GENERATION AND TOXICITIES OF HALOGENATED BENZO[a]PYRENS

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Introduction:

Many kinds of polycyclic aromatic hydrocarbons (PAHs) are discharged from various emission source in air environment and in aqueous environment[1]. PAHs discharged in the atmosphere are come into the aqueous environment through falling or rain water[2]. It seems PAHs are received the changes (oxidation, reduction, decomposition, substitution, conjugation etc.) in the aqueous environment by chemical reaction, photo-reaction, metabolism of the organism and various treatment processes. Especially, in the case of PAHs dissolved on the raw water for drinking water source, PAHs are exposed chlorine and then changed to various reaction products in the process for the disinfection by the reaction of oxidation and/or chlorine displacement. Additionally, it is afraid whether the reaction products effect on human health or not. There are few reports about behavior of PAHs during chlorine treatment in water treatment process and toxicities of halogenated PAHs.

In Dioxin 2011, we present the behaviour of benzo[a]pyren (B[a]P), most common and toxicic compound among PAHs, in the clorine water and toxicities of the chlorinated B[a]P. The chlorinated forms of PAHs were identified by gas chromatography with mass spectrometric detector in selected ion monitoring (SIM) mode after the extraction using solid phase cartridge from water sample. We have examined the cytotoxicity using cultured cells, the nutagenecityu using microorganism, the effect on the process of differentiation to the nervous cells at the expression level of the specific genes.

Materials and methods:

Chemicals

B[a]P (93.0%), dichloromethane and acetonitrile (pesticide residue PCB analysis grade), methanol (HPLC grade), sodium hypochlorite solution, and L(+)-ascorbic acid sodium salt were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Laboratory water was purified by aMilli-Q gradient A10 Elix system with an EDS polisher (Millipore, Bedford, MA, USA). The stock solutions were prepared to 1000 mg/L in acetonitrile. All stock solutions were stored at -20˚C, whereas working solutions were prepared fresh for each use by dilution of the standard stock solution.

B[a]P-Cl was chemically synthesized from B[a]P and N-chlorosuccinimide in propylene carbonate by the reflux for 1hr at 100C following the method by Dewhurst and Kichen[5]. B[a]P-Br was also ckemically synthesized B[a]P and N-bromosuccinimide in propylene carbonate by the reflux for 1hr at 100C. The standard sample was purified and concentrated by the recrystallization. The purities were identified B[a]P-Cl with 96.7%, B[a]P-Cl2 wth 94.6%, B[a]P-Br with 97.2% in NMR. It was estimated that containing impurity material was a solvent. B[a]P-Cl and B[a]P-Br were replaced with one chlorine or one bromide at the sixth position. B[a]P-Cl2 was replaced with two chlorines at the sixth/thirteenth or sixth/eleventh positions. So, B[a]P-Cl2 was mixture with B[a]P-6,13-Cl and B[a]P-6,11-Cl.

Condition of Chlorination

The pure water was fortified with 1ml of 0.1M phosphate buffer (pH 7.0) and was made the concentration of free residual chlorine ion to be 1ppm by adding sodium hypochlorite. Then, the solution of B[a]P was dropped into the chlorinated water to be 3.0μg/L final concentration. B[a]P was made to contact the chlorine ion at 20°C, while the water sample was stirred with stirrer.

Analytical procedure

L(+)-ascorbic acid sodium salt (Wako, Japan) was added to the sample water to 0.005% (w/v). The sample water adjusted to pH3.5 by 9% (v/v) nitric acid and then concentrated with solid phase extraction (SPE) method using Oasis HLB Plus Extraction Cartridges (Waters; Milford, MA, USA) prepacked with *N*-vinyl-pyrrolidone polymer resin as described previously. 1 liter of each sample was extracted with the SPE cartridges equilibrated

with 5ml dichloromethane, 5ml methanol and the 5ml pure water. Extraction of water samples was carried out at a 10ml/min flow rate. After passing the sample through the cartridges, cartridges were washed with 10ml pure water at a 5ml/min. Air was then pulled through the cartridge for 40min. The analytes were eluted from the cartridges with 3ml acetonitrile at a rate of 1-2 drops/sec. After evaporating the samples to near dryness under a gentle nitrogen stream, the compounds were transferred into a final volume of 1.0ml of acetonitrile for GC/MS analysis.

