Effects of Benzene and Its Metabolites on Global DNA Methylation in Human Normal Hepatic L02 Cells

Junjie Hu,[1,2] Huimin Ma,[1] Wenbing Zhang,[1] Zhiqing Yu,[1] Guoying Sheng,[1] Jiamo Fu[1]

[1]State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China

[2]Graduate School of the Chinese Academy of Science, Beijing, China

Received 6 April 2011; revised 25 August 2011; accepted 31 August 2011

ABSTRACT: Benzene is an important industrial chemical that is also widely present in cigarette smoke, automobile exhaust, and gasoline. It is reported that benzene can cause hematopoietic disorders and has been recognized as a human carcinogen. However, the mechanisms by which it increases the risk of carcinogenesis are only partially understood. Aberrant DNA methylation is a major epigenetic mechanism associated with the toxicity of carcinogens. To understand the carcinogenic capacity of benzene, experiments were designed to investigate whether exposure to benzene and its metabolites would change the global DNA methylation status in human normal hepatic L02 cells and then to evaluate whether the changes would be induced by variation of DNA methyltransferase (DNMT) activity in HaeIII DNMT-mediated methylation assay *in vitro*. Our results showed that hydroquinone and 1,4-benzoquinone could induce global DNA hypomethylation with statistically significant difference from control ($p < 0.05$), but no significant global DNA methylation changes were observed in L02 cells with benzene, phenol, and 1,2,4-trihydroxybenzene exposure. Benzene metabolites could not influence HaeIII DNMT activity except that 1,4-benzoquinone shows significantly inhibiting effect on enzymatic methylation reaction at concentrations of 5 μM ($p < 0.05$). These results suggest that benzene metabolites, hydroquinone, and 1,4-benzoquinone can disrupt global DNA methylation, and the potential epigenetic mechanism by which that global DNA hypomethylation induced by 1,4-benzoquinone may work through the inhibiting effects of DNMT activity at 10 μM ($p < 0.05$). © 2011 Wiley Periodicals, Inc. Environ Toxicol 29: 108–116, 2014.

Keywords: benzene; metabolites; epigenetic; global DNA methylation; hypomethylation; HaeIII DNA methyltransferase; carcinogenesis

Correspondence to: H. Ma; e-mail: mahuimin@gig.ac.cn or W. Zhang; e-mail: zhwb@gig.ac.cn

Contract grant sponsor: Foundation of State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences.

Contract grant number: GIGCX-09-01.

Contract grant sponsor: National Natural Science Foundation of China.

Contract grant number: NSFC-21007072.

Contract grant sponsor: Knowledge Innovative Program of The Chinese Academy of Sciences.

Contract grant number: KZCX2-YW-GJ02.

Contract grant sponsor: Special Scientific Research Start-up Fund for the Gainer of the Excellent PhD Dissertation Award or the President Scholarship of Chinese Academy of Sciences (2005).

Contract grant number: 0533041A07.

Published online 22 September 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.20777

INTRODUCTION

Benzene is an important industrial organic solvent and a common component of gasoline, engine exhaust, wood smoke, and tobacco smoke. It is reported that benzene is ubiquitous in the environment with air moving (Rappaport et al., 2009). The majority of human exposure occurs through the inhalation of benzene fumes from vehicular emissions, indoor sources, and occupational settings. Because of its carcinogenicity, benzene has been designated as a Group 1 carcinogen by International Agency for Research on Cancer (IARC, 1987). Epidemiological studies have also shown that benzene exposure is associated with acute myelogenous leukemia (AML) and other cancers (Schnatter et al., 2005). Despite control measures preventing exposure to high concentrations of benzene in some countries, it still presents a significant potential risk to both environment and public health.

Epigenetic modifications are believed to be crucial to understanding the toxic effects on mammals of exposure to chemicals, because these modifications can be reprogrammed by nutritional, chemical, and physical factors (Zhang et al., 2010). As epigenetic modifications regulate gene and protein expressions, they represent more stable biomarkers and fingerprints of exposure than altered genes or protein expressions (Zhang et al., 2010). DNA methylation, in mammals, is catalyzed by the family of DNA methyltransferase (DNMT), which transfers the methyl group from the ubiquitous methyl donor *S*-adenosyl-L-methionine (SAM) to the C5-position of cytosine ring. DNA methylation plays an important role in transcriptional regulation, X chromosome inactivation, genomic imprint, and mutagenesis (Attwood et al., 2002). Aberrant DNA methylations, including global DNA hypomethylation and gene-specific hypomethylation/hypermethylation, are widespread phenomena in AML (Kuang et al., 2008). Abnormal global DNA methylation is considered an epigenetic biomarker of early carcinogenesis (Gaudet et al., 2003).

The extant toxicity studies of benzene and its metabolites mostly focused on the toxicogenomic aspect (including genome-wide analyses of susceptibility gene, gene expression, and protein expression) and epigenomic (including DNA methylation, RNA noncoding, and protein modification). Based on the toxicogenomic data, many potential mechanisms of benzene toxicity have been proposed to explain the progress from cytotoxicity to carcionogenicity, but none of these hypotheses have been widely accepted. Researchers have found that benzene metabolites may be singularly, collectively, directly, and indirectly implicated in the progression of benzene related cytotoxicity to carcinogenicity (Atkinson, 2009). Studies based on the epigenomic data remain at an early stage (Zhang et al., 2010), especially the global DNA methylation level data. Only two reports using global DNA methylation level data have so far been published (Bollati et al., 2007; Ji et al., 2010). Bollati et al. (2007) demonstrated that genome-wide hypomethylation assessed by long interspersed nuclear element-1 was observed in gas station attendants and traffic police with low-dose of benzene exposure (about 22 ng/mL). However, the contribution of other traffic pollutants was not determined on the measured DNA methylation level (Bollati et al., 2007). Ji et al. (2010) reported that global DNA hypomethylation was observed in hydroquinone exposure human cells, but other benzene metabolites were not mentioned.

