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Critical role of cellular glutathione homeostasis for trivalent inorganic arsenite-induced oxidative damage in human bronchial epithelial cells

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ABSTRACT

Trivalent inorganic arsenic (iAs^{3+}) is a powerful carcinogen that enhances the risk of lung cancer. Paradoxically, iAs^{3+} also shows substantial efficacy in the treatment of lung tumors. However, the exact molecular mechanisms underlying iAs^{3+}-induced toxicity and therapeutic effect in lung remain unclear. In this study, the effects of iAs^{3+}, sodium arsenite ($NaAsO_2$) and arsenic trioxide (As_2O_3), on cell viability, apoptosis, genotoxicity and oxidative stress in cultured human bronchial epithelial cells were observed. Our results showed that $NaAsO_2$ and As_2O_3 exposure could result in defects in cell proliferation and greatly enhance the level of oxidative damage. To clarify the critical role of glutathione (GSH) homeostasis in oxidative damage induced by iAs^{3+}, we further measured the content of GSH, ratio of GSH to GSSG, and the activities of GSH-related enzymes involved in the process of GSH synthesis, recycling and utilization. Our data demonstrated that $NaAsO_2$ and As_2O_3 disrupted the balance of GSH homeostasis, and $NaAsO_2$- and As_2O_3-induced oxidative damage was closely associated with the imbalance in GSH synthesis, recycling and utilization. To better understand the physiologic significance of Nrf2 in maintaining GSH-homeostasis, the expression level of Nrf2 was measured after iAs^{3+} exposure. We found that the protein expression levels of Nrf2 were increased in both $NaAsO_2$- and As_2O_3-treated cells. Collectively, our findings suggest that disturbed Nrf2-regulated GSH-homeostasis is associated with the oxidative damage triggered by iAs^{3+}, and loss of GSH homeostasis might implicate in both the pathogenesis of iAs^{3+}-induced lung diseases and anticancer activity of iAs^{3+}.

1. Introduction

Trivalent inorganic arsenic (iAs^{3+}) is a toxic and carcinogenic environmental contaminant that humans are inadvertently exposed to every day through drinking water, food and air. Chronic exposure to iAs^{3+} is associated with various human disorders, including hypertension, cardiovascular disease and many types of cancer [1–3]. Paradoxically, iAs^{3+} is a novel anticancer agent that can be used to treat acute promyelocytic leukemia and some solid tumors [4]. Lung is one of the sensitive target organs for iAs^{3+} toxicity and increasing evidence, originating from studies in Taiwan [5], USA [6], Chile [7] and Argentina [8], indicates that ingestion or inhalation of iAs^{3+} enhances the risk of lung cancer. Intriguingly, iAs^{3+} is also under investigation as a treatment for lung tumors [4]. As a double-edged sword, iAs^{3+} induces both carcinogenic and anticancer effects in lung, and the underlying molecular mechanisms for its toxicity and therapeutic effect are still unclear. Oxidative stress was proposed to be a likely mechanism for the dual effects

Abbreviations: As_2O_3, arsenic trioxide; BCA, bicinchoninic acid; DCFH-DA, 2′7′-dichlorofluorescin diacetate; DMEM, dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FITC, fluorescein isothiocyanate; GCLC, catalytic subunit of γ-glutamylcysteine synthetase; GCLM, modifier subunit of γ-glutamylcysteine synthetase; γ-GCS, γ-glutamylcysteine synthetase; GR, glutathione reductase; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione disulfide; HBE, human bronchial epithelial cell line; iAs^{3+}, trivalent inorganic arsenic; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OTM, Olive tail moment; PI, propidium iodide; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase; $NaAsO_2$, sodium arsenite; Nrf2, nuclear erythroid-related factor 2.

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of iAs^{3+} in lung [2,9,10]. Evidence of increased oxidative stress induced by iAs^{3+}, such as DNA oxidation and enhanced free radical production, is observed both in cell culture systems and the biological samples of humans and rodents exposed to iAs^{3+} [9,11,12].