Gas chromatography with mass spectrometric detection (GC/MS)

GC was carried out using an HP6890 Series Gas Chromatograph system (Hewlett Packard, Wilmington, DE, USA) with an HP6890 Series auto-sampler and split/splitless injector. The analytical column was a DB-5 fused silica capillary column, 30 m×0.25 mm ID, 0.25 mm film thickness (J&W Scientific, Folsom, CA, USA). The oven temperature program was as follows: 100˚C initial temperature for 1 min, ramped at 10˚C/min to 220˚C, at 3˚C/min to 280˚C, holding for 5 or 10 min at 280˚C. The carrier gas (helium) flow was set to 1.2 mL/min. Pulsed splitless injection of a 2 μL volume was carried out at 250˚C. The MS was carried out using a 5973 Mass Selective Detector (Hewlett Packard, Wilmington, DE, USA) in electron ionization mode with an ionization voltage of 70 V and ion source temperature of 280˚C. The instrument was operated in selected ion monitoring (SIM) mode. The selected ion was monitored for identification by following mass number (*m/z*); B[a]P (m/z;252 and 253), mono-chlorinated B[a]P (m/z;286 and 288) and di-chlorinated B[a]P (m/z;320 and 322) .

Effect on the process of differentiation by the expression level of the specific genes

The growth medium for maintenance of the mouse ES cells was used Dulbecco's Modified Eagle's Medium (D-MEM) containing high concentration of glucose, added 20% (v/v) Fetal calf serum (FCS), 1/100 volume of the 100-fold concentration non-essential amino acid solution, 1mM sodium pyruvate, 0.1mM β-mercaptoethanol and 10^3 units of leukemia inhibitory factor. The serum was heated at 55 ^oC for 30min before using. The primary mouse fibroblast cells was cultured with D-MEM containing high concentration of glucose, added 10% (v/v) FCS, 1/100 volume of the 100-fold concentration non-essential amino acid solution, 1mM sodium pyruvate and 0.1mM β-mercaptoethanol. The mouse ES cells were cultured on the feeder cells that were seeded on the tissue culture's plastic dish covered gelatin. The feeder cells were seeded 4-8 x 10^4 cells/cm^2. The growth medium was changed every day and the mouse ES cells were transferred to the new dish attached feeder cells every three days. The dish covered gelatin was prepared by following procedures. The 0.1% gelatin solution was added into the tissue culture's plastic dish and discarded after keeping for 2 hrs in the CO_2 incubator at least and then the dish was washed twice with PBS. The feeder cells were prepared from the primary mouse fibroblast cells by treatment with 10 μg/mL mytomicin C for 2hrs at 37 ^oC for 2 hrs in the CO_2 incubator. The feeder cells were effective for the maintenance of the mouse ES cells for 5 days but their proliferation capacities were lost.

Results and discussion:

Chlorination of Benzo[a]pyrene

In the condition inthe Materials and Methods, non-chlorinated PAHs dissolved in water were recovered from 80% to 120% by a solid phase extraction. After contact of chlorine, B[a]P rapidly decreased and then reached to 6.6% of the initial concentration after 2 hour contact. The peak area of mono-chlorinated B[a]P (*t*=25.9min) increased depending on the contact time with chlorine. The concentration of mono-chlorinated B[a]P kept constantly during 24 hours or 48 hours after chlorin ecposure. The peak area of di-chlorinated B[a]P was detected at 2hours and increased until 4 hours. Di-chlorinated B[a]P was not changed from 4 hours and 48 hours at the constant concentration. However, Tri-chlorinated forms were not detected in this condition. From these results B[a]P rapidly is reacted with the chlorine and is changed to the chlorinated forms. First, B[a]P is changed to mono-chlorinated form and gradually changed to di-chlorinated form. In addition, it seems di-chlorinated form is substituted more with chlorine or is decomposed. If B[a]P of the dissolved state contains in the raw water, B[a]P might be changed chlorinated form in the water purification process, because B[a]P dissolved in water is difficult to remove in the general rapid filtration processing.

Cytotoxicity on the undifferential and differential mouse ES cells

It is difficult to obtain the standards of chlorinated B[a]P. So, previous time, we have prepared to extract chlorinated componds from chlorine water dissolved B[a]P. But, the extracted sample was problem about puritu and quantity. We have synthesized chemically these compounds. The purified samples were used for

