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Toxicological effects of benzo[a]pyrene on DNA methylation of whole genome in ICR mice

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Abstract

It has been well known that alterations in DNA methylation – an important regulator of gene transcription – lead to cancer. Therefore a change in the level of DNA methylation of whole genome has been considered as a biomarker of carcinogenesis. Previously, a large number of experimental results in genetic toxicology have showed that benzo[a]pyrene could cause DNA mutation and fragmentation. However, there was little to no studies on alterations in DNA methylation of genome directly result from exposure to benzo[a]pyrene. In this paper, possible mechanisms of alterations in whole genomic DNA methylation by benzo[a]pyrene were investigated using ICR mice after benzo[a]pyrene exposure. The blood, liver, pancreas, skin, lung and bladder of ICR mice were removed and checked after a fixed time interval (6 hours) of benzo[a]pyrene exposure, and whole genomic DNA methylation level was determined by high performance liquid chromatography (HPLC). The results exhibited tissue specificity, that is, the level of whole genomic DNA methylation decreases significantly in blood and liver, rather than pancreas, lung, skin and bladder of ICR mice. This study investigated the direct relationship between aberrant DNA methylation level and benzo[a]pyrene exposure, which might be helpful to clarify the toxicological mechanism of benzo[a]pyrene in epigenetic perspectives.

Key words: Benzo[a]pyrene, DNA methylation, HPLC, tissue specificity.

Introduction

A large number of studies over the previous two decades have reported the relationships between benzo[a]pyrene (see Figure 1) and cancers. However, it has been more difficult to relate cancers to specific benzo[a]pyrene sources, especially in human bodies, also it is difficult to quantify risks posed by various methods of exposure (i.e. inhalation or ingestion). Scientists recently discovered a link between vitamin A deficiency and lung cancer in smokers (1). Benzo[a]pyrene was found to be the carcinogenic factor, since it has been confirmed to induce vitamin A deficiency in mice. Previous studies have shown that benzo[a]pyrene and it metabolites result in DNA mutation and DNA breakage (2,3). Although there are many studies about benzo[a]pyrene toxicity, the carcinogenic mechanisms of benzo[a]pyrene still remain unclear (4).

DNA methylation is an important epigenetic modification, it is a chemical modification which is mediated by DNA methyltransferase for addition of a methyl group on the fifth carbon of cytosine base. DNA methylation has been closely implicated in numerous biological events, including transposable element silencing, genomic imprinting, and X chromosome inactivation. Demethylation of DNA is a process where the methyl group from 5-methylcytosine (5meC) is removed by certain enzymes such as DNA demethylases. DNA demethylation has been reported to play crucial roles in the epigenetic regulation of genes. Dysregulation of DNA demethylases is involved in many important diseases such as cancers, imprinting-related diseases, and psychiatric disorders. Many studies have shown that alterations in DNA methylation of genome is closely related to carcinogenesis (5), it is a very important step in cancer development. Therefore, the study on DNA methylation of whole genome has been concerned by more and more environmental scientists and engineers. Alterations in DNA methylation of genome may explain the toxicity and carcinogenesis mechanisms from the perspective of epigenetics.

Since epigenetics is a new scientific discipline in the 1980s, only a few literatures have reported alterations in DNA methylation caused by exposure to benzo[a]pyrene until now. For example, a population investigation found that exposure to low-dose benzo[a]pyrene

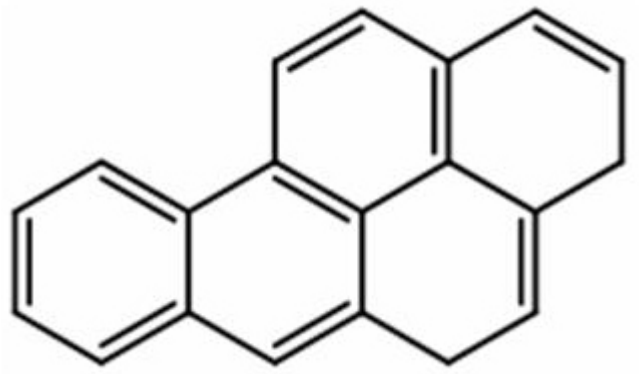

Figure 1. Chemical structure of benzo[a]pyrene.

