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The role of reactive oxygen species in arsenite and monomethylarsonous acid-induced signal transduction in human bladder cells: Acute studies

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ABSTRACT

Arsenicals are known to induce ROS, which can lead to DNA damage, oxidative stress, and carcinogenesis. A human urothelial cell line, UROtsa, was used to study the effects of arsenicals on the human bladder. Arsenite [As(III)] and monomethylarsonous acid [MMA(III)] induce oxidative stress in UROtsa cells after exposure to concentrations as low as 1 μM and 50 nM, respectively. Previous research has implicated ROS as signaling molecules in the MAPK signaling pathway. As(III) and MMA(III) have been shown to increase phosphorylation of key proteins in the MAPK signaling cascade downstream of ErbB2. Both Src phosphorylation (p-Src) and cyclooxygenase-2 (COX-2) are induced after exposure to 50 nM MMA(III) and 1 μM As(III). These data suggest that ROS production is a plausible mechanism for the signaling alterations seen in UROtsa cells after acute arsenical exposure. To determine importance of ROS in the MAPK cascade and its downstream induction of p-Src and COX-2, specific ROS antioxidants (both enzymatic and non-enzymatic) were used concomitantly with arsenicals. COX-2 protein and mRNA was shown to be much more influenced by altering the levels of ROS in cells, particularly after MMA(III) treatment. The antioxidant enzyme superoxide dismutase (SOD) effectively blocked both As(III)-and MMA(III)- associated COX-2 induction. The generation of ROS and subsequent altered signaling did lead to changes in protein levels of SOD, which were detected after treatment with either 1 μM As(III) or 50 nM MMA(III). These data suggest that the generation of ROS by arsenicals may be a mechanism leading to the altered cellular signaling seen after low-level arsenical exposure.

1. Introduction

Arsenical exposure may induce reactive oxygen species (ROS), which can lead to toxicity, DNA damage, oxidative stress, and carcinogenesis (Kitchin and Ahmad, 2003; Huang et al., 2004; Shi et al., 2004a,b). Previous studies were performed with concentrations of arsenite [As(III)] greater than 10 μM, but recent studies have emerged confirming the presence of ROS and oxidative stress associated with low-levels of As(III) as well as its metabolite, monomethylarsonous acid [MMA(III)] (Lantz and Hays, 2006; Cooper et al., 2007; Wang et al., 2007a,b).

Toxic ROS, including superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2), are generated from normal cellular respiration and aerobic metabolism or by exposure to exogenous oxidants such as arsenicals, resulting in oxidation of nucleic acids, proteins, and membrane lipids. Wang et al. (2007a,b) found that 0.2 μM of the trivalent arsenicals, As(III), MMA(III), and DMA(III) were more potent inducers of oxidative damage in lipids than the pentavalent arsenical species. As(III) was shown to generate detectable amounts of $O_2^{\bullet -}$ in U937 cells, human promonocytic cells, at concentrations ranging from 1 to 10 μM (Wang et al., 2007a,b). DNA damage in HaCat cells, a human keratinocyte cell line, was detected at levels of As(III) less than 5 μM (Shi et al., 2004b). This DNA damage could be blocked by the addition of the $^{\bullet}OH$ scavenger, sodium formate. This study found that As(III) first generated $O_2^{\bullet -}$ which was converted to H_2O_2 by SOD and was further converted to the highly reactive $^{\bullet}OH$ by Fe^{2+} (Shi et al., 2004b). These studies suggest that exposure to low-concentration trivalent arsenicals [(0–10 μM As(III); 0.2 μM MMA(III)/DMA(III)] induce oxidative stress in multiple cell types.

Abbreviations: As(III), arsenite; CAT, catalase; COX-2, cyclooxygenase 2; HaCaT, human keratinocyte cell line; HELF, human embryonic lung fibroblasts; H_2O_2, hydrogen peroxide; $OH^{\bullet}$, hydroxyl radical; mel, melatonin; MAPK, mitogen activated protein kinase; MMA(III), monomethylarsonous acid; PC12, cancer cell line derived from rat adrenal medulla; KI, potassium iodide; ROS, reactive oxygen species; $^1O_2$, singlet oxygen; Src, protein tyrosine kinase named after the Rous sarcoma virus; $O_2^{\bullet -}$, superoxide; SOD, superoxide dismutase; U937, human promonocytic cells; UROtsa, human urothelial cell line.