DNA, DNMT, and the methyl donor are three important factors in DNA methylation process in humans. Accordingly, there are three mechanisms by which the global hypomethylation induced by benzene and its metabolites may contribute to carcinogenesis: (a) DNA damage; (b) changes in expression and/or activity of DNMT; (c) one-carbon metabolism of SAM (*S*-adenosylmethionine) variety (Ji et al., 2010). Among the three mechanisms, however, depletion of SAM is not likely to happen in acute chemical exposure. The *in vivo* and *in vitro* experiments showed that hydroquinone and 1,4-benzoquinone can form a series of DNA adducts (Gaskell et al., 2005). The Sprague–Dawley mouse studies and epidemiological studies also demonstrated that benzene and its metabolites can induce the formation of 8-hydroxyguanine (Wan and Winn, 2007; Buthbumrung et al., 2008). These DNA adducts can cause DNA damage that may decrease the efficiency of hemimethylation DNA to accept methyl group from SAM (Maltseva et al., 2009). No literature has reported about alterations in the expression and/or activity of DNMT. Further studies are needed to determine if global DNA hypomethylation induced by benzene, and its metabolites could be caused by the inhibiting effects of benzene metabolites on DNMT activity.

This study was designed to investigate the effects of benzene and its metabolites on the genomic DNA methylation level in human normal hepatic L02 cells and then to evaluate the effects of benzene and its metabolites on DNMT activity in HaeIII DNMT-mediated methylation experiments *in vitro*. We chose HaeIII, because prokaryotic methyltransferase and catalytic domains of mammalian methyltransferase show similarities in high sequence homology, structure, and mechanism of methyl transfer (Jeltsch, 2002). This study sought to explain the mechanisms of benzene-induced epigenetic toxicity to improve our understanding of whether global DNA hypomethylation could be caused by direct benzene or its metabolites exposure, and of whether the potential mechanism of global DNA hypomethylation could work through the inhibiting effects of benzene metabolites on DNMT activity.

MATERIALS AND METHODS

Chemicals and Enzymes

HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Formic acid (HPLC-

grade), sodium acetate, isopropanol, ammonium acetate, ammonium bicarbonate, hydroquinone, 1,2,4-trihydroxybenzene, 1,4-benzoquinone, nuclease P1 (from *Penicillium citrinum*), snake venom phosphodiesterase I (from *Crotalus atrox*), and calf intestine phosphatase alkaline were purchased from Sigma (St. Louis, MO). HaeIII DNMT, SAM (32 mM), and 10× incubation buffer were obtained from New England Biolabs (Beverly, MA). Lambda DNA was obtained from Dingguo Biotechnology (Beijing, China). 5 mdC, 2′-deoxycytidine (dC), 2′-deoxyguanosine (dG), 2′-deoxyadenosine (dA), thymidine (T), cytidine (C), guanosine (dG), adenosine (dA), and uridine (T) were purchased from Amresco. Benzene, phenol, and other chemicals were of analytical reagent grade. Deionized water was prepared by using a Direct-Q water purification system (Millipore, Bedford, MA).

Cell Culture, Chemical Treatment, and DNA Isolation

The human normal hepatic cell line, L02, was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The L02 cells were cultured in high-glucose DMEM medium (Hyclone, Logan, UT) supplemented with 10% (v/v) fetal bovine serum (Gbico, San Diego, CA) at 37°C in a 5% CO_2 moist atmosphere. The culture medium was changed daily, and experiments were performed when cells reached about 80% confluence.

Benzene and its metabolites dissolved in dimethyl sulfoxide (DMSO) immediately before treatment for all experiments. The final concentration of DMSO present in cell cultures was 0.1% (v/v). L02 cells were seeded in a six-well plate at a density of 1×10^5 cells/mL and were treated with benzene, phenol, hydroquinone, 1,2,4-trihydroxybenzene, or 1,4-benzoquinone for 48 h at the 5, 10, 25, and 50 μM. The control group was incubated with 0.1% DMSO (v/v) only.

After the treatment, L02 cells were spun down and washed once with sterile 1× phosphate-buffered saline. The cells were collected and stored at −80°C until DNA isolation. Genomic DNA was isolated from L02 cells using TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China), following the manufacturer's protocol. The concentrations and purity of the DNA samples were assessed using the DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA). The DNA samples presented purity indices (A_{260}/A_{280} ratio) between 1.6 and 1.8, and then they were aliquoted and stored at −80°C until use.

