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Positive Signaling Interactions between Arsenic and Ethanol for Angiogenic Gene Induction in Human Microvascular Endothelial Cells

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Arsenic in the drinking water may promote vascular diseases in millions of people worldwide through unresolved mechanisms. In addition, little is known of the effects of coexposures to arsenic and other common vasculature toxicants, such as alcohol. To investigate signaling interactions between arsenic and alcohols, primary human microvascular endothelial (HMVEC) cells were exposed to noncytotoxic concentrations of arsenite (1–5μM) in the presence or absence of 0.1% ethanol (EtOH). Coexposure, but not exposure to either agent alone, rapidly increased active Fyn tyrosine kinase, tyrosine phosphorylation of a 109-kDa protein and serine phosphorylation of protein kinase C (PKC)δ. The 109-kDa protein was identified as PYK2, a regulator of vascular integrin signaling and an upstream activator of PKCδ. Membrane localization of phospholipase Cγ1 was increased by coexposure within 15 min, but not by either agent alone. In contrast, both agents equally increased membrane localization of Rac1–GTPase. Coexposure, but not exposure to either agent alone, induced transcript levels for the angiogenic genes, vascular endothelial cell growth factor (*Vegfa*) and insulin-like growth factor-1 (*Igf1*). However, EtOH inhibited arsenic-induced, nuclear factor-κB–driven interleukin-8 and collagen-1 expression. Differential effects of selective PKC inhibitors on induced gene expression combined with a lack of interaction for induction of hemeoxygenase-1 further demonstrated that arsenic-responsive signaling pathways differ in sensitivity to EtOH interactions. Finally, coexposure enhanced endothelial tube formation in *in vitro* angiogenesis assays. These data indicate that complex interactions occur between arsenic and EtOH exposures that functionally affect endothelial signaling for gene induction and remodeling stimuli.

Key Words: **arsenic; alcohol; endothelial; angiogenesis; cell signaling.**

Low to moderate exposures (10–200 ppb) of arsenic, especially trivalent arsenite, in drinking water may promote vascular diseases (Navas-Acien *et al*., 2005; Wang *et al*., 2007) and hypertension (Chen *et al*., 2007; Hsueh *et al*., 2005) in millions of exposed people worldwide. Animal studies revealed that drinking water containing levels of arsenic as low as 5 ppb increase inflammatory angiogenesis (Kamat *et al*., 2005; Soucy *et al*., 2005), tumor vascularization (Kamat *et al*., 2005; Soucy *et al*., 2003), and remodeling of the liver sinusoidal endothelium (Straub *et al*., 2007a, b). High exposures (> 1 ppm) enhance atherogenesis and loss of endothelial cell barrier function in susceptible genetic mouse models (Bunderson *et al*., 2004) and offspring of exposed mothers (Srivastava *et al*., 2007). Numerous studies in both vascular endothelial (Barchowsky *et al*., 1996, 1999b; Smith *et al*., 2001; Tsou *et al*., 2003) and smooth muscle (M. Y. Lee *et al*., 2005; P. C. Lee *et al*., 2005; Lynn *et al*., 2000; Soucy *et al*., 2004) cell cultures demonstrated that low μM or sub-μM concentrations of arsenic directly activate signaling, transcriptional activity, and gene expression. A substantial portion of this cellular activation involves stimulation of enzymes that generate reactive oxygen and nitrogen species (Barchowsky *et al*., 1999a; Bunderson *et al*., 2002; Lynn *et al*., 2000; Smith *et al*., 2001), but the molecular triggers that initiate arsenic-activated cell signaling remain unidentified.

It is unlikely that environmental exposure to arsenic occurs without exposure to other environmental or dietary xenobiotics that may influence the molecular actions of arsenic. While there are no prospective epidemiological studies that examine the health effects of their interactions, toxic coexposures to arsenic and alcohols have been documented to pose a significant cardiovascular health risk. Several past epidemics and case reports indicated that coexposures to arsenic and alcohols caused cardiovascular and liver diseases (Engel *et al*., 1994; Gerhardt *et al*., 1980; Klatsky, 2002; Narang *et al*., 1987). In England during the early 1900s, there were over 6000 cases and 70 deaths from heart disease attributed to drinking arsenic-contaminated beer (Klatsky, 2002). Peripheral vascular disease and cardiomyopathies were seen in a group of German vintners in the 1920s who drank wine fermented from grapes treated with arsenical fungicides (Engel *et al*., 1994). In the United States, arsenic-contaminated moonshine was implicated as the cause of a dozen cases of cardiovascular disease in Georgia (Gerhardt *et al*., 1980). Narang *et al*. (1987) found increased arsenic exposure in a group of patients suffering from

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alcoholic-related liver disease who had consumed a homemade alcoholic mixture. Epidemiological studies of health effects in large populations drinking arsenic-contaminated water often exclude individuals who drink alcohol to limit confounding data (Mazumder, 2005; von Ehrenstein *et al*., 2005). However, several human studies have demonstrated that alcohol consumption alters arsenic kinetics to favor higher levels of inorganic and monomethyl arsenic (Chiou *et al*., 1995; Hopenhayn-Rich *et al*., 1996; Hsueh *et al*., 2003; Tseng *et al*., 2005). Animal studies using EtOH as a coexposure demonstrated that EtOH affects arsenic kinetics by increasing its uptake and retention in the liver and kidneys (Flora *et al*., 1997). This increased accumulation was associated with increased liver pathology from very high arsenic levels (100 ppm) and 10% EtOH (Flora *et al*., 1997). Thus, significant cellular and molecular interactions between arsenicals and alcohols are likely to occur in the vasculature following coexposure to these two common environmental toxicants.

