1,4-benzoquinone-induced STAT-3 hypomethylation in AH1-1 cells: Role of oxidative stress

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1. Introduction

Benzene, a known occupational and environmental contaminant, is associated with increased risk of leukemia. The objectives of this study were to elucidate the regulatory mechanism of the hypomethylated STAT3 involved in benzene toxicity in vitro. As 1,4-benzoquinone (1,4-BQ) is one of benzene’s major toxic metabolites, AH1-1 cells were treated by 1,4-BQ for 24 h with or without pretreatment of the antioxidant α-LA or the methyltransferase inhibitor, 5-aza-2′ deoxycytidine (5-aza). The cell viability was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. ROS was determined via 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) flow cytometric assays. The level of oxidative stress marker 8-OHdG was measured by enzyme-linked immunosorbent assay. Methylation-specific PCR was used to detect the methylation status of STAT3. Results indicated the significantly increasing expression of ROS and 8-OHdG which accompanied with STAT3 hypomethylation in 1,4-BQ-treated AH1-1 cells. α-LA suppressed the expression of both ROS and 8-OHdG, simultaneously reversed 1,4-BQ-induced STAT3 hypomethylation. However, although the methylation inhibitor, 5-aza reduced the expression level of ROS and 8-OHdG, but had no obvious inhibiting effect on STAT3 methylation level. Taken together, oxidative stress are involved 1,4-BQ-induced STAT3 methylation expression.

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on 1,4-benzoquinone induced global DNA hypomethylation [9,14,24-26,33].

In this study, AHH-1 cells were treated by benzene metabolites 1,4-benzoquinone (1,4-BQ) for 24 h with or without pretreatment of the clinical application of antioxidant alpha lipoic acid (α-LA) or the methyltransferase inhibitor, 5-aza-2 deoxycytidine (5-aza) to explore the role of oxidative stress in 1,4-BQ-induced STAT3 methylation expression.

2. Materials and methods

2.1. Cell culture and 1,4-BQ exposure

The Homo sapiens B lymphocyte, AHH-1 (ATCC® CRL-8146™), obtained from the research group of Prof. Liu QL, were cultured in RPMI 1640 medium (HyClone, USA) with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% horse serum (Gibco, USA) at 37 °C in a humidified incubator with 5% CO2. For experiments, cells were seeded in culture plates and the varying concentrations of 1,4-BQ (Sigma–Aldrich, USA) (0, 10, 20, 40, 60, 80 μM) treated AHH-1 cell lines for 24 h and control was black vector (no any treatment). The study was approved by the Committees for Ethical Review of Research involving Subjects of Capital Medical University.

2.2. α-LA and 5-aza treatment

The antioxidant agent α-LA (Sigma, St. Louis, MO) and the methyltransferase inhibitor 5-aza (Sigma, St. Louis, MO) were dissolved in phosphate buffered saline (PBS) pH 6.8 and stored at −20 °C, respectively. After the exponentially grown AHH-1 cells were incubated in culture medium with 5-aza (5, 10, 20 μM) or α-LA (0.1, 1, 10, 100 μM) for one hour, 40 μM 1,4-BQ was added to culture medium for 24 h.

2.3. Cell viability assay

The cytotoxicity of 1,4-BQ was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was from AMRESCO (USA). Cell morphology was observed by light microscope (OlympusX81, Japan). Briefly, 1 × 10⁴ cells were seeded into a 96-well plate in a volume of 100 μl RPMI 1640 and incubated for 24 h at 37 °C. Cells were treated with varying concentrations of 1,4-BQ (0, 10, 20, 40, 60, 80 μM) for 24 h at 37 °C. After 24 h incubation, 10 μL MTT was added to each well and further incubated for 4 h. After which 150 μl dimethylsulfoxide (DMSO) was added and mixed thoroughly for 10 min. Optical density was then measured using a microplate reader (Thermo Multiskan MK3, USA) at 577 nm.