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in environment leaded to alterations in DNA methylation of human blood, Experimental data showed that concentration of moles of benzo[a]pyrene in air was significantly correlated with genome substitution - long interspersed nuclear elements (LINE-1) and aberrant DNA methylation of Alu gene (6). However, the effect of other pollutants on DNA methylation were not considered in the study, aberrant DNA methylation might result from other compounds in environment. There have been no any relevant reports on direct evidence of aberrant DNA methylation caused by exposure to benzo[a]pyrene, especially investigation of alterations in DNA methylation of whole genome due to benzo[a] pyrene exposure. In this study, the effects of benzo[a] pyrene on DNA methylation level of whole genome in vivo through acute exposure of benzo[a]pyrene to ICR mice. The toxicity and carcinogenesis mechanisms of benzo[a]pyrene were preliminarily discussed from the perspective of epigenetics.

Materials and methods

Chemical reagents and enzymes

HPLC grade methanol, acetonitrile and formic acid were purchased from Sinopharm Chemical Reagent (Shanghai) Co. Ltd. Ammonium hydrogen carbonate, ammonium acetate, 5-methyl deoxycytidine (5-mdC), deoxycytidine (dC), nuclease Pl, phosphodiesterase I were purchased from Dow Chemistry (USA) Co. Ltd. Alkaline phosphatase was purchased from Langsheng Biotechnology Co. Ltd. (Lanzhou, China). Benzo[a] pyrene (analytical pure) was purchased from Jilin Chemical Reagent Factory (China).

Animal experiments

SPF grade male ICR mice (age: 5-6 weeks, weight: 30-50 g) were purchased from Animal Center of Beijing Medical University. ICR mice were divided into the experimental group and the control group with three ICR mice in each group. Temperature and humidity were 25±1 °C and 55%-60%, respectively. Illumination time and dark time were both 12 h. There were no restrictions on drinking water and feeding. Toxic experiments started after a week of normal feeding, all ICR mice in both groups were acutely exposed to benzo[a]pyrene (dose: 600 mg/kg by bodyweight) by oral administration. The blood, pancreas, skin, liver, lung and bladder of ICR mice were collected after 6, 12, 18, 24, 32 hours of benzo[a]pyrene exposure.

DNA extraction and hydrolysis

DNA in the blood samples were extracted using D0033 DNA purification kit (Beyotime, China). Whole genomic DNA in skin, pancreas, liver, bladder and lung were extracted using AMP Biotech DNA purification kit (AMP Biotech, China). The A_{260}/A_{280} ratio of DNA in all samples was between 1.5 and 1.8. DNA hydrolysis was conducted according to Crain method (7). DNA sample (1 μg) was dissolved in 3 μL de-ionized water, the solution was immediately placed into ice bath followed by denaturation at 100 °C for 2-3 min. 1 μL 0.1 mol/L ammonium acetate (pH = 5.2) and 1 μL nuclease P1 (2 U/μL) were added. The mixture was reacted for 3 h at 50 °C, then 1 μL 0.1 mol/L ammonium bicarbonate (pH = 5.2) and 1 μL phosphodiesterase (0.002 U/μL) were added. 1 μL alkaline phosphatase (0.5 U/μL) was then added after the solution was reacted for 2 h at 35 °C. The solution was allowed to react for another 1 h at 35 °C. The completely hydrolyzed DNA samples were stored at -30 °C for analysis.

Determination of whole genome DNA methylation level by HPLC

Analytical SEC measurements were done on a system with a Waters 510 HPLC pump and a Waters 2410 differential refractometer (DRI) detector. chromatographic column: Atlantis dC_{18} (2.1 mm x 150 mm i.d, partical size 5 μm, Waters), guard column (2.1 mm x 20 mm i.d, partical size 5 μm, Phenomenon). The mobile phase was composed of aqueous solution (A) of formic acid with a volume fraction of 0.1% and methanol solution (B) of formic acid with a volume fraction of 0.1%. The elution sequence of moible phase is 0-25 min, 0%-18% B; the flow rate is 0.24 mL/min. Column temperature was 25°C and the sample injection volume was 10 μL. The wavelength of DAD detector was set to be 285 nm. The level of DNA methylation is expressed by w(5-mdC)/(w(dC)+w(5-mdC))%

Data analysis

Tongfang DA software (Version 2A.13B, Qinghua Tongfang Inc., China) was used for data processing. The experimental results were expressed by mean±SD. Single-factor Analysis of Variance (ANOVA) was used to compare the difference between the control group and the experimental group, $p<0.05$ indicates that there are no significant differences between the data.

