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Original Contribution

Citron Rho-interacting kinase mediates arsenite-induced decrease in endothelial nitric oxide synthase activity by increasing phosphorylation at threonine 497: Mechanism underlying arsenite-induced vascular dysfunction

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ABSTRACT

We reported that arsenite causes an acute decrease in nitric oxide (NO) production by increasing phosphorylation of endothelial NO synthase at threonine 497 (eNOS-Thr^{497}); however, the detailed mechanism has not yet been clarified. Here, we investigated the kinase involving in arsenite-stimulated eNOS-Thr^{497} phosphorylation. Although treatment with H-89, a known protein kinase A (PKA) inhibitor, inhibited arsenite-stimulated eNOS-Thr^{497} phosphorylation, no inhibition was found in cells treated with other PKA inhibitors, including Rp-8-Br-cAMPS or PKI. Based on previous reports, we also tested whether RhoA mediates arsenite-stimulated eNOS-Thr^{497} phosphorylation and found that arsenite causes an acute increase in RhoA activity. Ectopic expression of dominant negative (DN)-RhoA significantly reversed arsenite-stimulated eNOS-Thr^{497} phosphorylation. An *in vitro* phosphorylation assay also revealed that the well-known Rho effectors, Rho-associated protein kinase 1/2 (ROCK1/2), directly phosphorylate eNOS-Thr^{497}. Y27632, a selective ROCK inhibitor, reversed arsenite-stimulated eNOS-Thr^{497} phosphorylation. However, overexpression of a small interfering RNA (siRNA) against ROCK1/2 or DN-ROCK did not reverse arsenite-stimulated eNOS-Thr^{497} phosphorylation, thereby providing no conclusive evidence of a role for ROCK1/2. Knockdown of PKC-related protein kinase 1/2, another Rho effector, also did not reverse arsenite-stimulated eNOS-Thr^{497} phosphorylation. In contrast, we found that transfection with an siRNA against citron Rho-interacting kinase (CRIK), the other downstream effector of Rho, significantly reversed the arsenite-induced eNOS-Thr^{497} phosphorylation that was accompanied by restoration of eNOS enzymatic activity repressed by arsenite. Moreover, CRIK directly phosphorylated eNOS-Thr^{497} *in vitro*. Finally, we also found that arsenite increased eNOS-Thr^{497} phosphorylation and decreased acetylcholine-induced vessel relaxation in rat aortas. In conclusion, we demonstrate that arsenite acutely inhibits eNOS enzymatic activity and vessel relaxation in part by increasing the RhoA/CRIK/eNOS-Thr^{497} phosphorylation signaling axis, which provides a molecular mechanism underlying arsenite-induced impaired vascular diseases.

1. Introduction

Arsenic, in particular its trioxide, has been a consistent and serious threat to human health worldwide because excessive levels exist naturally in well water, groundwater and food supplies [1–4]. Several epidemiological studies have shown that chronic (and perhaps acute) exposure to arsenite is associated with increased risk for a variety of human diseases, including cardiovascular diseases (CVD) like arteriosclerosis, hypertension, and blackfoot disease [5]. Although the molecular mechanism by

Abbreviations: EC, endothelial cells; ROS, reactive oxygen species; NO, nitric oxide; eNOS, endothelial NO synthase; PP1, protein phosphatase 1; AMPK, AMP-activated protein kinase; PKC, protein kinase C; PP2A, protein phosphatase 2A; CRIK, citron Rho-interacting kinase; IBMX, 3-isobutyl-1-methylxanthine; PKA, protein kinase A; ROCK, Rho-associated protein kinase; PRK, protein kinase C-related protein kinase; DN, dominant negative; PSS, physiological salt solutions; S1PR, sphingosine-1-phosphate receptor

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which arsenite causes cardiovascular dysfunction has yet to be fully clarified, the immediate and strong binding of arsenite to the sulfhydryl groups of many important proteins in target cells, such as cardiac, smooth muscle, and endothelial cells (EC), is one of the proposed mechanisms [6,7]. Direct binding of arsenic to these proteins alters their physiological functions, consequently leading to malfunction. Another commonly suggested mechanism is EC dysfunction caused by arsenite. It is largely understood that the increased production of reactive oxygen species (ROS) plays an important role in disrupted function of key antioxidant enzymes, resulting in EC toxicity. Recently, several studies have also shown that acute and/or chronic arsenite exposure decreases nitric oxide (NO) production in EC, which is associated with arsenite-induced CVD development [4,8,9]. In this regard, we recently reported that arsenite results in an acute decrease in NO production by increasing the phosphorylation of endothelial NO synthase (eNOS) at threonine 497 (eNOS-Thr^{497}; in bovine sequence) [10]. Furthermore, this increased eNOS-Thr^{497} phosphorylation was shown to be mediated by the ROS/protein phosphatase 1 (PP1) signaling pathway, suggesting possible crosstalk between ROS and NO in arsenite-derived CVD development [10,11].

Endothelial NO has long been known as a key molecule regulating EC integrity. It serves as a vasodilator, and its dysregulation is therefore thought to contribute to the pathogenesis of vasodilation-related diseases, such as atherosclerosis and hypertension [12]. The production of NO is catalyzed by eNOS, and its activity is controlled mainly at the level of eNOS phosphorylation at specific sites [13]. Several sites of phosphorylation, such as eNOS-Ser^{1179}, eNOS-Thr^{497}, and eNOS-Ser^{116}, have been identified and evaluated [14,15]. Among those sites, phosphorylation of eNOS-Thr^{497} decreases eNOS activity and NO production [11,15]. Although several kinases have been reported to mediate eNOS-Thr^{497} phosphorylation, including AMP-activated protein kinase (AMPK) [16], protein kinase C (PKC) [17,18] and Rho-associated protein kinase (ROCK) [19,20], whether these kinases directly phosphorylate eNOS-Thr^{497} has yet to be determined. Furthermore, this phosphorylation site is also regulated by PP1 and PP2A, and as expected, dephosphorylation of eNOS-Thr^{497} also results in an increase in NO production [21,22].