Enzymatic Hydrolysis of DNA

DNA hydrolysis was performed following a procedure previously described (Ma et al., 2009). Briefly, 1 μg of DNA sample was firstly dissolved in 3 μL deionized water, denatured by heating at 100°C for 3 min, and then chilled in ice immediately. After adding one-tenth volume of 0.1 M ammonium acetate (pH 5.3) and 2 U of nuclease P1, the mixture was incubated at 45°C for 3 h. Then, 1/10 volume of 1 M ammonium bicarbonate and 0.002 U of snake venom phosphodiesterase I were added, and the incubation was continued at 37°C for 2 h. Last, the mixture was added with 0.5 U alkaline phosphatase and incubated for a further 1 h at 37°C. The solution was stored at −80°C until analysis. The final DNA hydrolysate was filtrated by Microcon centrifugal filter device (Millipore, Bedford, MA; YM-10, MW cut-off 10,000) to remove protein. The spin filter were first prerinsed with deionized water (100 μL) to remove glycerol, and then an aliquot (300 μL) of the DNA digest was transferred to Microcon centrifugal filter and centrifuged at 12,000 × g at 4°C for 30 min.

Preparation of Stock Solutions, Calibration Standard Solutions

Stock solutions of 5 mdC and dG were prepared at 1 mg/mL in deionized water. Standard working solutions of 5 mdC and dG (1, 10, 100, and 1000 ng/mL) were obtained by further dilution of stock solutions with deionized water. Calibration standard solutions ([5 mdC]/[dG]) were prepared at 1, 4, 6, 8, 10, 12, and 16%.

Liquid Chromatography

Digested DNA samples were analyzed using an Agilent 1100 series HPLC system equipped with a vacuum degasser, an autosampler, a quaternary pump, and a diode array detector. The chromatographic separation was performed at room temperature on a Waters (Milford, MA) Atlantis dC18 column (2.1 × 150 mm, 5-μm particle size) protected by a guard column (2.1 × 20 mm, 5-μm particle size). Solvent A (0.1% formic acid in deioned water) and solvent B (0.1% formic acid in methanol) were used as mobile phase. The column was eluted at a flow rate of 0.22 mL/min beginning with 100% A, followed by a linear gradient to 82% A over a period of 24 min. The injection volume was 10 μL.

Electrospray Ionization Tandem Mass Spectrometry

This method was modified from our previous work (Chen et al., 2004; Ma et al., 2005, 2009; Hu et al., 2010). The sample eluted from the HPLC system was introduced into a TurbolonSpray source installed on an API 4000 triple quadrupole mass spectrometer (Applied Biosystems). Analyst® Software version 1.3 (Applied Biosystems) was used for data acquisition and processing. The mass spectrometer was operated in the ESI^+ MS/MS mode. ESI conditions

were optimized by infusion of 10 μg/mL 5 mdC in deioned water at a flow rate of 220 μL/min. Nitrogen was used as curtain nebulizer gas, and the optimized electrospray ionization source parameters were turbo gas temperature 450°C; spray voltage 4500 V; declustering potential 40 V; entrance voltage 6 V; collision energy 15 V; collision cell exit potential 8 V; curtain gas (setting 20), gas 1 (setting 32), and gas 2 (setting 55). Tandem mass spectrometric analysis was performed using nitrogen as collision gas (CAD gas setting 6). Optimal multiple reaction monitoring conditions were obtained for nine channels: 5 mdC (*m/z* 241.9 → 126.3), dC (*m/z* 228.0 → 112.2), dG (*m/z* 268.1 → 152.3), dA (*m/z* 252.3 → 135.9), T (*m/z* 243.3 → 127.2), C (*m/z* 244.1 → 112.2), G (*m/z* 284.1 → 152.3), A (*m/z* 268.1 → 136.1), and U (*m/z* 245.1 → 112.9). A dwell time of 150 ms was used for each transition, and the quadrupoles Q1 and Q3 were set on unit mass resolution.

Effects of Benzene Metabolites on Enzymatic DNA Methylation Reaction *In Vitro*

In this experiment, HaeIII DNMT kit was used according to the HaeIII DNMT kit manufacturer's instructions. All the reaction mixtures were freshly prepared on ice. The freshly prepared solutions were then added in a sequential order to a 0.6 mL Eppendorf tube on ice: 1 μL of 10× incubation buffer, 2 μL of lambda DNA substrate (1.5 μg), 5 μL of water, 1 μL of (800 μM) SAM, and 1 μL of HaeIII DNMT (2.5 U). The final volume was 10 μL, and the control experiments were carried out by using the above mixture. In this study, the 5 μL of the benzene metabolites (added separately to the reaction mixture) was added to a final concentrations of 5, 10, and 25 μM. After incubating the mixture at 37°C for 3 h, 1 μL SAM (800 μM) was added to the mixture, and then the incubation was resumed at 37°C for another 3 h. The reactions were terminated by immediately placing the tubes on ice, and by adding 40 μL of 3 M cold sodium acetate (pH 5.3) and 400 μL of cold isopropanol. The mixture was kept at room temperature for 20 min, after which the methylated lambda DNA was collected by centrifugation at 14,000 rpm for 20 min. The pellets containing the methylated lambda DNA were then gently washed three times with 70% cold ethanol, and each wash was followed by centrifugation for 3 min at 10,000 × *g*.

The methylated lambda DNA was hydrolyzed by the protocol described earlier. Optimization conditions of HPLC-ESI-MS/MS were used to detect 5mdC concentrations.

Statistical Analysis

SPSS software (version 13.0, SPSS, Chicago) was used to perform the statistical analysis. The results were expressed as mean ± SD, and one-way analysis of variance was used to determine the difference between experimental and control groups. In all cases, $p < 0.05$ was regarded as statistically significant.

