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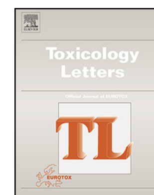

The protective role of Nrf2-Gadd45b against antimony-induced oxidative stress and apoptosis in HEK293 cells

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HIGHLIGHTS

- Sb_2O_3-induces apoptosis in a ROS-dependent manner in HEK293 cells.
- Nrf2 protects HEK293 cells against Sb_2O_3-induced apoptosis.
- Gadd45b drives activation of MAPKs upon Sb_2O_3 exposure.
- Nrf2 transcriptionally actives Gadd45b expression against Sb_2O_3-induced apoptosis.

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ABSTRACT

Antimony (Sb) is one of the most prevalent heavy metals and frequently causes biological toxicity. However, the specific mechanisms by which Sb elicits its toxic effects remains to be fully elucidated. In this study, we found antimony trioxide (Sb_2O_3) caused a dose-dependent cytotoxicity against HEK293 cells, and Sb_2O_3-induced excessive reactive oxygen species (ROS) was closely correlated with increased cell apoptosis. Mechanistic investigation manifested that nuclear factor NF-E2-related factor 2 (Nrf2) expression and nuclear translocation were significantly induced under Sb_2O_3 treatment in HEK293 cells, and Nrf2 knockdown aggregated Sb_2O_3-induced cell apoptosis. Moreover, elevated Gadd45b expression actives the phosphorylation of MAPKs upon Sb_2O_3 exposure, whereas Gadd45b knockdown diminished Sb_2O_3-induced activation of MAPKs and promoted cell apoptosis. In the meantime, however, the antioxidant N-acetylcysteine (NAC) was found to ameliorate Nrf2 expression and nuclear translocation as well as Gadd45b expression and MAPKs activation by repressing Sb_2O_3-induced ROS production. More importantly, we found Gadd45b was transcriptionally enhanced by Nrf2 through binding to three canonical antioxidant response elements (AREs) within its promoter region. Either Sb_2O_3 or TBHQ (a selective Nrf2 activator) treatment, Gadd45b expression was significantly increased by luciferase assay. Nrf2 inhibition greatly diminished Gadd45b expression due to reduced binding of Nrf2 in Gadd45b promoter under Sb_2O_3 treatment. To summarize, this study demonstrated the Nrf2-Gadd45b signaling axis exhibited a protective role in Sb-induced cell apoptosis.

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1. Introduction

Antimony (Sb) is a silvery-white metal that is ubiquitous throughout the environment as a result of natural processes and human activities (Sundar and Chakravarty, 2010). Due to mining and smelting processes, large quantities of Sb have been released resulting in serous Sb contamination of our living environments. Furthermore, coal combustion and Sb-associated products consumed in daily life are also important potential Sb contamination sources (He et al., 2012; Pierart et al., 2015). Environmental

Abbreviations: Sb, Antimony; NAC, *N*-acetylcysteine; Nrf2, NF-E2-related factor 2; ROS, Reactive oxygen species; Sb_2O_3, Antimony trioxide; TBHQ, Tertiary butylhydroquinone.

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exposure of Sb may cause respiratory irritation, pneumoconiosis, spotting on the skin and gastrointestinal symptoms (Sundar and Chakravarty, 2010). Although a large number of epidemiological studies of Sb exposure have been documented, its toxicity on health (e.g. nephrotoxicity) remains unknown and is of great concern.

Reactive oxygen species (ROS) production is believed to be the major mechanism underlying toxic metal's cytotoxicity to normal cells (Al-Gubory, 2014; Phaniendra et al., 2015). Even though excessive ROS production induced by Sb-containing compounds have been reported, a more complete characterization of its toxicity on normal cells is still limited (Lecureur et al., 2002a, 2002b; Losler et al., 2009; Mann et al., 2006). The transcription factor, NF-E2-related factor 2 (Nrf2), has been shown to regulate the expression of a network of cytoprotective enzymes for cellular defense against the environmental toxins and toxicants (Ma, 2013). Under normal conditions, the level of Nrf2 protein is present in the cytoplasm in association with its inhibitor Kelch-like ECH-asscociated protein 1 (Keap1), and binding of Keap1 to Nrf2 leads to biquitination of Nrf2 and proteosomal degradation. Upon exposure to endogenous activators (e.g. reactive oxygen species) or exogenous agents (e.g. heavy metals), Nrf2 dissociates from Keap1 and translocates into the nucleus. Through binding to antioxidant response elements (AREs), nucleus Nrf2 transcriptionally activates of its antioxidant target genes, such as glutathione S-tansferase, heme oxygenase-1, multidrug-resistance associated efflux pumps and NAD(P)H dehydrogenase quinone (NQO)-1 (Kobayashi and Yamamoto, 2005; Li and Kong, 2009). Until now, burgeoning evidence demonstrated the ability of Nrf2 to control cytoprotective adaptive response against environmental or industrial pollutions, such as arsenic, cadmium, chromium, copper, lead and mercury (Jiang et al., 2015; Lau et al., 2013; Simmons et al., 2011; Son et al., 2014; Toyama et al., 2011; Ye et al., 2015). However, whether Nrf2 also conducts cytoprotection against Sb-induced cell toxicity still largely unknown thus far.