Results and Discussion

Determination of HPLC/DAD to DNA methylation level

The liquid chromatogram for hydrolysis of DNA samples in ICR mice's blood was shown in Figure 2. Figure 2 shows that the baselines of 5-mdC and dC can be seperated under the condition of optimized chromatography. The retention times of dC and 5-mdC were 5.4 and 9.5 min, respectively.

The standard samples of 5-mdC and dC were mixed to prepare a series of standard solutions, the injection volume is 10 L based on above experimental conditions. Peak area and mass concentration of standard solutions were set to Y and X axis, respectively, standard curve

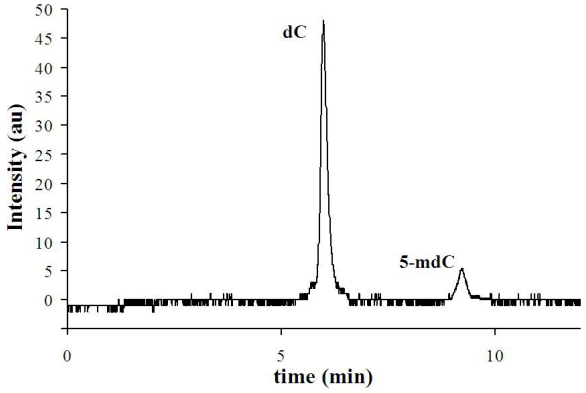

Figure 2. Liquid chromatographic separation of 5-mdC and dC.

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Table 1. Inter- and intra-day precision of 5-mdC and dC.

ρ (benzo[a]pyrene)	5-mdC		ρ (benzo[a]pyrene)	dC	
μg/L	Intra-day (%)	Inter-day (%)	mg/L	Intra-day (%)	Inter-day (%)
80	3.86	4.79	4	1.74	2.76
100	3.89	4.82	6	1.88	2.84
120	4.23	4.91	8	1.93	2.96
150	4.73	5.38	10	2.09	3.05
200	5.28	5.92	15	2.27	3.13

was plotted according to external standard method. Standard curves of 5-mdC and dC are y=584.51x-5.5227 and y=621.59x+68.591, respectively, correlation coefficents of standard curves are both greater than 0.99, linear ranges are 9-800 ng/mL for 5-mdC and 0.3-40 μg/mL for dC, and detection limits of 5-mdC and dC are 5 ng/mL and 6 ng/mL, respectively.

The average level of hydrolyzed calf thymus DNA methylation was determined to be 6.68%±0.20% (n=4) using the method mentioned above, which is consistent with previous result (8), indicating that this method is accurate. The samples were divided into low-concentration group and high-concentration group according to their mass concentrations. Tests of the samples in both groups were repeated for 3 consecutive times within one day and for 3 consecutive days. The result showed that the relative error of intra- and inter-day tests are less than 6% for 5-mdC and dC at different mass concentrations, suggesting that the precision of the method used in this study has good repeatability and reproducibility (see Table 1).

Effect of acute exposure to benzo[a]pyrene on DNA methylation of ICR mice

In order to confirm whether benzo[a]pyrene exposure could lead to changes in the level of DNA methylation of the whole genome, in this study a high dose of benzo[a]pyrene was administrated to male ICR mice, as a method of acute exposure to benzo[a]pyrene. During benzo[a]pyrene exposure, the body weight of ICR mice was not significantly changed, nor did the ICR mice exhibit obvious abnormal behaviors.

