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The effect of inorganic arsenic on endothelium-dependent relaxation: Role of NADPH oxidase and hydrogen peroxide

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ABSTRACT

Chronic arsenic ingestion predisposes to vascular disease, but underlying mechanisms are poorly understood. In the present study we have analyzed the effects of short-term arsenite exposure on vascular function and endothelium-dependent relaxation.

Endothelium-dependent relaxations, nitric oxide (NO) and endothelium derived hyperpolarizing factor (EDHF)-type, were studied in rabbit iliac artery and aortic rings using the G protein-coupled receptor agonist acetylcholine (ACh) and by cyclopiazonic acid (CPA), which promotes store-operated Ca^{2+} entry by inhibiting the endothelial SERCA pump. Production of reactive oxygen species (ROS) in the endothelium of rabbit aortic valve leaflets and endothelium-denuded RIA and aortic rings was assessed by imaging of dihydroethidium.

In the iliac artery, exposure to 100 μM arsenite for 30 min potentiated EDHF-type relaxations evoked by both CPA and ACh. Potentiation was prevented by catalase, the catalase/superoxide dismutase mimetic manganese porphyrin and the NADPH oxidase inhibitor apocynin. By contrast in aortic rings, that exhibited negligible EDHF-type responses, endothelium-dependent NO-mediated relaxations evoked by CPA and ACh were unaffected by arsenite. Arsenite induced apocynin-sensitive increases in ROS production in the aortic valve endothelium, but not in the media and adventitia of the iliac artery and aorta.

Our results suggest that arsenite can potentiate EDHF-type relaxations *via* a mechanism that is dependent on hydrogen peroxide, thus demonstrating that dismutation of the superoxide anion generated by NADPH oxidase can potentially offset loss of NO bioavailability under conditions of reduced eNOS activity. By contrast, selective increases in endothelial ROS production following exposure to arsenite failed to modify relaxations mediated by endogenous NO.

1. Introduction

In the cardiovascular system, exposure to arsenic accelerates the development of atherosclerosis and predisposes to hypertension and peripheral microvascular abnormalities such as Blackfoot Disease (Balakumar and Kaur, 2009; Prozialeck et al., 2008; States et al., 2009). Underlying mechanisms have been suggested to involve increased oxidant stress, because exposure of endothelial cells to arsenite at concentrations within the range found in contaminated drinking water (0.3–15 μM) causes excess production of the superoxide anion ($O_2^{\bullet-}$) by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Barchowsky et al., 1999; Smith et al., 2001; Qian et al., 2005; Straub et al., 2008). $O_2^{\bullet-}$ may contribute to vascular dysfunction through a rapid interaction with, and inactivation of, the potent vascular relaxing factor endothelium-derived nitric oxide (NO) (Lassègue and Griendling, 2010). Dismutation of $O_2^{\bullet-}$ by superoxide dismutase (SOD) also generates hydrogen peroxide (H_2O_2), and the production of both reactive oxygen species (ROS) increases within minutes of exposing endothelial cells to low concentrations of arsenite (5 μM) (Barchowsky et al., 1999; Smith et al., 2001). Notably, endothelium-derived H_2O_2 is now thought to participate in the physiological response to endothelium-dependent agonists and fluid shear stress (Matoba et al., 2002; Liu et al., 2011), and can compensate for the loss of NO bioavailability observed in experimental models of hypertension and diabetes and in patients with arterial disease (Karasu, 2000; Landmesser et al., 2003; Phillips et al., 2007; Larsen et al., 2009). One possible mode of action may be an ability of H_2O_2 to relax subjacent smooth muscle cells by acting as a freely diffusible endothelium-derived hyperpolarizing factor (EDHF) (Matoba et al., 2002; Liu et al., 2011). However, H_2O_2 may also promote depletion of the

Abbreviations: ACh, acetylcholine; CPA, cyclopiazonic acid; DHE, dihydroethidium; ER, endoplasmic reticulum; K_{Ca}, calcium-activated K^+ channels; NO, nitric oxide; H_2O_2, hydrogen peroxide; L-NAME, N^G-nitro-L-arginine methyl ester; $O_2^{\bullet-}$, superoxide anion; PE, phenylephrine; RIA, rabbit iliac artery; ROS, reactive oxygen species; SOD, superoxide dismutase.

