levels in both fractions were determined by Western blotting. The PKC α content in the membrane fraction is shown in (A) upper panel and the lower panel represents the Na⁺/K⁺ ATPase loading control for the membrane fraction. The percent change in PKC α content in the membrane fraction relative to the control (untreated) is represented in (B). The PKC α content in the cytosolic fraction is shown in (C) upper panel and the lower panel represents the GAPDH loading control for the cytosolic fraction. The percent change in PKC α content in the cytosolic fraction relative to the control is represented in (D). The images in (A) and (C) are representative of 3-6 independent experiments performed. The control (100%) is indicated by the dotted line in (B) and (D). Error bars represent mean $\pm$

SE for n=3-6. #, * and ** indicates significantly different from control with $p < 0.01$, 0.05 and 0.001, respectively.

FIG 10.

Effect of As(III) on endothelial permeability. Confluent HAECs were treated with 1, 5, and 10 μM As(III) for 6 h or pretreated with 250 nM Gö 6976 for 1 h prior to and during 10 μM As(III) treatment. Monolayer permeability was measured using FITC-dextran. The control (100%) is indicated by the dotted line. Error bars represent mean $\pm$ SE for $n=4-5$.