The members of Gadd45 gene family, Gadd45a, Gadd45b and Gadd45r, have been commonly implicated in stress signaling in response to physiological or environmental stressors, resulting in cell cycle arrest, DNA repair, cell survival and apoptosis (Salvador et al., 2013). Gadd45b has been implicated as an anti-apoptosis factor through activation MAPKs pathway in resistance to apoptosis initiated by a variety of stimuli (Ou et al., 2010; Salerno et al., 2012; Yang et al., 2009; Yoo et al., 2003). Nonetheless, we speculated whether Gadd45b could also provide protection from heavy metal induced cells apoptosis through MAPKs activation, and specifically, it is still unclear whether Gadd45b was regulated by Nrf2 under Sb-induced cell death.

In the present study, we uncovered a critical protective role of Nrf2-Gadd45b pathways against oxidative damage induced by Sb in human embryonic kidney (HEK) 293 cells. Our combined data therefore highlighted a crucial role of Nrf2-Gadd45b in modulating Sb-induced apoptotic cell death.

2. Materials and methods

2.1. Cell culture and reagents

The HEK293 cells were obtained from the Shanghai Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Cells were routinely maintained in 1640 medium (Hyclone) with 10% fetal bovine serum (Gibco) and 100 U/mL penicillin-streptomycin (Hyclone) at 37 °C in humidified atmosphere with 5% CO_2. Antimony trioxide (Sb_2O_3) and *N*-acetylcysteine (NAC) were purchased from Sigma-Aldrich. Tertiary butylhydroquinone (TBHQ) was obtained from Santa Cruz.

2.2. Cytotoxicity assessment

MTT assay was carried out to examine cytotoxicity upon chemical treatment following the instructions from manufacturer (Roche). In brief, cells were seeded in 96-well plates and then treated with Sb_2O_3 at different concentration. After treatment for 24 h, 20 μL of MTT (5 mg/mL) was added into complete culture media and cells were cultured for additional 4 h. Thereafter, the medium with MTT were aspirated, and 200 μL of DMSO was added into each well. Finally, 96-well plates were read at 490 nm on a microplate reader (Thermo).

2.3. Intracellular ROS level

Cells were cultured into 96-well pates and exposed to Sb_2O_3 in accordance with the manufacturer's instruction (Sigma-Aldrich). Dichlorofluorescein diacetate (DCF-DA) was added at a final concentration of 10 μM in the dark for 30 min before examination. Cells were then washed with PBS three times, and DCF fluorescence was then monitored using a microplate reader. The excitation and emission wavelength were 488 and 525 nm, respectively, and 0.1% H_2O_2 was used as a positive control to induce ROS in cells.

2.4. Apoptosis detection by flow cytometry analysis

Sb-induced apoptosis was determined by flow cytometry using the Annexin V-FITC apoptosis Detection Kit following the instructions from the manufacturer (BD Biosciences). In brief, cells after Sb_2O_3 treatment were collected and washed twice with PBS. Then, cells were stained with 5 μL of Annexin V-FITC and 5 μL of PI for 15 min. A total of 10,000 cells in each sample was analyzed by flow cytometry. The percentage distributions of early apoptosis and late apoptosis were calculated for comparison.

2.5. Western blot analysis

Whole cell proteins were extracted using ice-cold RIPA lysis buffer (Solarbio Biotechnology) supplemented with protease inhibitor cocktail (Roche). Nuclear or cytoplasmic protein were separated with the cytoplasmic and nuclear proteins extraction kit (Solarbio Biotechnology). Protein concentrations were determined with a Lowry protein concentration detection kit (Solarbio Biotechnology). About 50–150 μg of protein from each sample was denatured, electrophoresed, and transferred onto nitrocellulose membranes (Millipore). Western blotting analysis was performed with specific antibodies against p-JNK, JNK, p-ERK, ERK, p-P38 and P38 (Santa Cruz), caspase3, caspase7, Bax and Bcl2 (Cell signaling). Antibodies against Nrf2, Gadd45b, Histone 3 and β-actin were purchased from Proteinteach. The intensities of the autoradiograms were quantitated with the software Image J, and the band intensity was normalized to those of loading control.

2.6. qRT-PCR analysis

Total RNAs were isolated from cells with Trizol reagent following the manufacturer's instructions (Invitrogen). Approximately 2 μg of total RNAs was reverse-transcribed into cDNA with M-MLV reverse transcriptase (Promega). Gene expression levels were evaluated using SYBR Green qPCR master mix (Promega) on Mx3005P qPCR machine (Stratagene). β-actin was used as an internal control to determine the relative expression of target genes. The primer sequences for real-time PCR are shown in Supplemental data.

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