2.4. Intracellular reactive oxygen species (ROS) measurement

ROS were measured by flow cytometry using 2,7 dichlorofluorescin diacetate (DCFH-DA) (Jiancheng, China) according to the methodology (Cardiovascular toxicity evaluation of silica nanoparticles in endothelial cells and zebrafish model). 3 × 10⁴ cells were seeded into a 6-well plate for 24 h. After cells were exposed to series concentrations of 1,4-BQ (0, 10, 20, 40, 60, 80 μM) for 24 h, the cells were washed twice with PBS and co-incubated with serum-free RPMI 1640 containing 10 μM DCFH-DA for 30 min at 37 °C in dark. Subsequently, the 1 × 10⁶ cells were harvested and rinsed with PBS. Fluorescent intensities were measured at 488 nm excitation, 525 nm emissions using a flow cytometer (Becton Dickson, USA).

2.5. Oxidative stress marker 8-OHdG were detected

Cells were washed once with ice-cold PBS, and lysed in ice-cold RIPA lysis buffer containing 1 mM phenylmethylsulphonyl fluoride (PMSF) (DingGuo, China) and phosphatase inhibitor for 30 min after AHH-1 cell were exposed to 1,4-BQ for 24 h. After centrifuging the lysates at 12,000 rpm, 4 °C for 10 min and the supernatants were collected. Then, the supernatants were centrifuged and stored at −80 °C until use. The levels of oxidative stress marker 8-OHdG was measured by enzyme-linked immunosorbent assay (ELISA) kits (the minimum detection limit is 10 ng/L (Abcam, Catalog #KOG-200SE, Baltimore, MD 21230 USA) according to the manufacturer’s protocols. Briefly, 100 μL of supernatants were added in each well and incubated for 2.5 h at room temperature. Then, biotin antibody was added to each well and incubated for 1 h. After that, Streptavidin solution was added and incubated for 45 min. Next, the substrate reagent was added and incubated for 30 min. After the stop solution was added to each well, the absorbance at 450 nm was detected immediately using a micro-plate reader (Thermo Multiskan™ MK3; Thermo Fisher Scientific).

2.6. DNA isolation and methylation-specific PCR (MSP) to evaluate STAT3 methylation status

Genomic DNA was extracted in accordance with the protocols from EZNA-DNA kit (Omega). Then, 1 μg of the purified DNA was subjected to bisulfite modification which was performed using CpGenome DNA Modification Kit (Chemicon International, USA) according to the manufacturer’s instructions. CpGenome Universal Methylated DNA (Serologicals, Atlanta, USA) and normal human blood DNA were used as positive control for methylated and unmethylated status. Water blank was used as negative control. The following primer sets were used: for methylated DNA, MF-STAT3 (5′-TATCGTTTTTGTATGGTGATC-3′) and MR-STAT3 (5′-CCTACTTTAAACTCATTTACCTA-3′), and for unmethylated DNA, UF-STAT3 (5′-TTGTTTGTATGGTGATG-3′) and UR-STAT3 (5′-CCTACTTTAAACTCATTTACCTA-3′). Platinum Taq polymerase (Invitrogen, California, USA) was used. PCR reactions were performed in 20 μl volumes under the following conditions: 95 °C for 10 min, then 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and finally 7 min at 72 °C. The PCR products for methylated and unmethylated STAT3 were both 192 and 190 bp, respectively, and were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide and visualized under a UV illuminator. Distinct visible band of the amplification with methylation-specific primers was considered positive. All assays were performed in triplicate. The density of each band was analyzed with image analysis software (GelPro 4.5) for quantitation. STAT3 methylation level in each group was expressed as a fold of the band density relative to that of control.

2.7. Statistical analysis

Data were reported as means ± SDs. Differences between experimental and control groups were analyzed by Kruskal–Wallis test (SPSS 17 for Windows), P < 0.05 was considered as statistically significant. Each experiment was repeated for at least three times independently.

3. Results

3.1. 1,4-BQ induced cytotoxicity in a dose-dependent manner

AHH-1 cells were exposed to various doses of 1,4-BQ (0, 10, 20, 40, 60, 80 μM) for 24 h. MTT assays revealed the concentration-dependent toxic effects. Among them, cell viability in more than
Fig. 1. 1,4-BQ induced cellular toxicity in AHH-1 cells. The various concentrations of 1,4-BQ (0, 10, 20, 40, 60, 80 μM) treated AHH-1 cell lines for 24 h, then cell viability was measured by MTT assays. *P < 0.05, comparing with control group.

40 μM 1,4-BQ group were lower than 80% (Fig. 1). Therefore, 40 μM 1,4-BQ were used to consequent experiments.