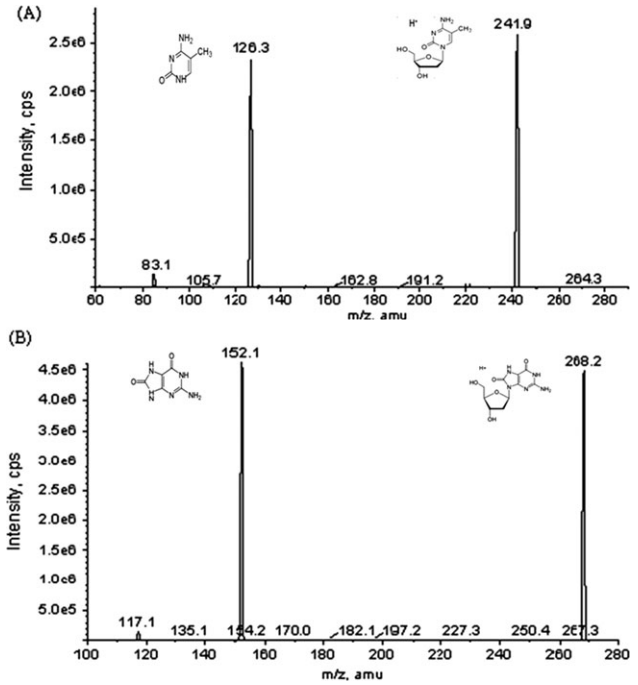

Fig. 1. Positive ESI product ion spectra of 5-mdC (A) and dG (B).

RESULTS

Performance of HPLC-ESI-MS/MS Method

The HPLC-ESI-MS/MS method, previously described by Song et al. (2005), was adopted for this experiment. The ratio of [5 mdC]/[dG] was chosen to express global DNA methylation level, based on the assumption that [dG] = [5 mdC]+ [dC] in genomic DNA. The full-scan spectra (ESI-MS spectrum) of 5 mdC and internal standard dG were obtained by direct infusion of a standard working solution, which contain 5 mdC and dG (5 μg/mL) in deionized water (0.1% formic acid, v/v), into the quadrupole mass spectrometer. The results were shown in Figure 1. This figure reports that the predominant ion species of 5 mdC and dG are *m/z* 241.9 and *m/z* 268.1, respectively. The main product ions of 5 mdC and dG are *m/z* 126.3 and *m/z* 152.3, respectively. Thus, the precursor/product ion pairs of *m/z* 241.9/126.3 for 5 mdC and *m/z* 268.1/152.3 for dG were used as MRM transitions, respectively.

Figure 2 presents the typical MRM ion chromatogram of the hydrolysate of DNA sample from L02 cells. The overall run time, including column washing and re-equilibration, was within 40 min. From the ion chromatogram, it is clear that the four deoxyribonucleosides, four ribonucleosides, and 5mdC were completely separated from each other and

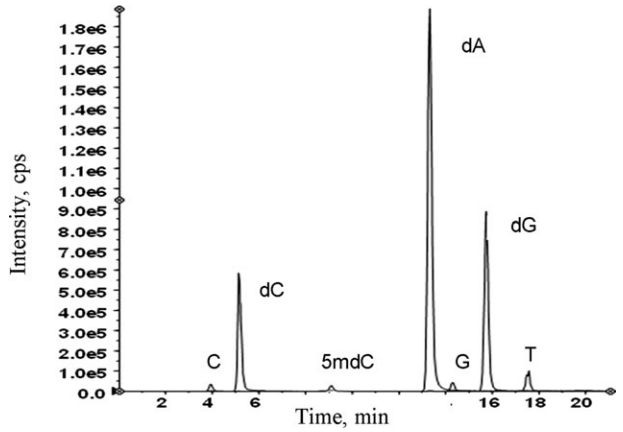

Fig. 2. HPLC-ESI-MS/MS chromatogram of normal and modified nucleosides.

the peek shapes were sharp. The retention times were 10.5 and 19.4 min for 5 mdC and dG, respectively.

Working solutions were freshly prepared daily by spiking different concentrations of 5 mdC (5, 10, 15, 20, 25, 30, 35, and 40 ng/mL) into a 500 ng/mL of dG. The ratio of [5 mdC]/[dG] ranges from 1 to 8%. The global DNA methylation calibration curve has good linearity with the correlation coefficient $R^2 = 0.9995$ (Fig. 3).

The interday and intraday precision of this method to assess global DNA methylation levels was less than 8%. The validation of this method was assessed by evaluating the global DNA methylation status of calf thymus DNA. Using 5 mdC/dG weight ratio as an indicator of global DNA methylation level, mean value of five replicates of these samples was 6.54% $\pm$ 0.23%. In literatures, this value ranged from 5.7% determined by GC/MS (Singer et al., 1979) to 6.65 determined by HPLC/UV (Kuo et al., 1980). These results suggest that the present method is precise and accurate.