Similar to the effects of arsenic, low to moderate levels of alcohol promote angiogenesis, while high concentrations are toxic to blood vessels (Burns and Wilson, 2003; Gu *et al*., 2001). Alcohol-induced angiogenesis may be protective in certain cardiovascular diseases (Gu *et al*., 2001), but excess alcohol-induced angiogenic potential has also been linked to increased tumorigenesis (Gu *et al*., 2005). Alcohol-induced angiogenesis is promoted by induction of VEGF and other proteins in the angiogenic program (Burns and Wilson, 2003; Gavin and Wagner, 2002; Gu *et al*., 2001). Similarities between arsenic and alcohol-induced angiogenesis extend beyond gene induction to possible similarities in cell signaling and stimulation of cytoskeletal changes that support endothelial cell migration (Qian *et al*., 2003, 2005). Despite these similarities in signaling potential, the interactions between these two toxicants at the level of functional signaling and gene expression in a relevant target cell have not been reported.

The following experiments were designed to test the hypothesis that coexposure to arsenite and EtOH produces positive signaling interactions that increase angiogenesis in human microvascular endothelial cells. The results demonstrate that the two agents interact positively at proximal steps in signaling cascades to induce of a number of angiogenic genes, including vascular endothelial cell growth factor (*Vegfa*) and insulin-like growth factor-1 (*Igf1*). However, the studies also reveal negative interactions in certain downstream signaling events and no interactions for induction of stress response genes. Functionally, the interactions between arsenic and EtOH lead to enhanced tube formation in an *in vitro* assay that models the initial steps in angiogenesis. Although these acute model studies in cultured vascular cells are limited in their ability to demonstrate pathogenic mechanisms for chronic vascular diseases, they do suggest that noncytotoxic arsenic exposures interact with other vascular toxicants to enhance signaling for inappropriate vascular remodeling.

MATERIALS AND METHODS

Cells and cell culture. Human lung microvascular endothelial cells (HMVEC; Lonza, Walkersville, MD) were cultured essentially according to the supplier's instructions at 5% CO_2 in complete MCDB 131 medium (MCDB 131; Invitrogen, Carlsbad, CA) supplemented with EGM-2 MV (SingleQuot, Lonza, Walkersville, MD). At confluence, the growth factors were reduced by a 1:5 dilution of complete medium with basal MCDB 131 and all experiments were conducted 24 h after placing the cells in reduced medium. All experiments used additions of sodium arsenite and EtOH to model common arsenic and alcohol exposures, respectively. In coexposure experiments, 0.1% EtOH was added to the culture medium 30 min prior to adding 1 or 5μM arsenite. In 24-h experiments, 0.1% EtOH was added at 12-h intervals to compensate for evaporation from the warm medium. In protein kinase C (PKC) inhibitor studies, either Gö6976 or GF109203x (EMD Bioscience, La Jolla, CA) were added 30 min prior to adding arsenite and/or EtOH.

XTT cell viability assay. Cell viability was measured using the XTT assay. HMVEC cells were grown to confluence in 96-well plates. The cells were then incubated with arsenite or EtOH for 24 h before removing the medium and incubating the cells with phenol free-basal EGM media (Lonza) containing XTT substrate solution. After 2 h, the amount of soluble formazan formed was measured spectrophotometrically at 450–650 nm and compared to the blank well containing XTT solution with no cells.

Membrane protein isolation. One day postconfluent cells grown in T-25 flasks were treated with appropriate agents and then rinsed twice with cold stop buffer (10mM Tris–HCl, pH 7.4, 10mM ethylenediaminetetraacetic acid [EDTA], 5mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 0.1M NaF, 0.20M sucrose, 100μM Na-orthovanadate, 5mM pyrophosphate). Cells were then scraped into membrane lysis buffer (20mM KH_2PO_4, pH 7.0, 1mM EDTA, plus protease inhibitor cocktail; EMD, La Jolla, CA) and Dounce homogenized with 160 strokes. Lysed cells were then spun at 29,000 g at 4°C for 30 min. Supernatant containing cytosolic proteins was removed and saved. The remaining pellet was washed with lysis buffer and spun at 29,000 × g at 4°C for 30 min. After removing the supernatant, membrane buffer (0.1M KH_2PO_4, 0.1M Na_2HPO_4, 0.025mM EDTA, 150mM sucrose, 5 mg/ml CHAPS, and protease inhibitor cocktail) was added and the pellet was solublized in a Dounce homogenizer. Protein content was assayed using Pierce Commassie Plus reagent according to the manufacturer's instructions and equal amounts of protein were separated by polyacrylamide gel electrophoresis (PAGE).

Western analysis. Western analyses for changes in protein abundance or phosphorylation state were performed, as previously described (Barchowsky *et al*., 1999b; Smith *et al*., 2001; Soucy *et al*., 2004). Antibodies used for immunoblotting included: anti-PLCγ1 (anti-phospholipase γ1) and anti-Fyn from Upstate/Millipore (Billerica, MA); anti-Rac1 and anti-pTyr-20-HRP from BD Biosciences (Bedford, MA); anti-pPKCδ (Thr505), anti-pSrc (Tyr 416), and anti-pPYK2 (Tyr 402) from Cell Signaling Technologies (Danvers, MA); anti-PKCδ from SantaCruz Biotechnology, Inc. (Santa Cruz, CA); and anti-b-actin (Sigma, St Louis, MO). Reacted bands were detected by horseradish peroxidase conjugated secondary antibodies and enhanced chemiluminescence substrates (Perkin Elmer, Boston, MA). Band densities were quantified using Image J software v1.38x (National Institutes of Health, Bethesda, MD). Band densities of phosphorylated proteins were normalized to the densities of the corresponding total protein or β-actin and densities for translocated proteins were normalized to β-actin.

