Arsenite stimulates plasma membrane NADPH oxidase in vascular endothelial cells

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Arsenite stimulates plasma membrane NADPH oxidase in vascular endothelial cells. Am J Physiol Lung Cell Mol Physiol 280: L442–L449, 2001.—Low-level arsenite treatment of porcine aortic endothelial cells (PAEC) stimulated superoxide accumulation that was attenuated by inhibitors of NADPH oxidase. To demonstrate whether arsenite stimulated NADPH oxidase, intact PAEC were treated with arsenite for up to 2 h and membrane fractions were prepared to measure NADPH oxidase activity. Arsenite (5 μM) stimulated a twofold increase in activity by 1 h, which was inhibited by the oxidase inhibitor diphenyleneiodonium chloride. Direct treatment of isolated membranes with arsenite had no effect. Analysis of NADPH oxidase components revealed that p67phox localized exclusively to membranes of both control and treated cells. In contrast, cytosolic Rac1 translocated to the membrane fractions of cells treated with arsenite or angiotensin II but not with tumor necrosis factor. Immunodepletion of p67phox blocked oxidase activity stimulated by all three compounds. However, depleting Rac1 inhibited responses only to arsenite and angiotensin II. These data demonstrate that stimulus-specific activation of NADPH oxidase in endothelial cells was the source of reactive oxygen in endothelial cells after nontoxic arsenite exposure.

Nicotinamide adenine dinucleotide phosphate oxidase; arsenic; p67phox; Rac1; angiotensin II; tumor necrosis factor

Epidemiological studies have shown that chronic exposure to low levels of arsenic through consuming contaminated drinking water results in many chronic diseases including peripheral vascular and cardiovascular disease as well as skin, lung, kidney, and bladder cancers (10, 15, 38). Several studies showed causal associations between high arsenic concentration in public drinking water in the United States and increased mortality rates due to arteriosclerosis (10, 19). An association between heart disease and arsenic-contaminated drinking water was also seen among residents in Utah whose drinking water sources were private wells (19). Furthermore, a recent study in New Hampshire showed that subpopulations in this state consume drinking water from domestic wells that have arsenic levels of up to 3.5 μM (30), which is significantly higher than the maximum contaminant level of 0.05 μM set by the Environmental Protection Agency (EPA).

The exact mechanisms by which arsenic exposure induces vascular diseases are unknown. However, these diseases are hypothesized to occur through common mechanisms such as reactive oxygen formation related to endothelial cell injury and clonal expansion of vascular cells (29). Exposure to the trivalent form of arsenic, arsenite, has been linked to increased formation of reactive oxygen species and oxidation of vicinal dithiols. Arsenite can bind to sulphydryl groups of proteins, such as dehydrogenases, resulting in their denaturation. This effect can inactivate antioxidant enzymes such as catalase or superoxide dismutase (SOD) and release iron from denatured proteins, leading to an oxidized intracellular state (18). As a result, high toxic levels of arsenite activate many stress-related responses, including induction of heat shock proteins (17) and cytochrome P-450 (18) and stimulation of mitogen-activated protein (MAP) kinase cascades (20). In addition, high levels of arsenite directly inhibit inhibitory κB kinase (32), which deprives the cell of nuclear factor-κB (NF-κB) transcriptional enhancement that is essential for cell survival after stressful stimuli. In contrast, previous studies from this laboratory established that low, nontoxic levels of oxidant formation occurred when primary porcine aortic endothelial cells (PAEC) were exposed to arsenite at levels of 5 μM or less (2). The predominant oxygen radical produced after arsenite exposure was identified as superoxide anion, which was confirmed by attenuation with SOD (3, 4). This low amount of reactive oxygen was sufficient to increase hydrogen peroxide accumulation and hydrogen peroxide-sensitive tyrosine phosphorylation and NF-κB activation (2–4) but not MAP kinase activity (4). In contrast to high arsenite levels, low-level exposure increased intracellular glutathione levels and did not overwhelm the cellular antioxidant defenses (2, 33). The sources of arsenite-stimulated oxidant formation in the vasculature are only now being realized. Pharmacological inhibitors of NAD(P)H oxidase, apocynin, and diphenyleneiodonium chloride (DPI), pre-