Intracellular glutathione (GSH), a biologically active thiol tripeptide, is the most abundant non-protein thiol with many biological functions in mammalian cells [13]. As a carrier of thiol, GSH typically acts as an antioxidant by interacting with free radicals, and the elevation of intracellular GSH level exerts a protective effect in counteracting oxidative stress [14]. Cells will become extremely vulnerable to oxidation triggered by iAs^{3+} when intracellular GSH is depleted [15,16]. Moreover, GSH plays an important role in biotransformation and efflux of iAs^{3+} [17–19]. Since GSH functions not only in antioxidant defense systems but also in the metabolic process of iAs^{3+}, it is not surprising that GSH has attracted much attention as a possible target for chemoprevention against iAs^{3+} toxicity. More importantly, regulation of GSH has also been suggested to be a novel therapeutic strategy to overcome the resistance to iAs^{3+} in cancer treatment [20].

GSH homeostasis is a vital and complicated system that regulates the cellular redox status and protects airway epithelial cells from oxidant-induced lung injury [21]. In general, GSH homeostasis is regulated by the following three aspects: (i) GSH is synthesized from glutamate, glucine and cysteine by glutathione synthetase and γ-glutamylcysteine synthetase (γ-GCS). (ii) GSH is oxidized to GSSG by the activity of GSH peroxidase (GSH-Px), thus regulating and maintaining cell redox status. (iii) GSSG is reduced to GSH with the mediation of GSH reductase (GR), by which a substantial portion of intracellular GSH is recycled back [22]. Given that GSH homeostasis is modulated by self-adjusting the balance among GSH synthesis, utilization and recycling, the disturbances of these processes may contribute to the pulmonary oxidant/antioxidant imbalance and the pathogenesis of lung diseases [14]. So far, it is not clear how iAs^{3+} affects the process of GSH homeostasis, and whether the maintenance of GSH homeostasis reduces iAs^{3+} toxicity and/or decreases the anticancer sensitivity of iAs^{3+} remain obscure.

Nuclear erythroid-related factor 2 (Nrf2) is a redox-sensitive transcription factor involved in transcriptional regulation of many antioxidant genes, including γ-GCS gene [23]. The activities of many GSH-related enzymes, such as GSH-Px and GR, are also up-regulated through the Nrf2-Keap1-ARE (antioxidant response elements) pathway [24]. Substantial studies suggested that Nrf2 exerts a protective role against iAs^{3+} toxicity, even at low concentrations, by which iAs^{3+}-induced pathological alterations, such as lung inflammatory response, are prevented [25]. However, whether and how Nrf2 modulates GSH homeostasis that governs iAs^{3+}-related lung carcinogenesis and therapeutic efficacy are poorly understood.

We previously reported that As_2O_3 and $NaAsO_2$ exerted cytotoxicity, genotoxicity and apoptosis-inducing effect in human lung adenocarcinoma A549 cells, and demonstrated that oxidative stress played a critical role in iAs^{3+}-induced anticancer effect [26]. In view of the importance of GSH homeostasis in counteracting oxidant-induced injury and increasing epidemiologic evidence between iAs exposure and lung diseases, such as bronchitis and bronchiectasis, which are related to the lesions of bronchial epithelial cells [27], in this study, we focus primarily on the alteration of intracellular GSH homeostasis following iAs^{3+} exposure, along with the effects of iAs^{3+} on oxidative stress, apoptosis, DNA and chromosomal breakage in human normal bronchial epithelial (HBE) cells. Our results would provide the first evidence of iAs^{3+} on the effect of GSH homeostasis in HBE cells which stem from human normal bronchial epithelial. In addition, the expression levels of Nrf2 after iAs^{3+} treatment are also presented in this study due to the possible role of Nrf2 in the regulation of cellular defense against oxidative stress.