Quantitative reverse transcription-PCR. Total RNA was harvested using Trizol and assayed for messenger RNA (mRNA) levels of VEGF, interleukin (IL)-8, collagen-1 (Col-1), and the housekeeping gene RPL13A, as previously described (Gao *et al*., 2004). Specific primer pairs used to amplify the specific complementary DNAs (cDNAs) included: VEGF forward 5′-CTTGCCTTG-CTGCTCTACCT-3′, reverse 5′-GCA AGG CCC ACA GGG ATT TT-3′; IL-8 forward 5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′, reverse 5′-TCTCA GCCCTCTTCAAAAACTTCTC-3′; Col-1 forward 5′-AGTGGTTACTACTG-GATTGACC-3′, reverse 5′-TTGCCAGTCTCCTCATCC-3′; RPL13A forward

5′-CGAGGTTGGCTGGAAGTACC-3′, reverse 5′-ATTCCAGGGCAACA-ATGGAG-3′and HO-1 forward 5′-CCAGCAACAAAGTGCAAGATTC-3′, reverse 5′-CTGCAGGAACTGAGGATGCTG-3′. QuantiTect Primer assay (Qiagen) was used to detect IGF-1 mRNA changes relative to RPL13A mRNA. Gene transcripts were quantified by realtime PCR in an Opticon 2 (Biorad) thermocycler using Sybr greener master mix (Invitrogen). The amount of cDNA product was compared to amplified standard cDNA and normalized to changes in RPL13A cDNA levels to determine the pg of normalized product per ml of PCR reaction.

Matrigel angiogenic tube formation assays. *In vitro* endothelial cell tube formation in three dimensional Matrigel cultures was used as a surrogate assay for angiogenic potential. Cells were cultured in reduced growth factor medium for 24 h before release with trypsin and replating onto 100-μl Matrigel cushions in eight well Labtec chamber slides (ThermoFisher, Pittsburgh, PA). A total 5000 cells in 100 μl of reduced medium were added onto each gel and allowed to equilibrate for 1 h before exposure to arsenite or EtOH. After 4 h of exposure, the cultures were fixed with 2% paraformaldehyde and then dried. After permeabilization with 0.1% Triton X-100, actin filaments were stained with rhodamine–phalloidin (Molecular Probes, Invitrogen). At least two images per gel were captured at 40× using a Nikon Microphot microscope equipped with an Olympus CCD camera and MagniFire v5.0 software. The number of tube branch points, an index of branching morphogenesis in the angiogenic process, was counted with the aid of MetaMorph v.7.0 software.

Statistics. One- or two-way analysis of variance (ANOVA) was used to identify significant differences ($p < 0.05$) between treatment groups and controls. The degree of significance between groups was then compared using Bonferroni posttest for multiple comparisons. All statistics were performed using GraphPad Prism, v4.0 software (GraphPad Software, San Diego, CA). Data are presented as means ± SEM of quantified values or fold relevant control.

RESULTS

Cell Viability

Metabolic activity assays using the tetrazolium XTT as a mitochondrial dehydrogenase substrate were used to demonstrate that the levels of exposure to arsenite and EtOH used in the studies were not cytotoxic. As seen in Figure 1, relative to the known cytotoxic agent staurosporine, exposure to either agent or coexposure to both agents for 24 h had no effect on cell viability. Shorter exposures to either agent or shorter coexposures showed no signs of toxicity (data not shown). These data indicate that effects on cell signaling, gene expression, and cell function observed in the following figures were not due to cytotoxic signaling.

Positive Interactions of Coexposures on Phosphorylation of Kinases

Cellular signal amplification in response to environmental stimuli involves sequential activation of kinase and GTPase cascades. Earlier studies demonstrated that exposure to arsenite stimulates Src family kinases (SFK) in endothelial cells (Barchowsky *et al.*, 1999b) and stimulates PKCδ to induce VEGF expression in smooth muscle cells (Soucy *et al.*, 2004). Therefore, the hypothesis that these signaling cascades were points of interactions in HMVEC coexposed to arsenite and EtOH was investigated. As shown in Figure 2, 15-min exposure to either arsenite or EtOH did not change the amount of activated, phosphorylated SFK or PKCδ, relative to the total amount of either protein (quantitation shown in Supplemental Fig. 1). After 4 h of exposure, EtOH alone increased phosphorylation of both kinases. In contrast, coexposure to arsenite and EtOH increased protein phosphorylation within 15 min of arsenite addition and the activated, phosphorylated states of the kinases were sustained for 4 h (Fig. 2). The activated SFK was identified as the 47-kDa isoform of Fyn based colocalization with anti-Fyn antibody binding and lack of binding of antibodies to the two other major endothelial SFK, Src, and Yes. In addition to PKC and Fyn, the tyrosine phosphorylation of a 109-kDa protein followed the same activation pattern (Fig. 2). This protein was identified as the SFK substrate and PKC regulator, Pyk2 and coexposure increased Pyk2 tyrosine402 phosphorylation (Fig. 2).