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vent arsenite-stimulated superoxide formation in this PAEC model (3). Recently, arsenite-stimulated reactive oxygen formation in smooth muscle cells was shown to require p22\textsuperscript{phox} and the activity of NADH oxidase to damage DNA (21). Therefore, this family of oxidases appears to be an important target of arsenite action. The regulation and character of NAD(P)H oxidase activity in vascular cells has been enigmatic compared with the well-characterized multicomponent enzyme complex in phagocytes, as reviewed by Griendling et al. (13) and Babior (1). The phagocyte oxidase complex consists of two membrane components (p22\textsuperscript{phox} and gp91\textsuperscript{phox}), which comprise the flavohemoprotein known as cytochrome b\textsubscript{558}. Three other components (p67\textsuperscript{phox}, p47\textsuperscript{phox}, and p40\textsuperscript{phox}) reside in the cytosol. On activation of the phagocyte, the cytosol components, along with Rac2, translocate to the membrane to become associated with cytochrome b\textsubscript{558}. Once activated, the complex resides in the membrane and generates superoxide anion as part of the oxidative burst of phagocytosis. The importance of this enzyme complex was realized with the discovery of the heterogeneous genetic disorder chronic granulomatous disease. This disease is found in children whose phagocytes are unable to mount an oxidative burst and therefore suffer from chronic bacterial and fungal infections.

Originally, it was thought that the oxidase was only found in phagocytes (37). However, recent studies have shown that this complex exists in nonphagocytic cells, including vascular cells (12, 13, 16, 26, 28). The identity of the nucleotide cofactors for the vessel wall oxidases remains controversial and may be cell specific (13). Jones and coworkers (16) showed that human umbilical vein endothelial cells generated basal levels of superoxide anion that were inhibited by SOD and DPI. In addition, these researchers demonstrated that endothelial cells expressed the mRNA for gp91\textsuperscript{phox}, p22\textsuperscript{phox}, p47\textsuperscript{phox}, and p67\textsuperscript{phox}. Gorlach et al. (11) used gp91\textsuperscript{phox}(−/−) mice to demonstrate that the endothelial NADPH oxidase is a major source of oxygen radical generation in the arterial wall. Together these studies suggest that an enzyme complex similar to the phagocyte NADPH oxidase exists in endothelial cells and that its regulation may be sensitive to arsenite.

Because our laboratory has shown previously that low-level arsenite treatment resulted in increased production of superoxide anion in PAEC, the objective of the present study was to determine whether arsenite directly stimulated the activity of a NAD(P)H oxidase. Immunodepletion studies provided novel and definitive demonstration that NADPH oxidase is the primary oxidase responding to nontoxic levels of arsenite in PAEC. However, in contrast to stimulation of smooth muscle NADH oxidase (21), the concentration relationship for NADPH oxidase activation is narrow, with higher arsenite concentrations causing diminishing amounts of stimulation. The results of this study also indicate that arsenite-induced stimulation is not direct and requires additional upstream effectors that are not present in isolated membrane preparations. This regulation may result from stimulus-specific recruitment of Rac1 to the cell membrane.