Effects of Benzene and Metabolites on Global DNA Methylation Levels in Human Normal Hepatic L02 Cells

In this experiment, we examined the effects of benzene and its metabolites on the global DNA methylation status using human normal hepatic L02 cells. No sign of cytotoxicity presented after the treatment of L02 cells with 5, 10, 25, or 50 μM of benzene or its metabolites for 48 h (data not shown). Slight global DNA methylation levels changes were observed at various concentrations of benzene, phenol or 1,2,4-trihydroxybenzene exposed. The values of global DNA methylation level relative to the control group were in the range of 93.1–105.5%, but no significant difference compared to the control group was observed ($p > 0.05$). However, hydroquinone and 1,4-benzoquinone dramatically induced global DNA hypomethylation ($p < 0.05$), and the average global DNA methylation values relative to the control group at 50 μM hydroquinone or 1,4-benzoquinone were 80.3% and 75.3%, respectively (Fig. 4). In comparison, 1,4-benzoquinone exerted a more potent decrease of global DNA methylation level. When the concentration of 1,4-benzoquinone arrived at 5 μM, the global DNA methylation level in L02 cells decreased to 84.5%, which showed significant difference compared with the control group ($p < 0.05$).

Effects of Benzene Metabolites on HaeIII DNMT-Mediated Methylation *In Vitro*

Optimized conditions were chosen for the *in vitro* DNA methylation. Based on the measurements, a common reaction condition was devised for HaeIII DNMT-mediated DNA methylation reactions (see Experimental section).

Table I shows the results of methylation levels of lambda DNA after 6 h enzymatic methylation reaction by HaeIII DNMT, where benzene metabolites were used as the substrate. It can be seen that the methylation levels of lambda DNA were not affected by benzene metabolites to some extent in the 5–25 μM range of concentrations. But 1,4-benzoquinone significantly affected the activity of HaeIII DNMT ($p < 0.05$) when the concentration was 5 μM, and the global DNA methylation levels decreased to 90.1% comparing to the control group.

DISCUSSION

We examine first the effects of benzene and its metabolites on global DNA methylation level in L02 cells, and then the activity of HaeIII DNMT inhibited by benzene metabolites was evaluated *in vitro*. Our data showed that hydroquinone and 1,4-benzoquinone could significantly decrease global DNA methylation level in L02 cells, and the potential mechanism may be relative to the inhibition activity of DNMTs *in vitro*. These results could promote our understanding about epigenetic mechanisms of benzene-induced carcinogenesis.

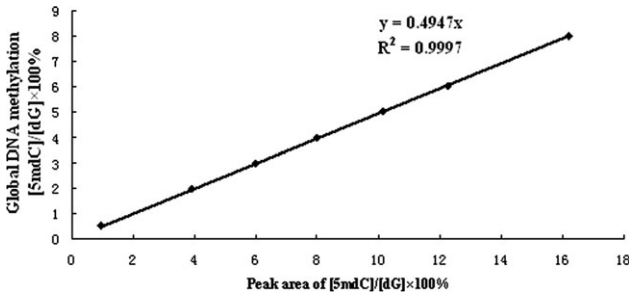

Fig. 3. Global DNA methylation calibration curves using 5-mdC (5–40 ng/mL) spiked into a 500 ng/mL solution of dG.