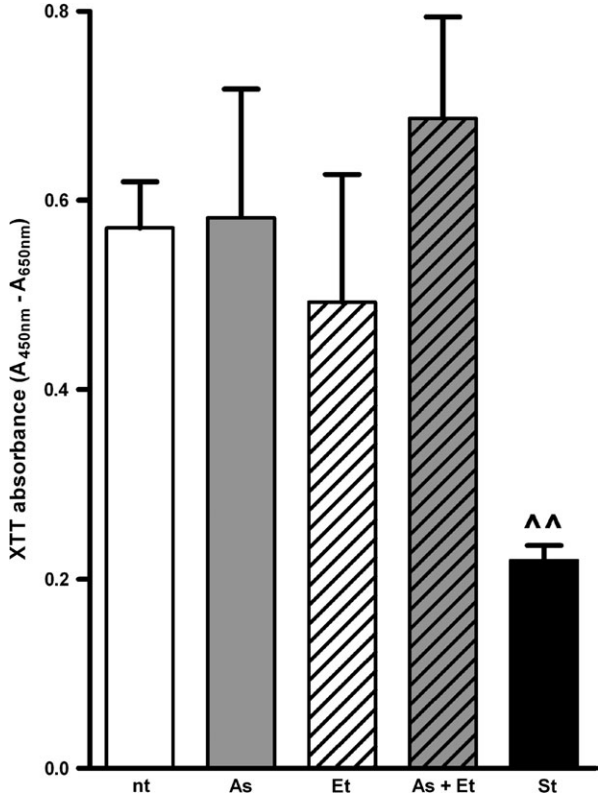

FIG. 1. Lack of toxicity of low level arsenic and/or ethanol exposures. Confluent HMVEC cells were left untreated or incubated with 0.1% EtOH for 30 min before incubating with or without 5.0μM arsenite for 24 h. To account for evaporation, EtOH was added twice during the exposure period. Control cells were left untreated (nt) or incubated for 2 h with a cytotoxic concentration (0.5μM) of staurosporin (St). At the end of the exposure period, the medium was changed to XTT substrate solution for 2 h and then the amount of formazan product formed was measured spectroscopically. Data are reported as mean ± SEM of product generated ($n = 4$). The data were analyzed for significant differences using one-way ANOVA followed by Bonferroni's posttest for multiple comparisons. ^^Designates reduction of mitochondrial dehydrogenase activity relative to activity in nontreated control cultures ($p < 0.01$).

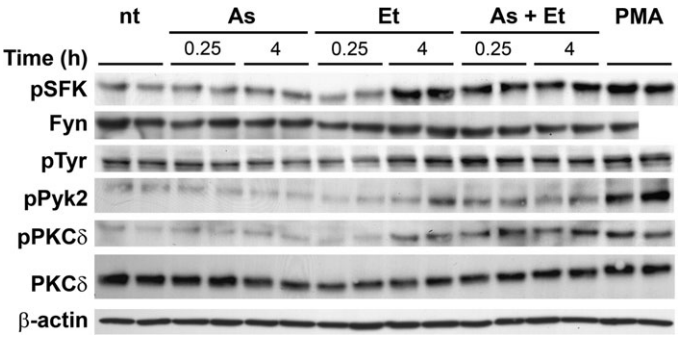

FIG. 2. Enhanced phosphorylation of kinases following coexposure to arsenite and EtOH. Cells were left untreated, exposed to 5.0μM arsenite (As), exposed to 0.1% EtOH (Et), or coexposed to arsenite and EtOH for either 15 min or 4 h. In coexposures, EtOH was added 30 min prior to arsenite. As a positive control, 20nM phorbol myristate acetate (PMA) was added to cells for 15 min. Total protein was isolated at the end of the exposure period and separated by PAGE. The images show the results of Western analysis for: Activated, tyr416 phosphorylated SFK; total Fyn protein; tyrosine phosphorylation of a 109-kDa protein (pTyr); activated, tyr402 phosphorylated Pyk2; activated, thr505 phosphorylated PKCδ; total PKCδ, and β-actin. Densitometric analysis and comparison of phosphorylation to total protein changes are presented in supplemental data. All lanes were proteins from separate cell cultures and representative of three experiments.

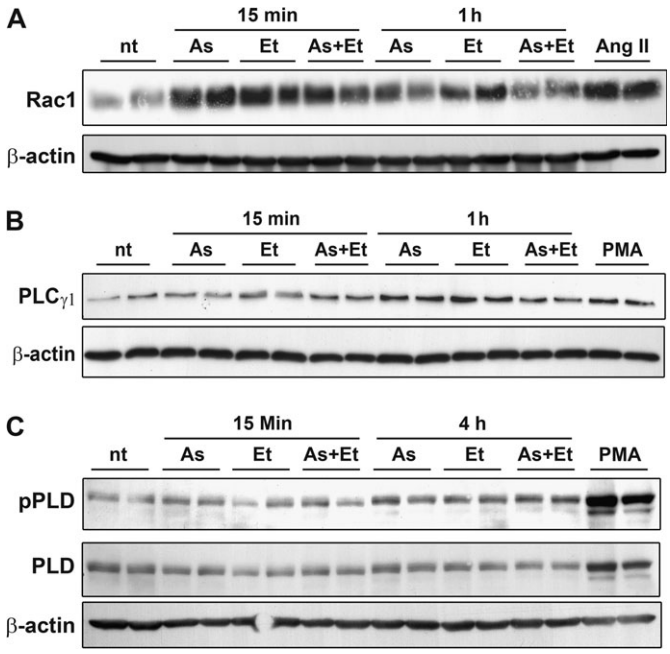

FIG. 3. Lack of interaction from coexposures on downstream signaling enzymes. In (A) and (B), cells were incubated for 15 min or 1 h with 5.0μM arsenite (As), 0.1% EtOH (Et), or arsenite/EtOH before total protein was isolated and separated into cytosolic and membrane fractions. The images show immunoblots of membrane associated Rac1 and PLCγ proteins as an index of their activation and translocation. The images in (C) present the amount of activated, ser561 phosphorylated PLD and total PLD, relative to β-actin, in total cell lysates. Fifteen min exposures to 100nM angiotensin II (Ang II) or 20nM PMA were used as positive controls for Rac1 or phospholipase activation, respectively. Densitometric analysis and comparison of membrane-bound or phosphorylated protein relative to total protein changes are presented in supplemental data. All lanes were proteins from separate cell cultures and representative of three experiments.