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endothelial endoplasmic reticulum (ER) Ca^{2+} store and amplify increases in cytosolic Ca^{2+} evoked by pharmacological stimulation of the endothelium (Hu et al., 2000; Edwards et al., 2008). This synergy can enhance the opening of calcium-activated K^+ channels (K_{Ca}) thereby allowing H_2O_2 to potentiate "EDHF-type" relaxations that are mediated by the spread of endothelial hyperpolarization into the arterial media *via* myoendothelial and homocellular smooth muscle gap junctions (Edwards et al., 2008; Garry et al., 2009). Recently it has been reported that EDHF-type responses to the endocannabinoid-like molecule N-oleoylethanolamine are modulated by H_2O_2 (Wheal et al., 2012).

The aim of the current study was to investigate how inorganic As^{III}, which is intrinsically more toxic than inorganic As^{V} (Vahter, 2002), affects EDHF-type and NO-mediated relaxations *via* the generation of $O_2^{\bullet-}$ and H_2O_2. Endothelium-dependent relaxations of rabbit iliac artery (RIA) and aortic rings were elicited by the G protein-coupled receptor agonist acetylcholine (ACh) and by cyclopiazonic acid (CPA), which promotes store-operated Ca^{2+} entry by depleting ER Ca^{2+} by inhibiting the endothelial SERCA pump (Fernandez-Rodriguez et al., 2009). In the RIA such relaxations consist of dual NO-mediated and EDHF-type gap junction-dependent components (Griffith et al., 2004, 2005; Chaytor et al., 2005), whereas in the aorta the EDHF-type component is negligible, so that the two mechanisms of relaxation can be dissociated (Ruiz et al., 1997; Fernandez-Rodriguez et al., 2009). The effects of arsenite were compared in the presence and absence of endogenous NO production, and the functional role of H_2O_2 investigated with catalase and a manganese-based SOD/catalase mimetic (Day et al., 1997). The role of NADPH oxidase was investigated with apocynin, which blocks the assembly of specific forms of this enzyme, and prevents the generation of $O_2^{\bullet-}$ and H_2O_2 in cultured endothelial cells treated with arsenite (Barchowsky et al., 1999; Touyz, 2008). Dihydroethidium (DHE) was used to assess ROS production in the different layers of the arterial wall (Zielonka and Kalyanaraman, 2010).

2. Methods

2.1. Mechanical responses

Iliac arteries, aortae and aortic valve leaflets (RAV) were obtained from male NZW rabbits (2–2.5 kg) killed by injection of sodium pentobarbital (150 mg/kg; i.v.) *via* the marginal ear vein and in accordance with local University guidelines. Rings of iliac artery or aorta 2–3 mm wide were mounted in a myograph (model 610M, Danish Myotechnology, Aarhus, Denmark) containing oxygenated (95% O_2; 5% CO_2) Holman's buffer (composition in mM: NaCl 120, KCl 5, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaC1_2$ 2.5, glucose 11, and sucrose 10) at 37 °C and maintained at a resting tension of 1 mN over a 60 min equilibration period, with frequent readjustments in baseline tension to correct for stress relaxation. To evaluate EDHF-type responses, preparations were incubated for 30 min with the eNOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) and the cyclooxygenase inhibitor indomethacin (10 μM) to inhibit prostanoid formation. Sodium arsenite (30 or 100 μM) was then added for 30 or 90 min prior to constriction with phenylephrine (PE, 1 μM). Once constrictor responses had reached a stable plateau, relaxation was studied by constructing cumulative concentration–response curves to CPA or ACh in the continued presence of arsenite. These curves were generally completed within ~60 min so that total cumulative exposure to arsenite was 90 min and 150 min in the two protocols. Preliminary experiments demonstrated that lower concentrations of arsenite (10 μM) did not affect relaxation under these experimental conditions. To evaluate the role of $O_2^{\bullet-}$ and H_2O_2, catalase (2000 units/ml, from bovine liver), manganese(III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP, 100 μM) or the NADPH oxidase inhibitor apocynin (1-(4-hydroxy-3-methoxyphenyl)ethanone, 100 μM) were co-administered with L-NAME and indomethacin.