**MATERIALS AND METHODS**

**Materials.** Cell culture medium (DMEM) with 1 g/l d-glucose and L-glutamine were purchased from Meditech. Trypsin-EDTA (0.25 mg/ml) and trypsin-neutralizing solution were purchased from Clonetics (San Diego, CA). A penicillin-streptomycin mixture was purchased from BioWhittaker (Walkersville, MD). Coomassie Plus protein reagent (Pierce, Rockford, IL) was used for the protein assays. The NAD(P)H oxidase assay used lucigenin (bis-N-methylacridinium nitrate) obtained from Molecular Probes (Eugene, OR) as the colorimetric reagent. NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form), bovine serum albumin, monoclonal antibody to β-actin, angiotensin II, and DPI were purchased from Sigma (St. Louis, MO). Tumor necrosis factor (TNF) was purchased from Roche Molecular Biochemicals (Indianapolis, IN). For the Western analysis, the secondary antibody used was donkey anti-rabbit IgG or sheep anti-mouse IgG horseradish peroxidase-linked whole antibody from Amersham Life Sciences (Piscataway, NJ). The polyclonial IgG rabbit antibody was purchased from Biocell (San Diego, CA). The primary antibody used was polyclonal specific for cytosolic p47\textsuperscript{phox}, p67\textsuperscript{phox}, and p40\textsuperscript{phox} from Transduction Laboratories (Lexington, KY).

**PAEC.** Intact, 1-day postconfluence endothelial cells in T75-cm\textsuperscript{2} flasks (Corning) were treated with arsenite at the concentration and times indicated in the figure legends. Control cells received no additions, whereas positive control cells were treated with either 100 nM angiotensin II (12, 27) or 10 ng/ml TNF (6) for 1 h. At the end of the exposure periods, all cells were washed twice with Hanks’ balanced salt solution without calcium or magnesium and once with cold PBS and were then scraped and lysed in 600 μl of lysis buffer [20 mM Tris-Cl, pH 7.4, 0.5% (w/v) Triton X-100, 150 mM NaCl, 10 mM EDTA, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF)]. To ensure at least 90% rupture of the cells, lysates were homogenized with 160 strokes of a dounce homogenizer, and then particulate and soluble fractions were separated by centrifugation at 20,000 g for 20 min at 4°C. The resulting supernatant was removed, and the pellet was rinsed with 600 μl of lysis buffer and centrifuged again. The final pellet was suspended in 250 μl of oxidase assay buffer (50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 150 mM sucrose, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.5 mM PMSF) with a dounce homogenizer. The protein concentrations of the cellular fractions were determined using the Coomassie Plus protein reagent and BSA as the standard according to the manufacturer’s instructions for microassay format.

**Determination of plasma membrane protein markers.** Plasma membrane enrichment and purity were measured by assaying all cellular fractions for 5′-nucleotidase activity, a known enzyme marker for the plasma membrane. The 5′-nucleotidase activity was determined using a Sigma Diag-
nostic Kit (Sigma) according to the manufacturer’s instructions except that 1 ml of assay solution and 0.67 ml of sample were used instead of the recommended volumes. Results are expressed as units per liter per microgram of protein. The fractionation procedure outlined previously resulted in enrichment of 5′-nucleotidase in the particulate fraction (0.32 ± 0.08, 0.25 ± 0.06, and 0.09 ± 0.06 U·l⁻¹·µg protein⁻¹ for total homogenate, particulate fraction, and supernatant, respectively). As a result, the particulate fraction is referred to as the membrane fraction for the remainder of the paper.