Varied Temporal Activation of Proximal Signaling Enzymes by Arsenite and EtOH

Signal amplification is mediated by membrane translocation of enzymes that generate second messengers, such as lipids and reactive oxygen species. In keeping with the posttranslational modifications seen in Figure 2, membrane localization of PLCγ was not increased within 15 min by either arsenite or EtOH (Fig. 3B). Coexposure increased membrane PLCγ by approximately 1.5-fold within 15 min (quantitation shown in Supplemental Fig. 2). In contrast, when added alone for 1 h, both agents increased membrane PLCγ, but membrane PLCγ levels were no different than basal levels following a 1-h coexposure (Fig. 3B). Membrane translocation and activity of monomeric GTPases, such as Rac1 and Cdc42 (Qian *et al*., 2005; Smith *et al*., 2001), is required for arsenic-stimulated superoxide generation by nicotinamide dinucleotide phosphate (reduced) (NADPH) oxidase. In contrast to their effects on kinases and other signaling enzymes, arsenite, EtOH, or coexposure equally promoted translocation of Rac1 to the membrane fraction of the HMVEC within 15 min (Fig. 3A; quantitation shown in Supplemental Fig. 2). In other experiments, arsenite-stimulated translocation of Rac1 correlated with increased GTPase activity of the enzyme (data not shown). The responses to the three exposures were equally diminished at 1 h relative to the 15-min response. This is consistent with the transient time course reported for arsenic-stimulated NADPH oxidase activity in endothelial cells (Smith *et al*., 2001). Finally, delayed serine phosphorylation of phospholipase D (PLD), which is known to be downstream of PKC activation (Brizuela *et al*., 2007; Hoek *et al*., 1992), was slightly elevated at both time points by arsenite and EtOH. A positive interaction, however, was observed with a 4 h coexposure (Fig. 3C).

Differential Interactions of Arsenite and EtOH at the Level of Angiogenic Gene Induction

As mentioned in the introduction, arsenite and EtOH both stimulate angiogenesis and vascular remodeling, as well as angiogenic gene induction. In contrast to earlier observations in smooth muscle cells (Soucy *et al*., 2004), the data in Figure 4 indicate that 4h exposures to arsenite (1 or 5μM) alone does not increase VEGF transcript levels in HMVEC cultures. EtOH (0.1%) was also ineffective in inducing VEGF. However, coexposures produced two- to threefold increases in VEGF mRNA levels that were not dependent on the concentration of arsenite in this range. A similar positive interaction was seen with increases in IGF-1 mRNA levels. In contrast, negative interactions were seen for induction of *IL8* and *Col1*, two nuclear factor kappa B (NF-κB)–driven genes. *IL8* induction was responsive to the concentration of arsenite, whereas no *IL8*