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Upon investigating the role of oxidative stress associated with increased cellular proliferation after low-concentration As(III) treatment, Yang et al. (2007) found that 0.5 μM As(III) stimulated cellular proliferation of human lung embryonic fibroblasts (HELF), while higher concentrations (5–10 μM) actually inhibited cell growth. There was a significant positive correlation between the ROS level and the concentration of As(III) administered. For low-concentration As(III), SOD activity was significantly increased over controls, but for high-concentration, SOD activity was inhibited (Yang et al., 2007). This provides evidence that there is a concentration-dependent relationship between the level of arsenical and the amount of ROS produced. These data suggest that $O_2^{\bullet -}$ plays a role the stimulation of cellular proliferation following exposure of HELF cells to 0.5 μM As(III).

$O_2^{\bullet}$ appears to serve as a growth signal in different cells via activation of the rac/ras-MAPK signaling pathway (Kumagai and Sumi, 2007; Buetler et al., 2004; Yang et al., 2002). Over expression of SOD has been shown to inhibit proliferation, whereas inhibition of SOD by pharmacologic means increased $O_2^{\bullet -}$ levels and stimulated cellular proliferation. Down regulation of SOD by antisense oligonucleotides blocked apoptosis when associated with a moderate increase in $O_2^{\bullet -}$. A large increase in $O_2^{\bullet -}$ led to apoptosis, suggesting that a graded increase in the $O_2^{\bullet -}$ level may be the deciding factor between growth and differentiation of cells and apoptosis (Buetler et al., 2004).

Low-concentrations of arsenicals have also been shown to stimulate growth and differentiation of cells by activating specific signaling pathways. In PC12 cells, a rat tumor cell line, As(III) was shown to activate c-Jun N-terminal kinase, p38, and extracellular signal regulated kinase (ERK). This activation could be blocked by the addition of N-acetyl cysteine, suggesting a role for oxidative stress in the increased signaling observed. It has also been suggested that As(III) can bind cysteine rich areas of epidermal growth factor receptor (EGFR) leading to Ras and subsequent ERK activation. The activation of the EGFR-ERK pathway is important in mediating gene expression related to the regulation of cellular proliferation (Simeonova et al., 2002; Luster and Simeonova, 2004).

MAPK signaling is altered in multiple types of cancers, including bladder (Wadhwa et al., 2005; Eschwège et al., 2003). It is important to investigate if As(III) and MMA(III) can induce the MAPK pathways in bladder cells and determine if the induction of the MAPK pathway plays a role in the malignant transformation of cells exposed chronically to As(III) or MMA(III). Numerous studies support the effect of arsenicals on MAPK signaling. Exposures to 0.1–5 μM trivalent arsenicals led to an increased AP-1 DNA binding activity, increased phosphorylation of ERK, but not increased phosphorylation of c-jun or p38 kinases (Drobna et al., 2002). He et al. (2007) found that low concentration As(III) (0.1 and 0.5 μM) stimulated cellular proliferation in HELF cells via activation of JNK and ERK 1/2. Studies performed utilizing pharmacologic inhibitors suggest that EGFR/ErbB2, Src activation, ERK 2 phosphorylation, and PI3K activation to play important roles in the increased expression of COX-2 following 50 nM MMA(III) exposure in UROtsa cells (Eblin et al., 2007).

In the present study, an immortalized, non-tumorigenic human urothelial cell line (UROtsa) was utilized to study the effects of arsenical induced ROS generation and the subsequent activation of the MAPK signaling pathway. The contribution of specific ROS to the increased MAPK signaling was investigated utilizing both enzymatic and non-enzymatic antioxidants. Although the MAPK pathway has been shown to be activated by both ROS and arsenicals, the correlation between ROS increase caused by arsenicals leading to the activation of the MAPK pathway has not been made. In addition, little research has been undertaken to determine if there is a difference in ROS generated between the arsenical species, As(III) or MMA(III), and what roles these differences play in MAPK activation.