2.2. Detection of superoxide/hydrogen peroxide

RAV leaflets, and endothelium-denuded rings of iliac artery and aorta were incubated with arsenite (100 μM), apocynin (100 μM) or both for 60 min in oxygenated Holman's buffer containing L-NAME (300 μM) and indomethacin (10 μM) at 37 °C. To assess the production of reactive oxygen species (ROS) dihydroethidium (DHE, 5 μM) was then added for a further 30 min, following which the preparations were washed and fixed in 4% paraformaldehyde and images collected with a Leica SP5 confocal microscope (excitation 514 nm, emission 560–630 nm). This protocol was designed to match the total exposure of rings preincubated with 100 μM arsenite for 30 min in mechanical experiments in which it took a further ~60 min to construct full concentration–relaxation curves. It should be noted that oxidation of DHE can generate two products, ethidium and 2-hydroxyethidium, which possess overlapping emission spectra and whose fluorescence is enhanced by binding to DNA (Zielonka and Kalyanaraman, 2010). Although H_2O_2 does not oxidize DHE directly and the formation of 2-hydroxyethidium is specific for $O_2^{\bullet-}$, H_2O_2 may promote the formation of ethidium in the presence of peroxidase activity or haem proteins so that increased fluorescence in DHE-loaded vascular smooth muscle/endothelial cells may reflect production of both $O_2^{\bullet-}$ and H_2O_2 (Fernandes et al., 2007; Ray et al., 2011). The RAV was used to circumvent the complicating effects of signals transmitted from subjacent smooth muscle to the endothelium. All imaging data presented were acquired in the presence of L-NAME in order to avoid potentially confounding effects of NO which has been reported to promote the formation of ethidium in the presence of molecular oxygen (Zielonka and Kalyanaraman, 2010).

Table 1
Effects of exposure to arsenite for 30 min on endothelium-dependent relaxations to CPA and ACh in the iliac artery.

CPA	*n*	pIC_{50}	R_{max}, %
Control	9	4.99 ± 0.07	94.4 ± 2.8
Arsenite 100 μM		4.98 ± 0.10	91.6 ± 3.3
L-NAME + indo	6	4.76 ± 0.07	82.7 ± 3.9
L-NAME + indo + arsenite 30 μM		4.85 ± 0.05	79.8 ± 3.3
L-NAME + indo	22	4.85 ± 0.05	82.1 ± 1.8
L-NAME + indo + arsenite 100 μM		5.16 ± 0.08***	83.1 ± 1.7
ACh	***n***	**pEC_{50}**	**R_{max}, %**
Control	12	7.32 ± 0.10	91.3 ± 2.2
Arsenite 100 μM		7.52 ± 0.10	91.1 ± 2.3
L-NAME + indo	12	6.83 ± 0.09	72.7 ± 4.1
L-NAME + indo + arsenite 100 μM		7.08 ± 0.09***	72.4 ± 4.0

Experiments were performed in the absence (control) and presence of L-NAME (300 μM) and indomethacin (10 μM). Potency (negative log IC_{50} or log EC_{50}) and maximal responses (R_{max}) are given as mean ± SEM. *n* denotes the number of animals studied.
*** $P < 0.001$ compared with time-matched preparations not exposed to arsenite.

2.3. Statistics

The maximal percentage reversal of PE-induced tone (R_{max}) by CPA or ACh and concentrations giving 50% reversal of this constrictor response (IC_{50} for CPA) or 50% of maximal relaxation (EC_{50} for ACh) were determined for each experiment. The use of IC_{50} rather than EC_{50} was necessary to allow for small initial constrictor responses to CPA that were observed in many experiments and can be attributed to an effect of CPA on smooth muscle Ca^{2+} stores (Chaytor et al., 2005). All metrics are given as mean ± SEM and compared using paired or unpaired Student's *t*-tests or one-way ANOVA followed by a Bonferroni's post-test as appropriate. Significance was accepted at $P < 0.05$; *n* denotes the number of animals studied in each experimental group.

2.4. Reagents

Pharmacological agents were purchased from Sigma–Aldrich (UK), except CPA (Ascent Scientific) and MnTMPyP (Calbiochem), and were dissolved in Holman's buffer, except apocynin and indomethacin (absolute ethanol), and CPA and DHE (DMSO).

3. Results

3.1. Effects of arsenite on responses to CPA and ACh in the RIA

3.1.1. EDHF-type relaxations

Responses evoked by CPA in the presence of L-NAME/indomethacin were unaffected by exposure to 30 μM arsenite for 30 min, whereas exposure to 100 μM arsenite for 30 min caused a leftward shift in the concentration–relaxation curve, such that pIC_{50} increased from ~4.8 to ~5.2 without change in R_{max} (Fig. 1A; Table 1). EDHF-type relaxations evoked by ACh were similarly potentiated by exposure to 100 μM arsenite for