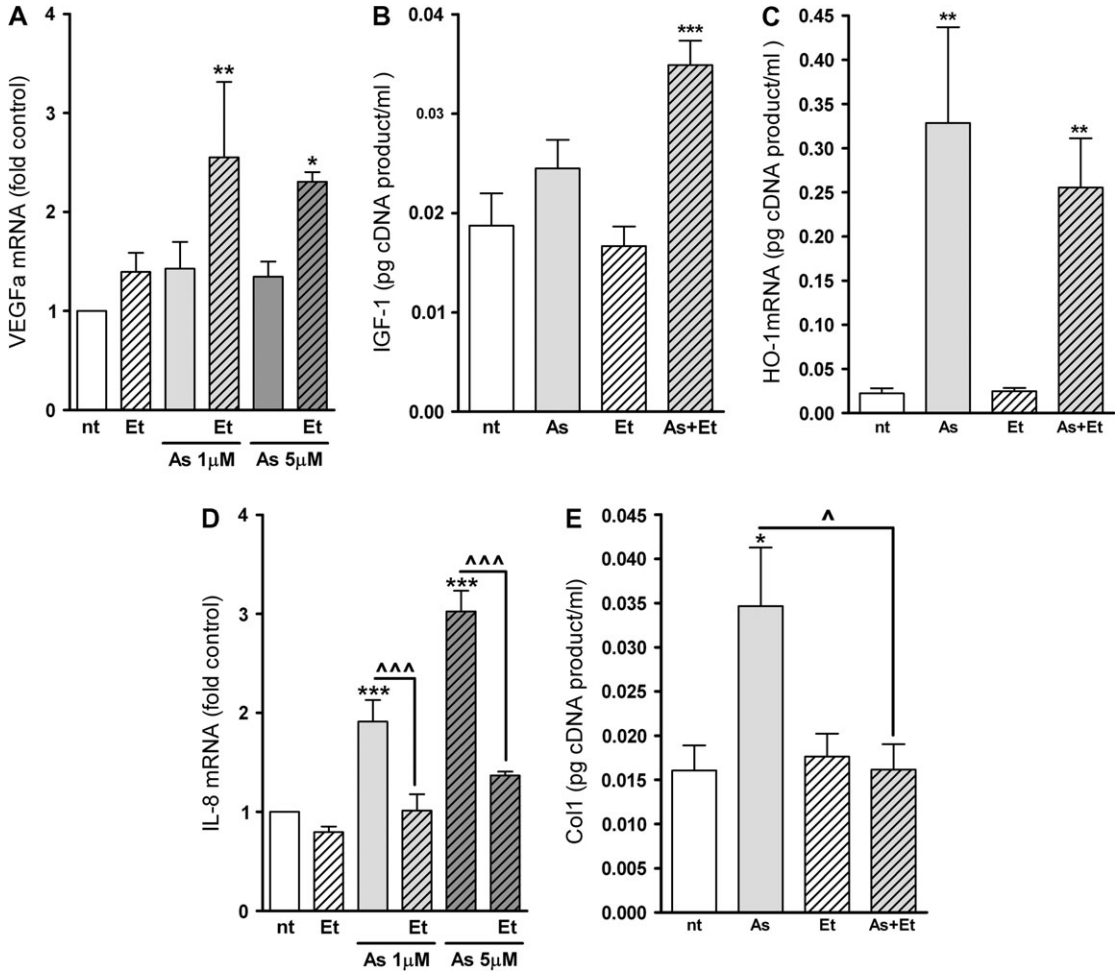

FIG. 4. Differential effects of coexposure to arsenite and EtOH on arsenite-stimulated gene transcription. Total RNA was isolated from cells exposed to 1 ($n = 6$) or 5 ($n = 3$) μM arsenite (As), 0.1% EtOH (Et), or arsenite/EtOH for 4 h. mRNA was reverse transcribed, amplified by PCR, and the amount of product was quantified as described in methods. All values obtained for the indicated gene transcripts were normalized to values obtained for transcripts of the housekeeping gene *RPL13A*. The data in (A) and (C) are presented as mean ± SEM for fold change of treated of treated samples relative to control in order to compare separate experiments that used 1 or 5μM arsenite. The data in (B), (D), and (E) were generated using 1 μM arsenite and are presented as the mean ± SEM abundance of the indicated cDNA product normalized to the abundance of RPL13A transcripts. The data were analyzed for significant differences using one-way ANOVA followed by Bonferroni's posttest for multiple comparisons. Significant increases relative to control are designated by *,** or *** for $p < 0.05$,0.01 or 0.001 respectively. Significant differences from arsenite-stimulated transcript levels are designated by ^and ^^^for $p < 0.05$ or 0.001, respectively ($n = 3$).

induction occurred in control or arsenic-exposed cells that were treated with EtOH (Fig. 4). Similarly, EtOH inhibited arsenite-induced Col1 mRNA levels. Finally, EtOH did not affect basal or arsenite-induced hemeoxygenase-1 (HMOX1) mRNA levels (Fig. 4).

Differential Effects of PKC Inhibitors on VEGF and IL-8 Gene Expression

Because PKC activation appeared to be a main point of interaction (Fig. 2), we examined the effect of selective PKC inhibitors on the positive and negative interactions between 1μM arsenite and 0.1% EtOH on changes in VEGF and IL-8 mRNA levels. Both Gö6976, an inhibitor of classic PKC isoforms, and GF109203x, a preferential inhibitor of novel PKC isoforms, prevented arsenite and EtOH coexposures from inducing VEGF mRNA levels (Fig. 5). In contrast, GF109203x, but not Gö6976, inhibited arsenite-induced IL-8 mRNA levels (Fig. 5). These data suggested that a novel PKC, such as PKCδ, mediated arsenite-induced IL-8 expression and that activation of multiple PKCs were involved in the effect of coexposure on *VEGFa* transcripts. Neither PKC inhibitor affected arsenite-induced levels of HO-1 mRNA levels (data not shown) indicating independence from PKC signaling.

Effect of Coexposures on Angiogenic Potential

The functional effect of arsenite and EtOH coexposures on angiogenic potential was tested in three dimensional Matrigel tube forming assays. In these assays, the number of tube branch

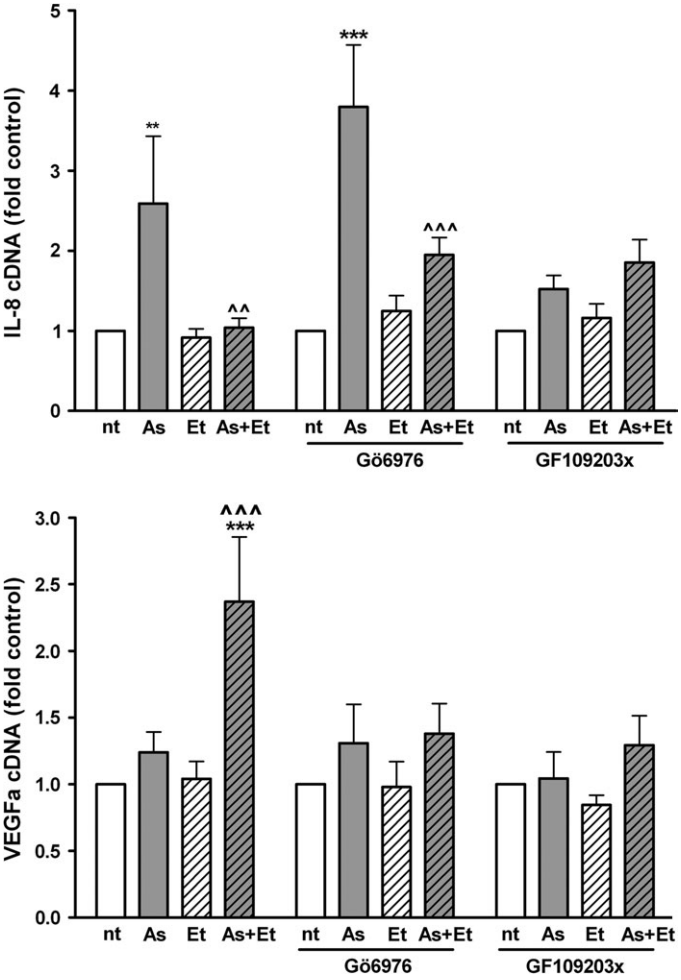

FIG. 5. Effect of selective PKC inhibition on arsenite and EtOH induced VEGF and IL-8 mRNA levels. Cells were left untreated or treated for 30 min with 0.5μM of either a selective inhibitor of classic PKCs (Gö6976) or an inhibitor of novel PKCs (GF19203x). The cells were then exposed for 4 h to 1.0μM arsenite and 0.1% EtOH, alone or in combination. Total RNA was isolated an analyzed by quantitative reverse transcription PCR for VEGF, IL-8, or RPL13A transcripts. Data are presented as the mean ± SEM of the fold increase of the indicated cDNA product normalized to amplified RPL13A transcripts relative to basal transcript levels ($n = 4$). Statistical analysis was made by comparing the effects of arsenite and EtOH within the different pretreatment groups. The data were analyzed for significant differences using two-way ANOVA followed by Bonferroni's posttest for multiple comparisons. Significant increases from the respective control group are designated by **, or *** for $p <$ 0.01, or 0.001, respectively. Significant differences from the arsenite exposures are designated by ^^ or ^^^for $p <$ 0.01, or 0.001, respectively.