Thus, the goal of this study was to determine if it is the parent chemical As(III), the metabolite MMA(III), or the secondary generation of ROS that leads to the increased MAPK signaling seen after low-concentration arsenical exposure in human bladder cells. Cells were exposed to sub-cytotoxic, environmentally relevant levels of arsenicals [1 μM As(III) (74 ppb) and 50 nM MMA(III) (4.5 ppb)]. In arsenical exposed human populations in Romania, 50 nM MMA(III) has been detected in the urine (Aposhian et al., 2000). In addition to being environmentally relevant, these concentrations were specifically chosen since both 1 μM As(III) and 50 nM MMA(III) have been shown to malignantly transform UROtsa cells (Sens et al., 2004; Bredfeldt et al., 2006). By utilizing these concentrations, these acute studies can be linked with studies into the malignant transformation that occurs in UROtsa cells following chronic exposure to the same concentrations of arsenicals used in this study.

2. Methods

2.1. Chemicals

Sodium arsenite, protease inhibitor cocktail, epidermal growth factor (EGF), insulin, peg-superoxide dismutase, peg-catalase, melatonin, and potassium iodide were purchased from Sigma Chemical Company (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Modified Eagle Medium: Ham's F12 (DMEM:F12), fetal bovine serum (FBS), antibiotic–antimycotic, and 1× trypsin–EDTA (0.25%) were acquired from Gibco Invitrogen Corporation (Carlsbad, CA). Diiodomethylarsine (MMA(III) iodide, CH_3AsI_2) was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) using the method of Millar et al. (1960). Water used in studies was distilled and de-ionized. Ras Assay Reagent consisting of Raf-1 RBD and agarose, Anti-Ras clone RAS10, Mg^{2+} Lysis/Wash Buffer, GTPγS (10 mM), and GDP (100 mM) were all purchased as part of the Ras Activation Assay Kit (Upstate, Temecula, CA).

2.2. Cells

UROtsa cells were a generous gift from Drs. Donald and Maryann Sens (University of North Dakota). Cell culture conditions were previously described by Bredfeldt et al. (2004). Prior to experimentation, cells were fed a serum-free growth medium made up of 1:1 mixture of DMEM and Ham's F-12 supplemented with insulin (5 μg/ml), hydrocortisone (36 ng/ml), and epidermal growth factor (10 ng/ml).

2.3. MTT assay for cytotoxicity of ROS antioxidants (non-enzymatic)

This colorimetric assay was derived from Wang et al. (2007a,b). Antioxidant-induced toxicity was measured by comparing treated cells with untreated control cells, measuring the ability of mitochondrial reductase enzymes to catalyze the reduction of the yellow 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) compound to a purple formazan. Healthy control cells would have the highest activity of enzymes, so comparison between treatment groups can be performed. Mitochondria are a location of ROS generation, so this specific assay was chosen to monitor the health of the mitochondria following the various treatments. For the MTT assay of UROtsa cells, approximately 8×10^3 cells per well were seeded in a 96-well plate and incubated overnight. Cells were treated with several concentrations of ROS inhibitors for 24 h followed by the addition of 20 μl of 2 mg/ml MTT directly into the medium. After incubation (37 °C for 0.5–3 h), the plate was centrifuged and the medium removed. 100 μl of isopropanol/HCl was added into each well and crystals were dissolved by shaking the plate at room temperature. Absorbance was measured by a plate reader at 570 nm. Triplicate wells were used for each sample and the experiments were repeated at least three times to get means and standard deviations.

2.4. Trypan blue exclusion assay for cytotoxicity of ROS antioxidants and arsenicals in combination

This assay was derived from a previously described method by Bredfeldt et al. (2006). Cells were plated in 6-well plates at a density of 2×10^5 cells per well and allowed to grow for 24 h. To determine toxicity associated with these compounds, 5 mM KI, 2.5 mM melatonin, 200 units/ml peg-SOD, or catalase were added to a well for 2 h. Next, 1 μM As(III) or 50 nM MMA(III) was added to each well for a period of 24 h. Cell density for UROtsa cells treated with ROS antagonists was obtained via trypan blue exclusion assay.