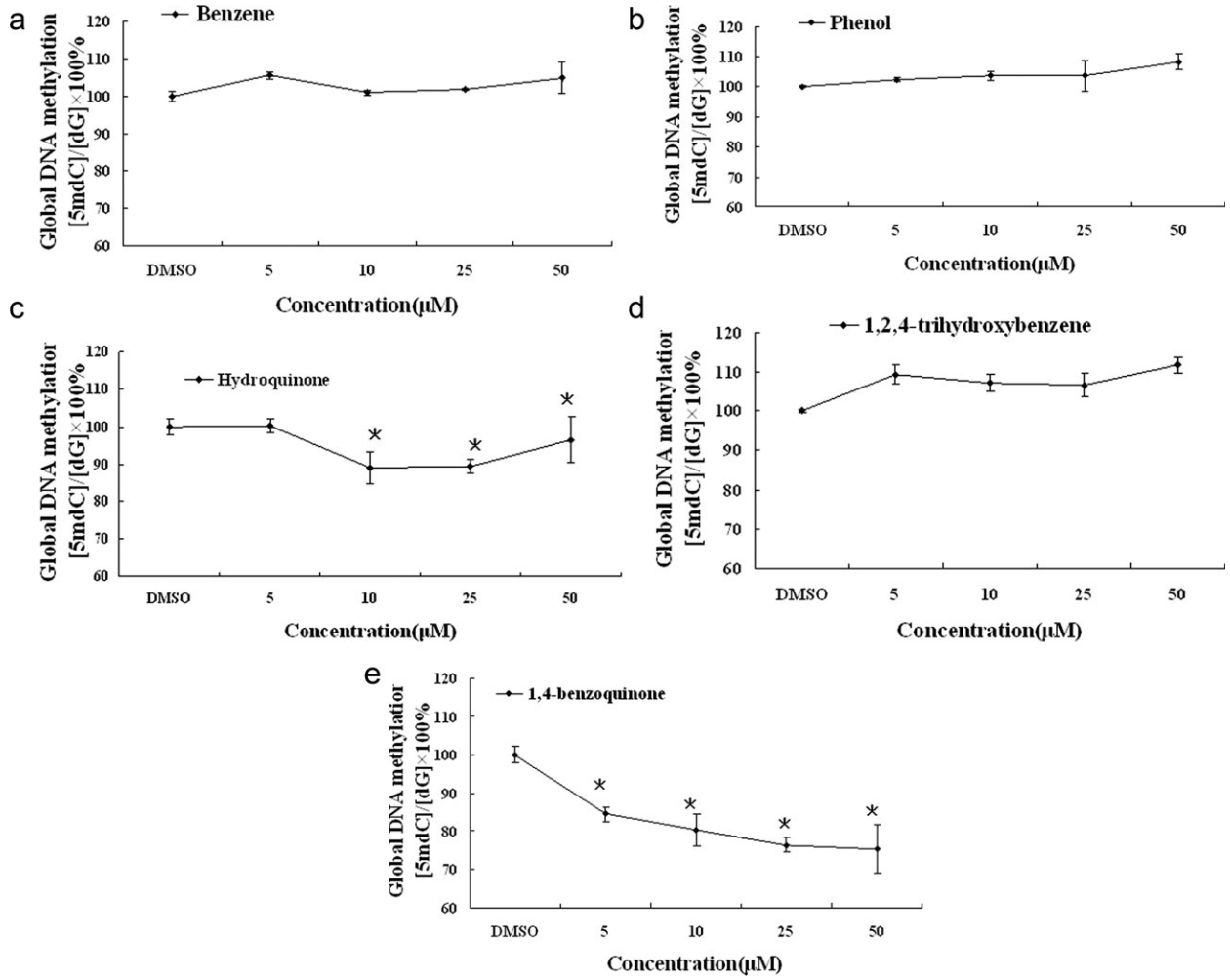

Fig. 4. Effects of benzene and its metabolites on global DNA methylation in human normal hepatic L02 cell. The level of global DNA methylation is expressed as a percentage of the value for the untreated control, which is set at 100%, and data are presented as mean value ± SD, $n = 4$. * Significantly different from control, $p < 0.05$.

In spite of studies over several decades, the mechanisms underlying benzene-induced toxicity and leukemogenicity are not yet fully understood. Attention to the field of epigenetics, such as DNA methylation alterations and histone modification (Bollati et al., 2007; Xing et al., 2010; Gao et al., 2010), has increased. Based on our previous experiments result, global DNA hypomethylation levels occurred in liver tissues in benzene-exposed SD rats (Hu et al., 2010). So, in this study, L02 cells were used to determine whether benzene, and its metabolites would affect global

TABLE I. Effects of benzene's metabolites exposure on HaeIII DNMT-mediated methylation *in vitro* assay

	5-Methyl-2′-deoxycytidine content in lambda DNA[a](%)			
Concentration (μM)	Phenol	Hydroquinone	1,2,4-Trihydroxy benzene	1,4-Benzoquinone
5	100.66 ± 0.02	106.68 ± 0.01	99.54 ± 0.00	93.90 ± 0.03
10	104.00 ± 0.02	101.70 ± 0.03	103.01 ± 0.01	**90.10 ± 0.02**
25	100.59 ± 0.04	98.23 ± 0.06	99.93 ± 0.04	88.53 ± 0.07

Note: Value set in boldface are significantly different from control, $p < 0.05$.

[a]The level of global DNA methylation is expressed as percentage of the value for the control, which is set at 100%, and data are presented as mean value ± S.D., $n = 3$.

DNA methylation in a cell culture model. Dramatic decrease of global DNA methylation was observed after treatment of L02 cells with 5–50 μM of hydroquinone or 1,4-benzoquinone, even when the exposed dose of 1,4-benzoquinone was 5 μM, the level of global DNA methylation decreased to 84.5% ($p < 0.05$). However, no significant changes of global DNA methylation were found in L02 cells with the same dose of benzene, phenol, or 1,2,4-trihydroxybenzene exposure. The results confirm the fact that benzene metabolites play an important role in the mechanism of benzene-induced toxicology. Ji et al. (2010) indicated that hydroquinone can induce global DNA hypomethylation in human lymphoblastoid cell TK6 in a dose-dependent manner. Gene-specific hypomethylation can also be induced by hydroquinone, such as MAGEA1 hypomethylated frequently found in hydroquinone exposed TK6 cells (Zhang et al., 2010). Moreover, epidemiological study showed that RUNX3, a gene whose altered expression is associated with myeloproliferative disorders, is hypomethylated at three different CpG sites among benzene-exposed workers (Muller et al., 2008).

Ji et al. (2010) further proposed three mechanisms by which global DNA hypomethylation induces carcinogenesis: chromosome instability, reactivation of transposable elements, including intragenomic endoparasitic DNA or transposons, and loss of imprinting. Further research is needed to determine which mechanisms can be used to understand how global DNA hypomethylation induced by benzene and its metabolites causes cancers.

Several proposed mechanisms of how benzene and its metabolites interfere with global DNA methylation are as follows: (1) NO, a by-product of benzene metabolism, induces a posttranscriptional increase of DNMT activity; (2) reactive oxygen species and oxidize DNA may reduce binding affinity of the methyl-CpG binding protein 2; (3) DNA strand breaks reduce the affinity between DNMT and CpG island (Bollati et al., 2007). Formation of DNA adducts may also be an influencing factor. For example, the DNA adduct, trans-anti-benzo[a]pyrene-N2-dG flanking a target dC in the CpG dinucleotide on its 5′-side, has a great adverse impact on methylation catalyzed by SssI and HhaI methyltransferases, because bulky benzo[a]-pyrenyl residue block the normal contact of the methyltransferase catalytic loop with the B[a]PDE-modified DNA (Subach et al., 2006). *In vivo* and *in vitro* experiments have shown that hydroquinone and 1,4-benzoquinone can form a series of DNA adducts (Gaskell et al., 2005), such as (3′-hydroxy)-3, N4-benzetheno-2′-deoxycytidine 3′-monophosphate (Pongracz et al., 1990), (3′′-hydroxy)-1, N2-benzetheno-2′-deoxyguanosine 3′-monophosphate (Pongracz and Bodel, 1991), and (3′,4′-dihydroxy)-1,N2-benzetheno-2′-deoxyguanosine 3′-monophosphate (Levay et al., 1991). In addition, the common oxidative DNA damage product 8-hydroxyguanine at or adjacent to the recognition site can weaken the affinity of DNMT to DNA and dramatically inhibit methylation (Maltseva et al., 2009). In cells and Sprague–Dawley mice, the epidemiological studies have demonstrated that benzene and its metabolites can induce the formation of 8-hydroxyguanine (Buthbumrung et al., 2008; Wan et al., 2007). 8-Hydroxyguanine induced by benzene and its metabolites may influence the binding of DNMT to CpG islands in the promoter region, changing DNA methylation patterns.