In this study, we attempted to identify the kinase responsible for directly phosphorylating eNOS-Thr^{497} upon arsenite exposure. We found that citron Rho-interacting kinase (CRIK) is a novel kinase phosphorylating eNOS-Thr^{497} that is associated with decreased eNOS enzymatic activity and *ex vivo* vessel relaxation caused by arsenite.

2. Materials and methods

2.1. Materials

Sodium arsenite ($NaAsO_2$, used as arsenite) was purchased from VWR International (West Chester, PA, USA). H-89 was obtained from Calbiochem (Nottingham, UK), and 3-isobutyl-1-methylxanthine (IBMX) and Y27632 were purchased from Sigma (St. Louis, MO, USA). 8-Bromoadenosine 3′,5′-cyclic monophosphothioate (Rp-8-Br-cAMPS), PKA inhibitor (PKI), and lysophosphatidic acid (LPA) were purchased from Santa Cruz Biotech (La Jolla, CA, USA). VPC23019 was obtained from Tocris Bioscience (Bristol, UK). Recombinant bovine eNOS was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and recombinant human His-tagged ROCK1, ROCK2, and GST-tagged CRIK were supplied by SignalChem (Richmond, BC, Canada). Antibodies against eNOS (cat#610297), p-eNOS-Ser^{1179} (cat#612393), p-eNOS-Thr^{497} (cat#612706), ROCK1 (cat#611136), ROCK2 (cat#610623), and PKC-related protein kinase 1 (PRK1) (cat#610686) were purchased from BD Transduction Laboratories (Lexington, KY, USA). Antibodies against Rho (cat#2117) and PKC-related protein kinase 2 (PRK2) (cat#2612) were obtained from Cell Signaling Technology (Boston, MA, USA). Antibody against p-eNOS-Ser^{116} (cat#07-357) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA), and antibodies against myc (cat#sc-40) and β-actin (cat#sc-1616), and all corresponding secondary antibodies were from Santa Cruz Biotech. Antibodies against GST (cat#MA4-004) and His (cat#MA1-21315) were obtained from Thermo Scientific Inc. (Waltham, MA, USA). Lipofectamine 2000, minimal essential medium (MEM), Dulbecco's phosphate-buffered saline (DPBS), newborn calf serum (NCS, cat#16010-159), penicillin–streptomycin antibiotics, L-glutamine, trypsin–EDTA solution, and plasticware for cell culture were obtained from Gibco-BRL (Gaithersburg, MD, USA). All other chemicals were of the purest analytical grade.

2.2. Cell culture and drug treatments

Bovine aortic EC (BAEC) were isolated and cultured as described previously [23] and maintained in MEM supplemented with 5% NCS at 37 °C under 5% CO_2 in air. EC were confirmed by their typical cobblestone configuration when viewed under light microscopy and by a positive indirect immunofluorescence test for von Willebrand factor VIII. Cells between passages 5 and 10 were used for all experiments. When BAEC were grown to confluence, the cells were further maintained for the indicated times in MEM with 5% NCS containing 30 μM sodium arsenite. In some experiments, the cells were treated with various chemicals for 0.5 h before arsenite treatment.

2.3. Transfection of dominant negative RhoA or ROCK, or knockdown of expression of Rho effector genes using small interfering RNA

Transfection was performed as previously described [24]. Dominant negative (DN)-RhoA, a pcDNA3.1 mammalian expression vector-containing cDNA encoding RhoA-N19, an activity-dead RhoA construct, is a kind gift from Professor In-San Kim, Kyungpook National University, Daegu, Korea. DN-ROCK, a pEF-BOS mammalian expression vector-containing cDNA encoding mutant ROCK2 possessing deletion of kinase domain and point mutations of Asn at 1036 and Lys at 1037 to Thr in the Rho binding domain, is a kind gift from Professor Jae Ho Kim, Pusan National University, Busan, Korea. DN-ROCK is known to abolish the binding activity of GTP-Rho in both ROCK1 and ROCK2 [25,26]. Each DN-construct (3 μg) was transfected into cells grown to 70% confluence in 60-mm dishes using Lipofectamine 2000, according to the manufacturer's instructions. As a control, equal amounts of pcDNA3.1 or pEF-BOS vector were also transfected. For knockdown of expression of Rho effector genes, small interfering RNA (siRNA) oligonucleotides against each effector gene and a scramble siRNA oligonucleotide were purchased from Dharmacon Inc. (Lafayette, CO, USA). BAEC grown to 70% confluence in 60-mm culture dishes were transfected with 100 nM of each siRNA oligonucleotide using DharmaFECT 1 Transfection Reagent (Dharmacon Inc.), according to the manufacturer's instructions. All transfections were incubated for 5 h at 37 °C, and the transfected cells were further incubated in MEM containing 5% NCS for 24 h.

2.4. Western blot analysis

For Western blot analysis, BAEC treated with sodium arsenite in the absence or presence of various chemicals were washed with ice-cold DPBS and lysed in lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA) containing 1 × Protease Inhibitor Cocktail™ (Roche Molecular