Figure 3 showed the changes of the total genomic DNA methylation levels in the blood, skin, pancreas, liver, bladder and lung of male ICR mice over observation time after acute benzo[a]pyrene exposure. As can be seen from the figures, the levels of DNA methylation in blood, liver, bladder and lung of the exposed group exhibited a downward trend compared with the blank group. However, there was a slight increase observed in the pancreas. For the exposed group, the levels of DNA methylation in blood and liver of ICR mice significantly decreased ($p<0.05$) at some points of observation time. Blood DNA methylation level decreased to 81.7% of the blank group after 12 h of benzo[a]pyrene exposure, and after 32 h, blood DNA methylation level decreased to 58.1% ($p<0.05$) of the blank group. A significant decrease ($p<0.05$) in level of DNA methylation were observed in the liver of ICR mice after 6 h of benzo[a]pyrene exposure, and the levels of DNA methylation in the liver were 71.6% and 64.5% ($p<0.05$) after 12 and 32 h of benzo[a]pyrene exposure, respectively.

As a pollutant, benzo[a]pyrene commonly exists in the environment, benzo[a]pyrene exposure to human body may cause skin cancer, lung cancer and other diseases. However, the mechanisms of the causation of diseases by benzo[a]pyrene have not been completely revealed. Recently, some researchers have confirmed that the change of DNA methylation pattern may be the pathogenic mechanisms of some environmental carcinogens (9). Hermann et al (6) found that there was a significant correlation between the mass concentration of benzo[a]pyrene in the environment and the level of DNA methylation in the blood of people who have special occupations (i.e. gas station workers and traffic police), however the study did not consider the effect of other pollutants in the environment. In this study, the effect of benzo[a]pyrene exposure on ICR mice was investigated, the experimental results showed that benzo[a]pyrene exposure can change the level of ICR mice's DNA methylation. Comparing with blood, liver DNA methylation level is more susceptible to benzo[a]pyrene exposure, suggesting that the change of DNA methylation by benzo[a]pyrene exposure has tissue specificity, liver and blood are the main targets for the effects of benzo[a]pyrene on the whole genomic DNA methylation. This is consistent with the conclusion that the target sites of benzo[a]pyrene are hematopoietic system and hepatic tissue, as found in previous human and animal experiments (10-13).

So far, the mechanisms of abnormal DNA methylation due to benzo[a]pyrene exposure are still unclear. Hermann et al (6) gave three possible mechanisms: I. nitrogen oxides, as a by-product of benzo[a]pyrene metabolism, increases the activity of DNA methylase after inducible transcription; II. benzo[a]pyrene exposure leads to oxidative damage of reactive oxygen species and DNA, which dereases the binding ability of methyl CpG to protein, causing the change of epigenetic properties; III. cleavage of DNA chain affects the binding ability of DNA methylase and CpG island. Furthermore, two other possible mechanisms should also be considered. I. DNA adducts formed adjacent to CpG island will inhibit the binding of DNA methylase to DNA. However the metabolites of benzo[a]pyrene, such as hydroquinone and 1,4-benzoquinone are both able to form a series of adducts with DNA (14), these adducts are likely to hinder the combination of DNA methylase and CpG. II. Epidemiological investigations have shown that both animal and cellular experiments have demonstrated that benzo[a]pyrene can cause oxidative damage of DNA and the formation of 8-hydroxy-2-deoxyguanosine (8-OHDG), a DNA oxidative damage marker. 8-OHDG near the DNA methylase recognition domain can decrease the affinity of DNA methylase and CpG island, and DNA methylation is inhibited accordingly