**NADPH oxidase assay.** The NADPH oxidase activity was measured by superoxide-dependent lucigenin chemiluminescence as previously described (12, 27) with some modifications. Briefly, 35 µg of membrane protein and 400 µM lucigenin were added to wells of a 96-well luminometer plate. Oxidase assay buffer was added to each well for a final volume of 250 µl. The reaction was started by adding NADPH (100 µM) to each well, and relative light units (RLU) of chemiluminescence were read in a microtiter luminometer (Dynatech ML2250, Dynatech Laboratories, Chantilly, VA, or PerkinElmer Life Sciences, Branford, CT). Only the initial 1 min of enzyme activity was monitored because this was the linear portion of luminescence generation. To show specificity of the luminescence, lucigenin reduction was determined in wells containing membrane fraction but no NADPH or with all assay reagents but no membrane fraction. Luminescence levels under either of these conditions were not above background. Therefore, the background chemiluminescence of the buffer was always subtracted from each reading before data calculations. The specificity of the assay for superoxide was demonstrated by inhibiting the reaction with SOD (120 U/ml). The SOD-inhibitable portion of the chemiluminescence, which defined the reported oxidase activity, was determined by subtracting the luminescence from the wells with SOD from the luminescence of the wells without SOD. The results are expressed as RLU per minute per microgram of protein. Inhibitors such as 400 µM DPI, a 1:1,000 dilution of polyclonal antibody to p67phox (a generous gift from Dr. Mark Quinn [31]), or a 1:1,000 dilution of monoclonal antibody to Rac1 (Transduction Laboratories, Lexington, KY) were added 5 min before the reaction was started. For the antibody inhibition studies, whole molecule rabbit or mouse IgG was used as a nonspecific control (ChromPure rabbit or mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, PA).

**Western immunoblotting.** Postconfluent T75 flasks (Corning) were treated with 5 µM arsenite for 1 h and washed twice with stop buffer (10 mM Tris·HCl, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.1 M NaF, 0.20 M sucrose, 100 mM sodium orthovanadate, 5 mM pyrophosphate, 0.5 mM PMSF, 10 µg/ml aprotinin, 0.5 µg/ml leupeptin, and 0.7 µg/ml pepstatin). The cells were lysed in 600 µl of lysis buffer and ruptured with a dounce homogenizer. The membrane and supernatant were obtained, homogenized, and separated into membrane and soluble fractions. The initial oxidase activity in the total homogenate (H), the membrane fraction, with and without arsenite treatment of PAEC resulted in superoxide anion accumulation that was sensitive to pharmacological inhibitors of NADPH oxidases (2–4). To confirm the presence and location of NADPH oxidase activity in PAEC, membranes were prepared from untreated cells. In these studies, NADPH oxidase activity was defined as SOD-inhibitable lucigenin reduction. The data in Fig. 1 validate that endothelial cells generate a basal level of superoxide anion in the presence of 100 µM NADPH as represented by the assayed oxidase activity in the homogenate and membrane. In addition, the largest proportion of assayed oxidase activity is found in the 29,000-g membrane fraction, with no activity detected in the supernatant.

**RESULTS**

Membrane fractions contained the greatest specific activity for NADPH oxidase. All cell types in the vessel wall, including endothelial cells, contain a phagocyte-like NADPH oxidase (13, 23). We have shown previously that arsenite treatment of PAEC resulted in superoxide anion accumulation that was sensitive to pharmacological inhibitors of NADPH oxidases (2–4). To confirm the presence and location of NADPH oxidase activity in PAEC, membranes were prepared from untreated cells. In these studies, NADPH oxidase activity was defined as SOD-inhibitable lucigenin reduction. The data in Fig. 1 validate that endothelial cells generate a basal level of superoxide anion in the presence of 100 µM NADPH as represented by the assayed oxidase activity in the homogenate and membrane. In addition, the largest proportion of assayed oxidase activity is found in the 29,000-g membrane fraction, with no activity detected in the supernatant.

**Fig. 1.** Specific activity of NADPH oxidase of fractionated aortic endothelial cells. One-day postconfluent aortic endothelial cells were harvested, homogenized, and separated into membrane and soluble fractions. The initial oxidase activity in the total homogenate (H), the 29,000-g membrane pellet (P), and supernatant (S) was then assayed during the first minute of activity initiated by injection of 100 µM NADPH. Activity is expressed as relative light units (RLU)·min⁻¹·µg protein⁻¹, and the bars represent means ± SD of the assay of superoxide dismutase (SOD)-inhibitable activity; n > 3. The activity of the soluble supernatant fraction was not detectable.
Time and dose dependencies of the NADPH oxidase activity. Because the membrane contains the greatest amount of oxidase activity, this fraction was used to examine the time and dose relationships of the arsenite-induced effect on the NADPH oxidase activity. The data in Fig. 2 show the time course for arsenite stimulation of the oxidase. Arsenite addition resulted in a significant increase in total enzymatic activity within 1 h that returned to control levels by 2 h. The data in Fig. 3 demonstrate that the effects of arsenite were dose dependent over the range of 0–20 μM. The biphasic nature of this relationship was consistent with previous data demonstrating that concentrations of arsenite of greater than 5 μM are toxic to the endothelial cells (2, 4).