As detailed earlier, the changes to global DNA methylation occurred after treatment of various concentrations of benzene and its metabolites in L02 cells, and statistically significant differences were observed at high dosages of hydroquinone and 1,4-benzoquinone. However, little is known about the relationship between alteration of DNMT activity and global DNA hypomethylation induced by benzene and its metabolites (Ji et al., 2010). Previous research has suggested that DNMT may be involved in the changes of DNA methylation in benzene exposed subjects (Bollati et al., 2007). So, we hypothesize that the changes to global DNA methylation induced by benzene or its metabolites in L02 cells are affected by the inhibiting effects of benzene metabolites (such as hydroquinone and 1,4-benzoquinone) on DNMT activity. Based on the sensitivity and stability of HaeIII DNMT to chemical contaminants, an optimized *in vitro* DNA methylation assay by using HaeIII DNMT was performed to investigate whether benzene metabolites could influence HaeIII DNMT activity *in vitro*. The results showed that benzene's metabolites, including phenol, hydroquinone, and 1,2,4-trihydroxybenzene cannot induce changes of activity of HaeIII DNMT, whereas 1,4-benzoquinone significantly induces global DNA hypomethylation level at the concentrations of 5 μM. We propose that the potential mechanism by which benzene metabolites induced global DNA hypomethylation in L02 cells may work through inhibiting DNMT activity.

Despite the significant relationship between 1,4-benzoquinone concentration and HaeIII DNMT activity, HaeIII DNMT cannot fully replace other mammalian DNMTs, such as DNMT1, DNMT3a, and DNMT3b, which are also important factors during the process of DNA methylation. Further studies are needed to understand the effects of other DNMT activity on benzene-induced global DNA hypomethylation.

In conclusion, our study showed that both hydroquinone and 1,4-benzoquinone exposure can significantly decrease the global DNA methylation level in L02 cells, and this change may due to the inhibition activity of DNMT. However, further studies are still needed in the future for fully understanding the mechanisms of benzene-induced DNA methylation change.

This work was financially supported by the Foundation of State Key Laboratory of Organic Geochemistry, Guangzhou Institute of

Geochemistry, Chinese Academy of Sciences (GIGCX-09-01), National Natural Science Foundation of China (NSFC-21007072), Knowledge Innovative Program of The Chinese Academy of Sciences (KZCX2-YW-GJ02), the National Basic Research Program of China (2008CB418205), Special Scientific Research Start-up Fund for the Gainer of the Excellent PhD Dissertation Award or the President Scholarship of Chinese Academy of Sciences (2005) (0533041A07). The authors are grateful to the editors and the reviewers of the Environmental toxicology.

REFERENCES

Atkinson TJ. 2009. A review of the role of benzene metabolites and mechanisms in malignant transformation: Summative evidence for a lack of research in nonmyelogenous cancer types. Int J Hyg Environ Health 2121:1–10.

Attwood JT, Yung RL, Richardson BC. 2002. DNA methylation and the regulation of gene transcription. Cell Mol Life Sci 592:241–257.

Bollati V, Baccarelli A, Hou LF, Bonzini M, Fustinoni S, Cavallo D, Bynu HM, Jiang JY, Marinelli B, Pesatori AC, Bertazzi PA, Yang AS. 2007. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res 673:876–880.

Buthbumrung N, Mahidol C, Navasumrit P, Promvijit J, Hunsonti P, Autrup H, Ruchirawat M. 2008. Oxidative DNA damage and influence of genetic polymorphisms among urban and rural schoolchildren exposed to benzene. Chem Biol Interact 172:185–194.

Chen HY, Zhang WB, Song RF, Ma HM, Dong YL, Sheng GY, Fu JM. 2004. Analysis of DNA methylation by tandem ion-pair reversed-phase high-performance liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 18:2773–2778.

Gao A, Zuo X, Liu QJ, Lu X, Guo W, Tian L. 2010. Methylation of PARP-1 promoter involved in the regulation of benzene-induced decrease of PARP-1 mRNA expression. Toxicol Lett 1952-3:114–118.

Gaskell M, McLuckie KIE, Farmer PB. 2005. Genotoxicity of the benzene metabolites *para*-benzoquinone and hydroquinone. Chem Biol Interact 153:267–270.

Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R. 2003. Induction of tumors in mice by genomic hypomethylation. Science 300:489–492.

Hu JJ, Ma HM, Zhang WB, Cai YM, Yu ZQ, Sheng GY, Fu JM. 2010. Effects of an acute exposure of benzene on DNA methylation in Sprague-Dawley rats. Ecol Environ. 19:2902–2905.