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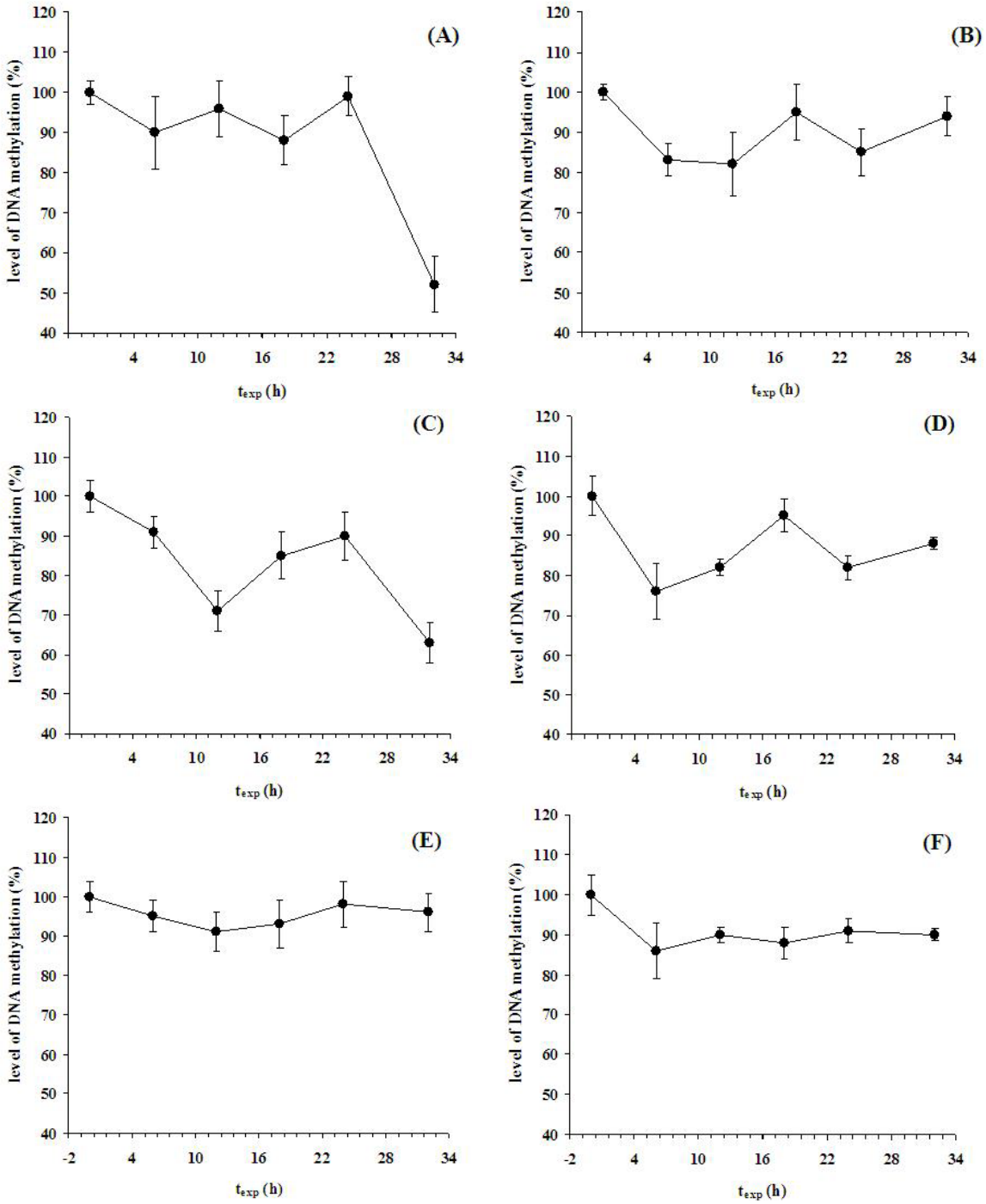

Figure 3. Changes of whole-genomic DNA methylation levels versus time in blood (A), pancreas (B), liver (C), lung (D), skin (E) and bladder (F) of dosed male ICR mice.

(15,16).

In this study, only single-dose acute exposure was applied to investigate the effect of benzo[a]pyrene on whole genomic DNA methylation level of male ICR mice. It has been found that the levels of DNA methylation in liver and blood decreased significantly, while the levels in pancreas, bladder, skin and lung tissues did not reduce dramatically. Future work are needed to further investigate the changes of DNA methylation level in pancreas, bladder, skin and lung tissues after longer exposure time. In addition, changes of DNA methylation level in ICR mice under a condition of chronic toxicity should be studied to accomplish a more comprehensive investigation on the epigenetic toxicity of benzo[a] pyrene.

This study proved that benzo[a]pyrene exposure can change the DNA methylation level in male ICR mice by animal experiments, and the changes are statistically different. The changes of DNA methylation level in male ICR mice by benzo[a]pyrene exposure showed tissue specificity, the level of whole genomic DNA methylation decreases significantly in blood and liver, rather than pancreas, lung, skin and bladder of ICR mice.

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