3.2. 1,4-BQ induced intracellular ROS generation in AHH-1 cells

The induction of oxidative stress might be involved in the cytotoxic effects. To get a closer insight into cytotoxicity induced by 1,4-BQ, we measured the ROS generation through DCFH-DA. As shown in Fig. 2, after AHH-1 cells were exposed to 1,4-BQ for 24 h, the intracellular ROS levels in all treated groups were increased gradually. The fluorescence intensity in 40 μM and 60 μM 1,4-BQ treated groups was significantly higher than that of control, respectively.

Fig. 3. 1,4-BQ induced STAT3 hypomethylation in AHH-1 cells. The various concentrations of 1,4-BQ (0, 10, 20, 40, 60, 80 μM) treated AHH-1 cell lines for 24 h, then DNA was extracted and STAT3 methylated expression were detected by methylation-specific PCR method. *P < 0.05, comparing with control group; #P < 0.05, comparing with 1,4-BQ group.

8-OHdG is an internationally recognized biomarker evaluating oxidative DNA damage and oxidative stress [31]. We speculated that 1,4-BQ-induced ROS generation could cause oxidative damage followed by the production of 8-OHdG. After the AHH-1 cell were exposed to 1,4-BQ for 24 h, cells were lysed and the supernatants were used to detect the level of oxidative stress marker 8-OHdG by ELISA assay. As shown in Fig. 2, the level of 8-OHdG was mild elevated in 1,4-BQ group compared to control.

3.3. 8-hydroxy deoxyguanosine adduct (8-OHdG) level elevated in 1,4-BQ-treated AHH-1 cells

Our published study has shown that the aberrant hypomethylated STAT3 might be a potential biomarker of benzene hematoxity in workers occupationally exposed to benzene [31], but the potential mechanisms are not yet clear. In the current study, the various doses of 1,4-BQ (0, 10, 20, 40, 60, 80 μM) treated AHH-1 cells for 24 h, then STAT3 methylation status was detected using MSP method. As shown in Fig. 3, STAT3 methylation levels decreased in 1,4-BQ-treated AHH-1 cells in a dose-dependent manner which implicated that 1,4-BQ activated the expression level of STAT3 encongene. The result in vitro is in accordance with our previous published population study occupationally exposed to benzene [34].

3.4. 1,4-BQ lead to STAT3 methylation status alternation in AHH-1 cells

In vitro exposure of AHH-1 cells to the 1,4-BQ led to intracellular ROS generation. The α-LA, the natural and strongest antioxidant, has been widely used in the prevention and treatment of clinical disease. In this study, we observed that the ROS scavenger α-LA only at lower dose (0.1 μM) markedly reduced 1,4-BQ-induced ROS level (Fig. 4A). Interestingly, the higher dose (more than 1 μM) α-LA cannot continuously inhibit ROS level. In addition, the effect of α-LA on 8-OHdG was simultaneously investigated, result indicated that α-
LA attenuated 1,4-BQ-induced 8-OHdG level in a dose-dependent manner (Fig. 4B).

3.6. 1,4-BQ-induced STAT3 methylation status was influenced by α-LA in AHH-1 cells

Oxidative stress is one key toxic mechanisms of benzene [3,11,17,20,23,28,29]. To elucidate the role of oxidative stress in 1,4-BQ-induced STAT3 hypomethylation, clinical application of antioxidant α-LA pretreated cells at 0.1, 1, 10, 100 μM for 1 h, then 40 μM 1,4-BQ treated AHH-1 cell lines for another 24 h, MSP was used to detect the methylation status of STAT3. Results indicated that the ROS scavenger α-LA at low dose reversed the expression level of STAT3 methylation induced by 1,4-BQ in AHH-1 cells (Fig. 5), which suggested that oxidative stress negatively regulated 1,4-BQ-induced STAT3 methylation expression. Interestingly, 0.1 μM α-LA showed the most obvious inhibiting effect which might exist in the low dosage-hormesis effect.

3.7. Effect of STAT3 methylation status on 1,4-BQ-induced oxidative stress

To further elucidate the role of oxidative stress in 1,4-BQ-induced STAT3 hypomethylation, After the methyltransferase inhibitor 5-aza pretreated cells at 5, 10, 20 μM for 1 h, 40 μM 1,4-BQ treated AHH-1 cell lines for another 24 h, then ROS, 8-OHdG and the expression level of STAT3 methylation were detected. Results indicated that 10 μM 5-aza significantly decreased 1,4-BQ-induced ROS levels (Fig. 6A). 5-aza in all dose groups inhibited 8-OHdG expression induced by 1,4-BQ (Fig. 6B). However, the methylation inhibitor 5-aza had no obvious inhibiting effect on the expression level of STAT3 methylation (Fig. 7).