points was enumerated as an angiogenic index of branching morphogenesis. Arsenite was added at a concentration of 1μM to reduce toxicity in the subconfluent cultures as the angiogenic assays are generally inhibited above this exposure level (Barchowsky *et al*., 1996; Kao *et al*., 2003; Soucy *et al*., 2003). As seen in Figure 6, arsenite, but not EtOH, increased tube formation and branching relative to control. Coexposure to both arsenite and EtOH further increased the response relative to arsenite alone. The Matrigel assay was extremely sensitive to arsenite, since similar responses were seen with 10 fold reductions in both arsenite and EtOH concentrations (data not shown).

DISCUSSION

The signaling interactions between arsenic and other environmental contaminants or exposures have not been extensively investigated, despite case reports and reports from epidemics that such coexposures pose significant cardiovascular health risks. The current study investigated the effects of arsenic and EtOH coexposures in microvascular endothelial cells to provide mechanistic evidence of pathogenic interactions. In these studies, noncytotoxic concentrations of arsenite and EtOH were used to reveal signaling relevant to common exposures and disease promotion, instead of tissue injury. The data are the first to demonstrate that these two agents interact at the level of kinase signaling cascades to regulate expression of inflammatory and vascular remodeling genes. Further, the data in Figure 6 indicate that these changes in cell signaling produce functional increases in endothelial cell angiogenic potential, which is known to contribute to vascular disease and tumorigenesis.

The data in Figure 2 suggest that positive interactions between arsenite and EtOH occurred early in cell kinase activation cascades. A major finding was that coexposure caused rapid increases in the active phosphorylation state of Fyn, Pyk2, and PKCδ. The effect of combined exposure cannot be described as synergy, since only one concentration of the agents was used in these studies. Nonetheless, the level of arsenite used had no effect on the phosphorylation of these kinases and 0.1% EtOH increased phosphorylation only after 4 h of exposure. Others have demonstrated a signaling axis whereby activated SFK tyrosine phosphorylate Pyk2 and subsequently regulate vascular PKCδ activity (Joseloff *et al*., 2002; Taniyama *et al*., 2003). Arsenite exposures activate Src in aortic endothelial cells (Barchowsky *et al*., 1999b) and uroepithelial cells (Simeonova *et al*., 2002), but arsenic effects on the SFKs in microvascular cells have not been reported. Although human microvascular endothelial cells contain predominantly Src, Yes, and Fyn, only the 47-kDa isoform of Fyn was tyrosine phosphorylated in response to coexposure (Fig. 2). Werdich and Penn demonstrated that of the three kinases, only Fyn mediates integrin signaling required for VEGF-induced tube formation in human retinal microvascular cells (Werdich and Penn, 2005). Thus, a positive interaction at the level of Fyn activation (Fig. 2) may account for the enhanced tube formation observed following coexposure of HMVEC to arsenite and EtOH (Fig. 6). Activation of Src cannot be ruled out, since the analysis in Figure 2 does not directly measure kinase activity. Phosphorylation at tyrosine 416 is not required for increased SFK enzyme activity, but it is required for full activity (Thomas and Brugge, 1997).

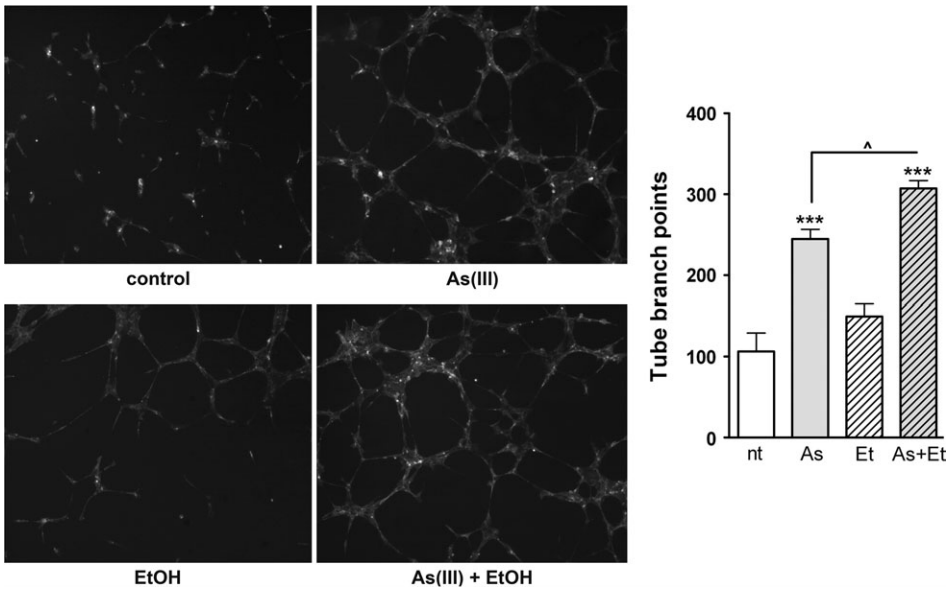

FIG. 6. Effect of arsenite and EtOH on endothelial cell tube formation. HMVEC were plated on Matrigel cushions and allowed to equilibrate for 1 h prior to addition of 1μM arsenite with or without 0.1% EtOH. The cultures were incubated for 4 h and then fixed with paraformaldehyde. After drying, actin filaments in endothelial cell tubes were stained with rhodamine–phalloidin and two 40× fluorescent images were captured from each gel. The vessel branch points in each field were enumerated and the duplicates averaged. The data in the graph are the mean ± SEM number of branch points in four separate cultures. The data were analyzed for significant differences using one-way ANOVA followed by Bonferroni's posttest for multiple comparisons. Statistical difference from control is indicated by *** for $p < 0.001$. Coexposure to arsenite and EtOH significantly increased arsenite-stimulated tube branching ($p < 0.05$).