IARC. 1987. IARC monographs on the evaluation of carcinogenic risks to humans. Supplement/World Health Organization, International Agency for Research on Cancer. France. 1–42:120–122.

Jeltsch A. 2002. Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. Chembiochem 35:382–382.

Ji Z, Zhang L, Peng V, Ren X, McHale CM, Smith MT. 2010. A comparison of the cytogenetic alterations and global DNA hypomethylation induced by the benzene metabolite, hydroquinone, with those induced by melphalan and etoposide. Leukemia 245:986–991.

Kuang SQ, Tong WG, Yang H, Lin W, Lee MK, Fang ZH, Wei Y, Jelinek J, Issa JP, Garcia-Manero G. 2008. Genome-wide identification of aberrantly methylated promoter associated CpG islands in acute lymphocytic leukemia DNA Methylation in ALL. Leukemia 22:1529–1538.

Kuo KC, McCune RA, Gehrke CW, Midgett R, Ehrlich M. 1980. Quantitative reversed-phase high-performance liquid-chromatographic determination of major and modified deoxyribonucleosides in DNA. Nucleic Acids Res 820:4763–4776.

Levay G, Pongracz K, Bodell WJ. 1991. Detection of DNA adducts in HL-60 cells treated with hydroquinone and para-benzoquinone by P-32 postlabeling. Carcinogenesis 127:1181–1186.

Ma HM, Zhang WB, Song RF, Zhou Z, Chen HY, Sheng GY, Zhou Z, Fu JM. 2005. Characterization of methylated and unmethylated CpG oligodeoxynucleotides by electrospray ionization tandem mass spectrometry. Eur J Mass Spectrom 116:581–589.

Ma HM, Zhang WB, Hu JJ, Yu ZQ, Chen Y, Luo Q, Shi XL, Zhang YY, Song RF, Zhou Z, Sheng GY, Fu JM. 2009. Analysis of global DNA methylation levels in human blood using high-performance liquid chromatography/tandem electrospray ionization mass spectrometry. Eur J Mass Spectrom 154:555–561.

Maltseva DV, Baykov AA, Jeltsch A, Gromova ES. 2009. Impact of 7, 8-dihydro-8-oxoguanine on methylation of the CpG site by Dnmt3a. Biochemistry 486:1361–1368.

Muller CI, Luong QT, Shih LY, Jones LC, Desmond JC, Kawamata N, Tcherniantchouk O, Liu Q, Ito Y, Tefferi A, Vos SD, Koeffler HP. 2008. Identification of marker genes including RUNX3 AML2 that discriminate between different myeloproliferative neoplasms and normal individuals. Leukemia 229: 1773–1778.

Pongracz K, Bodel WJ. 1991. Detection of 3′-hydroxy-1, N^6-benzetheno-2′-deoxyadenosine 3′-phosphate by phosphorus-^{32}postlabeling of DNA reacted with *para*-benzoquinone. Chem Res Toxicol 42:199–202.

Pongracz K, Kaur S, Burlingame AL, Bodell WJ. 1990. Detection of 3′-hydroxy-3, N^4-benzetheno-2′-deoxycytidine-3′-phosphate by p-32 postlabeling of DNA reacted with p-benzoquinone. Carcinogenesis 119:1469–1472.

Rappaport SM, Kim S, Lan Q, Vermeulen R, Waidyanatha S, Zhang LP, Li GL, Yin SN, Hayes RB, Rothman N, Smith MT. 2009. Evidence that humans metabolize benzene via two pathways. Environ Health Perspect 1176:946–952.

Schnatter AR, Rosamilia K, Wojcik NC. 2005. Review of the literature on benzene exposure and leukemia subtypes. Chem Biol Interact 153:9–21.

Singer J, Schnute WC, Shively JE, Todd CW, Riggs AD. 1979. Sensitive detection of 5-methylcytosine and quantitation of the 5-methylcytosine-cytosine ration in DNA by gas chromatography ion monitoring. Anal Biochem 942:297–301.

Song LG, James SR, Kazim L, Karpf AR. 2005. Specific method for the determination of genomic DNA methylation by liquid chromatography electrospray ionization tandem mass spectrometry. Anal Chem 77:504–510.

Subach OM, Baskunov VB, Darii MV, Maltseva DV, Kirsanova OV, Kolbanovskiy A, Kolbanovskiy M, Johnson F, Bonala R. 2006. Impact of benzo [a] pyrene-2-deoxyguanosine lesions on methylation of DNA by SssI and HhaI DNA methyltransferases. Biochemistry 45:6142–6159.

Wan J, Winn LM. 2007. Benzene's metabolites alter c-MYB activity via reactive oxygen species in HD3 cells. Toxicol Appl Pharmacol 2222:180–189.

Xing C, Wang QF, Li B, Tian H, Ni Y, Yin S, Li G. 2010. Methylation and expression analysis of tumor suppressor genes p15 and p16 in benzene poisoning. Chem Biol Interact 184:306–309.

Zhang LP, McHale CM, Rothman N, Li GL, Ji ZY, Vermeulen R, Hubbard AE, Ren XF, Shen M, Rappaport SM, North M, Skibola CF, Yin SN, Vulpe C, Chanock SJ, Smith MT, Lan Q. 2010. Systems biology of human benzene exposure. Chem Biol Interact 1841–2:86–93.