All of the arsenite-induced increases in the oxidase activity of whole cells were blocked by in vitro incubation of the membrane fractions with 400 μM DPI, a known inhibitor of the phagocyte NAD(P)H oxidase (Figs. 2 and 3). This in vitro inhibition indicated that arsenite activated a phagocyte-like NAD(P)H oxidase in the endothelial cells. In contrast, in vitro addition of arsenite to membrane fractions from control cells failed to increase oxidase activity (567 ± 241 and 524 ± 223 RLU·min⁻¹·μg⁻¹ of untreated and arsenite-treated membrane proteins, respectively). These data indicate that arsenite required signaling events that are proximal to the membrane oxidase to activate the enzyme complex.

Selective translocation of the Rac1 subunit of NADPH oxidase in response to arsenite. The presence of functional NADPH oxidase subunit proteins in endothelial cells has been difficult to measure (13, 26). To confirm that the PAEC have a phagocyte-like NAD(P)H oxidase, Western analysis was used to determine the presence of the essential components for phagocyte NAD(P)H oxidase, p67phox and Rac1. Figure 4A confirms that endothelial cells contain p67phox protein only in the membrane fractions of control and treated cells. One-hour exposures of the cells to arsenite, angiotensin II, or TNF did not change the amount of the p67phox protein in the membrane. The data in Fig. 4B demonstrate that Rac1 is found in both the membrane and soluble fractions of the cell. In contrast to p67phox, the amount of membrane-bound Rac1 increased following exposure to arsenite or angiotensin II. Treatment with TNF only marginally affected Rac1 translocation.
These data demonstrate that at least two critical NADPH oxidase components are present in the membrane fractions that contain arsenite-inducible activity. In addition, the complex appears to be constitutively assembled in the resting cell and translocation of Rac1 may represent an arsensite-sensitive means of increasing enzyme activity.

Functional confirmation that arsenite activated NADPH oxidase in aortic endothelial cells. To demonstrate that the proteins detected by Western analysis in Fig. 4 are part of a functional enzyme complex, membrane fractions from control or arsenite-stimulated cells were immunodepleted of p67phox or Rac1 before assay for enzyme activity. Immunodepletion with either antibody completely inhibited any arsenite-induced effects (Fig. 5). In contrast, immunodepletion with isotype-matched IgG fractions had no effect on membrane oxidase activity (data not shown). The data in Fig. 5 also demonstrated that angiotensin II and TNF stimulated NADPH oxidase activity in this endothelial cell model. Stimulation in response to these positive controls was also inhibited by in vitro incubation with DPI (data not shown) or immunodepletion with the antibodies for p67phox (Fig. 5A). However, immunodepletion with antibody to Rac1 did not inhibit TNF-stimulated oxidase activity. This finding is consistent with minimal Rac1 translocation in response to TNF (Fig. 4B). Taken together, these results demonstrate that arsenite stimulated a membrane-bound NADPH oxidase enzyme complex associated with p67phox and Rac1. In addition, these data provide a novel demonstration of stimulus-specific regulation of the endothelial cell oxidase by arsenite, angiotensin II, and TNF.