The positive interactions between arsenite and EtOH at the levels of PLCγ activation might explain the downstream positive interactions for increased PKCδ and PLD activation, as well as gene induction. PLC activity is known to be stimulated by EtOH and is a point of synergy for EtOH and dopamine stimulated PKCδ and PKCϵ activation (Gordon *et al*., 2001). The mechanism for EtOH effects on PLC appears to be at the level of guanine nucleotide exchange by G proteins, especially those that are pertussis toxin sensitive (Garic-Stankovic *et al*., 2005; Hoek *et al*., 1992). Placement of PLC above PKC in the signaling cascade is consistent with the fact that phorbol myristate acetate (PMA), a PKC activator, failed to stimulate PLCγ membrane translocation (Fig. 3). Oxidants stimulate SFK-mediated PLCγ phosphorylation to increase PLCγ-catalyzed diacylglycerol generation (Wang *et al*., 2001) that is required for PKC activation. Thus, arsenite could activate PLCγ by first stimulating Fyn. This is not consistent, however, with the observation that no interaction occurred between arsenite and EtOH for Rac1–GTPase activation (Fig. 3). Reactive oxygen generation in vascular cells exposed to arsenite is mediated by Rac1-dependent NADPH oxidase activity (Qian *et al*., 2005; Smith *et al*., 2001) and the rapid and transient time course for Rac-1 activation (Fig. 3) is consistent with arsenic-stimulated activity of the oxidase (Smith *et al*., 2001). In cancer cells, inhibition of PLC activity prevents arsenic from increasing reactive oxygen formation (Chen *et al*., 2002) suggesting that these signaling sequences may be cell specific. Since exposing the cells to either arsenite or EtOH equally activated Rac1 within 15 min, but neither activated PLCγ at the same time point, it is unlikely that positive interactions seen with coexposures involve either Rac1 or NADPH oxidase generated reactive species.

Previous studies in vascular smooth muscle cells demonstrated that arsenic increased diacylglycerol levels to activate PKCδ that signals for increased *VEGFa* expression (Soucy *et al*., 2004). This PKC activity, as well as activity of classic PKC isoforms, was also required for positive interactions between arsenite and EtOH for induction of *VEGFa* expression in the microvascular endothelial cells (Fig. 5). It was interesting that only a novel PKC, such as PKCδ, was required for induction of *IL8* (Fig. 5), which along with induction of *Col1* was negatively affected following coexposure to arsenite and EtOH. These data are consistent with arsenic and EtOH positively interacting through sequential activation of PLC, PKCδ, and then PLD. PLD is likely downstream of PKC since it is phosphorylated in response to PMA (Fig. 3) and previous reports indicate that PLD activation is often a late PKC-mediated signaling event (Brizuela *et al*., 2007; Hoek *et al*., 1992). Inhibition of arsenic-induced IL-8 and Col1 mRNA levels may result from the fact that EtOH and other short chain alcohols compete with H_2O in PLD catalysis of phospholipids. This competition results in formation of the corresponding phosphatidylalcohol instead of phosphatidic acid (Hoek *et al*., 1992). PLD generated phosphatidic acid is required for PKC phosphorylation of IκB that is essential for NF-κB nuclear translocation and transactivation of NF-κB driven genes, such as *IL8* and *Col1* (Cummings *et al*., 2002; Jonsson and Palmblad, 2001; Saeed *et al*., 2004). Finally, it is interesting to

note that there was no effect of EtOH on basal or arsenite-induced HO-1 mRNA levels (Fig. 4). Also, neither protein kinase inhibitor affected arsenite-induced *HMOX1* (data not shown). These data indicate that the mechanism for arsenite induction of *HMOX1* is completely separate from the signaling pathways for angiogenic gene expression and that induction of the angiogenic genes and the angiogenic response are not mediated by stress activated signaling.

The ultimate question was whether the complex positive and negative cell signaling and gene expression interactions functionally impacted the angiogenic potential of the microvascular cells. The results presented in Figure 6 closely match the positive interactions of arsenite and EtOH on signaling cascades and angiogenic genes expression. The studies were not designed to investigate the breadth of the angiogenic response nor which specific gene product(s) regulated increased tube formation and branching following coexposure. It is likely that the signaling interactions caused by coexposure reprogram the cell phenotype through a number of gene product interactions and dissecting these interactions is well beyond the scope of a single study. However, the results in Figure 6 do reveal signaling pathways and genes not involved in the angiogenic response. The data support the conclusion that signaling pathways and gene expression that is inhibited by EtOH, such as PLD, NF-κB transactivation, and IL-8, are not likely to be involved in arsenite-stimulated angiogenesis or angiogenic responses following coexposure.

In conclusion, coexposure to noncytotoxic levels of arsenite and EtOH stimulated positive signaling interactions in human microvascular endothelial cells that increased expression of angiogenic genes and enhanced branching vascular tube formation. Pathogenic angiogenesis and vascular remodeling contribute to the etiology of both vascular disease and tumorigenesis. Thus, the public health implications of these potentially synergistic interactions are that moderate alcohol consumption may increase the incidence of or exacerbate both cardiovascular diseases and tumorigenesis that result from exposure to arsenic in drinking water. Further *in vivo* animal and epidemiological studies will be required to both prove this hypothesis and investigate the full heath impacts of coexposures to these two common environmental hazards.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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