2. Materials and methods

2.1. Chemicals and reagents

$NaAsO_2$, purity ≥ 99.0%, was obtained from Fluka Chemical Co. (Buchs, Switzerland), and As_2O_3 was purchased from YiDa Pharmaceutical Co. Ltd. (Harbin Medical University, Heilongjiang, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), low-melting agarose, normal-melting agarose, acridine orange, ethidium bromide were all purchased from Amresco Co. (Ohio, USA). 2′7′-dichlorofluorescin diacetate (DCFH-DA) was from Applygen Technologies Inc. (Beijing, China), and the apoptosis detection kit was obtained from KeyGen Biotechnology (Nanjing, China). Glutathione (GSH) and oxidized disulfide (GSSG) assay kit, GSH reductase (GR) assay Kit, GSH peroxidase (GSH-Px) detection kit and cell lysis buffer were all from Beyotime Institute of Biotechnology (Jiangsu, China). γ-glutamylcysteine synthetase (γ-GCS) detection kit and superoxide dismutase (SOD), malondialdehyde (MDA) assay kits were purchased from Research Institute of Nanjing Jiancheng Bio-engineering (Nanjing, China). Rabbit polyclonal GCLC, GCLM and Nrf2 antibodies were obtained from Abgent Inc. (San Diego, CA, USA). Mouse monoclonal β-actin antibody and horseradish peroxidase conjugated secondary antibody were purchased from ZSGB Bio. (Beijing, China). Dulbecco's modified Eagle's medium (DMEM), trypsin and fetal bovine serum were all purchased from Gibco Life Technologies. (Grand Island, NY, USA).

2.2. Cell culture

Human bronchial epithelial (HBE) cell line was generously provided by Stem Cells and Tissue Engineering Laboratory, State Key Laboratory of Biotherapy, Si Chuan University (Chengdu, China). Cells were routinely maintained in high glucose DMEM containing 100 units/ml penicillin and 100 μg/ml streptomycin supplemented with 10% (v/v) fetal bovine serum in a humidified incubator at 37 °C with 5% CO_2. The culture medium was replaced with fresh medium every 2–3 days.

2.3. Arsenic exposure

Cells were plated onto culture plates at a proper density with complete DMEM media. After overnight culture, cells were treated with desired concentrations of As_2O_3 or $NaAsO_2$ for up to 24 h, and then used for assays.

2.4. Cytotoxicity measured by MTT assay

The cytotoxicity of As_2O_3 or $NaAsO_2$ was determined by MTT assay according to the protocol previously described by Saravanan et al. [28] with some modifications. Briefly, after treatment, cells were incubated with MTT solution (0.5 mg/ml, 100 μl/well) at 37 °C for another 4 h. Subsequently, the supernatant was discarded and dimethyl sulfoxide (DMSO, 100 μl/well) was added into wells to dissolve the formed formazan crystals. The absorbance was measured on a Bio-Rad micro-plate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Additionally, DMSO was served as a blank control, and experiments were conducted in twelve replicates for each treatment. Cell viability (%) was expressed as a percentage of optical density in treatment group compared to untreated control.

2.5. Measurement of intracellular reactive oxygen species (ROS)

The production of intracellular ROS was detected by an oxidation-sensitive fluorescent probe (DCFH-DA) as described previously [29]. After exposure to As_2O_3 or $NaAsO_2$, cells were incubated with DCFH-DA at a final concentration of 10 μM at 37 °C in the dark for 1 h. Afterward, cells were washed with D-hanks' solution and visualized under a fluorescence microscope (DMLB2, Leica, Wetzlar, Germany). The fluorescence intensity was calculated according to the following formula: average fluorescence intensity = sum of integrated optical density (IOD)/sum area. The values of sum (IOD) and sum area were obtained by the Image-Pro® Plus 6.0 software (Media Cybemetics Inc., USA).

2.6. Measurement of cellular superoxide dismutase (SOD) activity and level of lipid peroxidation

Total intracellular SOD activity and MDA content were detected by commercial assay kits, respectively. Briefly, the treated cells were harvested and lysed, followed by centrifugation at 12,000 × *g* at 4 °C for 10 min. Subsequently, the supernatant was collected and further analyzed for SOD activity or MDA content according to the manufacturer's protocols. The absorbance was measured at a wavelength of 550 nm (SOD activity) or 532 nm (MDA content) using a spectrometer (V-1100D, Mapada instruments Co., Ltd. Shanghai, China). In addition, the SOD activity and MDA content were normalized for the protein concentration by a Bicinchoninic Acid (BCA) protein assay kit (Cwbio Biotech Co., Ltd., Beijing, China).