DISCUSSION

The results of this study, combined with our previous observations (2–4), demonstrate that environmentally relevant levels of arsenite activated a NADPH-dependent oxidase in PAEC. This PAEC oxidase was located in the plasma membrane (Fig. 1), was dependent on exogenous NAD(P)H for activity, and shared some of the critical subunits with the better described NAD(P)H oxidase complexes in phagocytes, smooth muscle cells, and vascular adventia (1, 12, 27). Whereas the composition and regulation of the endothelial cell oxidase are still controversial (1, 9, 23), it is clear from the data presented that both the p67phox and Rac1 subunits are essential for arsenite-stimulated superoxide formation. In addition, translocation of Rac1 may represent a major regulatory mechanism for stimulation of NADPH oxidase activity by arsenite. The data suggest that the site of arsenite action is proximal to the enzyme complex because arsenite cannot stimulate activity directly in isolated membranes.

The data presented in Figs. 2 and 3 demonstrate that peak stimulation of NADPH oxidase occurs within 1 h of arsenite exposure and is dose dependent. These results are significant in several ways. First, the time course and concentration range for activating the oxidase are consistent with arsenite-induced increases in intracellular oxidations, accumulation of superoxide and hydrogen peroxide, and cell signaling seen in endothelial cells (2–4). Second, the concentration of arsenite needed to activate the oxidase and oxidant-sensitive endothelial cell signaling is within the range of concentrations found in contaminated drinking water in the United States (10, 19, 30) and in drinking water where vascular diseases are prevalent (5, 19, 35). Finally, these concentrations are not toxic to the PAEC (2–4). This may be due to the ability of these cells to increase their glutathione synthesis (2, 33) and inability to increase their levels of intracellular free calcium or reactive nitrogen in response to this level of arsenite exposure (3). Higher arsenite concentrations are toxic to endothelial cells because these levels deplete glutathione, collapse the mitochondrial electron gradient, increase free calcium, and promote protein and DNA damage from peroxynitrite (14, 22).

In smooth muscle cells, peak oxidant production occurs in response to 10 μM arsenite but with a prolonged time course over 4 h (21). This prolonged increase was inhibited by antisense oligomers that target p22phox expression and was related to activation of
NADH but not of NADPH oxidase. Furthermore, this prolonged oxidant generation was associated with increased DNA damage (21). In contrast, the time course for arsenite effects on the endothelial cell oxidase was more transient (Fig. 2 and Ref. 2), and concentrations of arsenite greater than 5 μM had a negative effect on activity (Fig. 3). In an earlier study, the same concentration-response relationship was shown for the effects of arsenite on PAEC proliferation (2). This suggests that in the endothelial cell, NADPH oxidase may be the source of mitogenic reactive species but not the source of toxic species. The difference between the sensitivity of the endothelial and smooth muscle cell enzymes to the inhibitory effects of arsenite may relate to the known differences in subunit composition (26). In particular, only the endothelial cell enzyme contains gp91phox (11, 13, 26), which is directly inhibited by more potent arsenicals such as phenylarsine oxide (8, 34).

The Western analysis in Fig. 4 confirms that the PAEC express significant levels of two critical regulatory subunits of NADPH oxidase, p67phox and Rac1. This confirmation is important because it has been difficult to demonstrate that the endothelial cells have detectable levels of the phagocyte oxidase proteins despite several reports of measurable mRNA levels (13). These data are also important because they confirm that regulation of the PAEC oxidase differs significantly from that of the phagocyte enzyme complex. In unstimulated phagocytes, the p67phox subunit is located in the cytosol, with only a portion of the pool translocating to the membrane on cell activation (7, 24, 31). In contrast, the PAEC p67phox is found only in the membrane of control cells, and the amount associated with the membrane does not appear to change when arsenite-induced oxidase activity is maximal (Fig. 4A). The difference in subunit location between the endothelial cell and phagocytes appears to be partially true for Rac1. Rac1 translocation is independent of p67phox and is a major determinant of phagocyte oxidase activation (31). The data in Fig. 4B indicated that there was a constitutive pool of Rac1 in the PAEC membrane that increased on exposure to arsenite or angiotensin II but not as readily when TNF was added. Arsenite cannot activate oxidase activity if the cell is disrupted. Thus similar to angiotensin II, the major mechanism for arsenite-induced activity may be activation of Rac1 and increased association with its effector p67phox. The structural basis for this interaction and the interactions between Rac1 and p67phox and the rest of the oxidase complex have been well characterized (25). However, it is not known how arsenite might activate Rac1 or even whether Rac1 is a direct target of arsenite. It is quite possible that stimulation of a proximal effector, such as a kinase or guanine exchange factor, may be the rate-limiting event in the activation cascade.