4. Discussion

In this study, the oxidative stress-mediated STAT3 hypomethylation appears to play a key role in the mechanism of benzene toxicity in the AHH-1 cell line. Benzene is an established human hematotoxicant and leukemogen [37–40]. 1,4-benzoquinone (1,4-BQ), one of benzene’s key toxic metabolites, is widely used to evaluate the effects of benzene-induced toxicity and the potential epigenetic mechanism in vitro [9,14,24–26]. In this study, the result of STAT3 methylation induced by 1,4-BQ in vitro is consistent with that of chronic benzene poisoning patients, which implicates that the decreasing STAT3 methylation levels plays an important role in benzene hematotoxicity.

The transcription factor STAT3 regulates gene expression through epigenetic mechanisms [35]. STAT3 activation has been as an oncogene to promote tumorigenicity [16,18]. Our recently published study has shown that the aberrant hypomethylated STAT3 might be a potential biomarker of benzene hematotoxicity in chronic benzene poisoning patients [34].

Aberrant DNA methylation is produced in the early stage of the disease, and has an important significance in the early diagnosis of the disease [10]. The study on the role of epigenetics in benzene toxicity was hot point. Bollati et al. studied the genomic methylation status in workers exposed to the lower dose of benzene and found the markedly methylation patterns change, the whole genomic hypomethylation and accompanied with some regional (CpG Island) hypermethylation [7].

The role of oxidative stress in the toxicity of low dose benzene has been recognized by many domestic and foreign scholars [4,11,28]. Research has shown that benzene metabolism induces the reactive oxygen species [4], thus causing an increase in free radicals and attacks DNA molecules, resulting in oxidative damage and DNA strand breaks [2,5]. Our data are consistent with Badham’ result.
of genetic material, such as methylation, acetylation, phosphorylation.

Alpha lipoic acid, called “super antioxidant”, is an antioxidant agent with “the largest and most active function” among all antioxidants [15]. Lipoic acid can exert an antioxidant action. In Europe, research has found that the lipoic acid as antioxidant can protect liver and heart damage, inhibit cancer cells in vivo and alleviate the allergic asthma and arthritis caused by inflammation. Some substances at low doses exists the exciting effect which are called hormesis, which may display the promotion of growth, prolong life, enhance immune function and improve fertility. But lipoic acid has the harmful effects at high doses [15].

In this study, we found that the ROS scavenger α-LA only at lower dose (0.1 μM) markedly reduced 1,4-BQ-induced ROS level. Further, to elucidate the role of oxidative stress in 1,4-BQ-induced STAT3 hypomethylation, clinical application of antioxidant α-LA pretreated cells, results indicated that the ROS scavenger α-LA at low dose reversed the expression level of STAT3 methylation induced by 1,4-BQ in AHH-1 cells, which suggested that oxidative stress was involved in 1,4-BQ-induced STAT3 methylation expression. Interestingly, 0.1 μM α-LA showed the most obvious inhibiting effect which might exist in the low dosage–hormesis effect.

It is well known that DNA methyltransferase (DNMT) inhibitor is the pivotal epigenetic modification reagents. DNA methylation inhibitor 5-aza can reverse DNA methylation patterns from hypermethylation to normal state and shows potent antitumor activity, it suggests its usefulness as novel cancer therapeutic drug. 5-aza significantly decreased 1,4-BQ-induced ROS and 8-OHdG levels. However, the methylation inhibitor 5-aza had no obvious inhibiting effect on the expression level of STAT3 methylation. 5-aza decreased ROS and 8-OHdG levels induced by 1,4-BQ, which elevated the 1,4-BQ-induced STAT3 methylation expression, all these counteracted the inhibiting effect of 5-aza on 1,4-BQ-induced STAT3 methylation expression.

In summary, it was firstly reported that the role of oxidative stress in 1,4-BQ-induced STAT3 methylation expression.

Competing interests

These authors have no any competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2015.05.013

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