The data in Fig. 5 provide further novel information regarding the unique regulation of endothelial cell NADPH oxidase. In addition to demonstrating that p67phox and Rac1 are essential for arsenite-induced activation of the enzyme complex, these data demonstrate that angiotensin II and TNF stimulate the enzymatic capacity of the endothelial cell oxidase. The most striking difference between these data and data from previous reports is the time course for oxidase activation. Agonist-induced increases in NADPH oxidase activity in vascular smooth muscle cells (6, 13, 36) or adventitial fibroblasts (27) requires hours compared with 1 h for the endothelial cells (Fig. 5). This difference may result from the need for increased expression of the oxidase subunits to increase smooth muscle oxidase activity (6, 13). Also, the endothelial enzyme does not need to achieve full catalytic capacity before arsenite stimulates significant superoxide and hydrogen peroxide accumulation, which occurs within 10 min and is maximal by 1 h (2–4). The rapid increase in reactive oxygen formation after arsenite addition might argue against a regulatory role for subunit expression in the endothelial cell. However, endothelin-1 has been demonstrated recently to activate human umbilical vein endothelial cell NAD(P)H oxidase activity by inducing a transient, 1-h increase in gp91phox expression (9).

The demonstration that TNF-induced signaling differs from that of arsenite and angiotensin II was not unexpected. However, the mechanisms for TNF-stimulated oxidase activity in the endothelial cell are not well defined. In this study, TNF stimulated total enzyme capacity but minimal Rac1 translocation (Fig. 4B). This increase in enzyme capacity was not inhibited by the addition of antibody to Rac1 (Fig. 5B). These data illustrate the main difference of the response to TNF, which is known to differ from angiotensin II signaling. TNF signals through a ceramide-dependent pathway to activate the oxidase in other cell types (13). It is possible that this ceramide signaling pathway effects posttranslational modification of one of the constitutively assembled oxidase subunits to increase enzyme activity.

The alternative pathway of posttranslational modification of the individual oxidase subunits has been proposed as plausible mechanisms for increasing enzyme activity in other vessel wall oxidases (1, 13). For example, calcium- and protein kinase C-dependent phosphorylation of the subunits activate oxidase activity in several cell types including phagocytes (1, 13). This mechanism may not explain the current data for arsenite-induced activity because the arsenite concentrations used in these studies were insufficient to affect intracellular free calcium levels (3). However, a role for calcium-independent protein kinase C isoforms cannot be ruled out. Again, full evaluation of the regulatory mechanisms in the PAEC response to arsenite will require further experiments with additional pig-specific reagents to determine protein expression and posttranslational modification.

In summary, these studies provide novel demonstration of both the mechanisms involved in low-level arsenite stimulation of vascular endothelial cell reactive oxygen formation and the functional regulation of the endothelial NADPH oxidase. The data confirm previ-
ous pharmacological determinations that arsenite stimulates oxidant formation by activating the plasma membrane NADPH oxidase. They also demonstrate differences between the endothelial and smooth muscle oxidase in the concentration and time relationships for arsenite-induced stimulation. In addition, the data indicate that activation of the endothelial cell oxidase is stimulus specific. Further investigation will be required to define the rate-limiting step in arsenite-dependent activation of this key enzyme complex involved in regulating the oxidant tone of the vasculature.

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