examination for the biological evaluation. Cytotoxicity was estimated using Cell-Titer-Glo Luciferase Cell Viability Assay kit (Pormega) to measure ATP value in the living cells by luciferase.
B[a]P-Cl and B[a]P-2Cl showed very weak cytotoxicity for mouse ES cells on 24 hrs and 72hrs after exposure. B[a]P showed cytoptoxicity on 72hrs after exposure but did not show with dependence of exposed concentration. B[a]P and haloganated B[a]Ps shows a weak cytotoxicity on the undifferential mouse Embyionic stem (ES) cells. B[a]P-Cl and B[a]P were exposed at the concentration of 21.2 mM and B[a]P-Br was exposed at the concentration of 22.4 mM in the differentiation process to induce nerve cells. B[a]P was exposed 10 mM. At the those concentration, the differentiat-ing mouse cells were alive 70-80% in the number on the differentiation start. On twelfth day after the induction of differentiation, axons were observed under the optical microscope. On twentieth day, the axon of neuron cells were complexly linked each other and the complicated network structure was formed. There was morphologically no change on the cells exposed by B[a]P and halogenated B[a]P in comparison with the control cells. There was also no significant difference on the viability. In the case using stable established cell lien, HepG2 cells, there was also morphologically no change on the cells exposed by both compounds in comparison with the control cells. There was no significant difference on the viability.

Effect of haloganated B[a]P for expression of nerve specific expression genes

The effect on the gene expression of B[a]P and halogenated B[a]P in the nerve cell differentiation process was examined using RT-PCR on the basis of the expression activity of the gene expressed particularly in the differentiation process. All of B[a]P, B[a]P-Cl and B[a]P-Br showed a littele the different expression profile of the nestin, musashi, neurospecific enolase(NES) genes which are the nerve specific cell marker in comparison with the control cells. B[a]P-Cl was supressed more the nerve specific cell markers than B[a]P and B[a]P-Br between tenth and fourteenth day after the induction of differentiation. B[a]P-Cl also supress ERK2, Ah receptor, CYP1A1 and CypB1B genes since tenth day after the induction of differentiation. These results indicate B[a]P-Cl and B[a]P-Br caused the supression of the nerve specific gene expression in the mouse nerve cell differentiation process. B[a]P-Cl supressed more heavier than B[a]P-Br. From suppression of AhR related genes and signal transduction system genes, it is worried the possibility of affecting the nerve development.

Effect of haloganated B[a]Ps in differntiating process using the transformed cells

We have established the transformed cell lines, which stably contain the constructed plasmid inserted the regulatory region of nerve specific expressed gene, Neuron-specific enolase (NSE-prom mouse ES cells) and microtuble-associted protein 2 (MAP2-prom mouse ES cells) , upstream of the luciferase gene. Five microM B[a]P, B[a]P-Cl, B[a]P-cl2 mix and B[a]P-Br were exposed to NSE-prom mouse ES cells and MAP2-prom mouse ES cells to estimate the effect in the differentiation process to nerve cells by these compounds.

By the exposure of B[a]P, the gene expression controlled by the reguratory region of NES was suppression. B[a]P did not show visually the cytotoxicity but affected the process to nerve differentiation on the gene expression. Whereas, the gene expressioncontrolled by MAP2 was strongly suppressed by exposure of B[a]P-Cl and B[a]P-Br, but not B[a]P and B[a]P-Cl2 mix. These results indicate halogenated B[a]Ps were affected for the process to nerve cells without morphological change in some periods. About the activity of the reguratory region of MAP2, the inhibitory activity was higher in order of B[a]P-Br>B[a]P-Cl>B[a]P-Cl2 mix> B[a]P. B[a]P replacing one chlorine atom showed stronger suppression activity than B[a]P replacing two chlorine atoms. Type of the halogen atom, replacing position and number might affect the suppression activity.

Mutagenecity

The mutagenicity was not dected in Ames test using TA100 and TA98 under the condition without metabolic activation. However, under the condition with metabolic actvation, B[a]P-Cl showed mutagenecity more than 10.6 μg/ml in TA100 strain and 6.5 μg/ml in TA98 strain. Similarly, B[a]P-Br showed mutagenecity more than 8.4 μ g/ml in TA100 strain and 9.8 μ g/ml in TA98 strain. B[a]P-Cl2 mix showed mutagenecity more than 1.4 μ g/ml in TA100 strain and 4.5 μg/ml in TA98 strain. B[a]P-Cl showed more mutagenic activity in TA98 strain than in TA100 strain, but B[a]P-Br and B[a]P-Cl2 showed more mutagenic activity in TA100 strain than in TA98 strain. In TA100 strain, the mutagenec activity was the order of B[a]P-Cl2>B[a]P-Br>B[a]P-Cl. In TA98 strain, the mutagenecity was the order of B[a]P-Cl2>B[a]P-Cl>B[a]P-Br. These results indicate that halogenated B[a]Ps show the mutagenecity with metabolic actibvation.

Smmary:

The chlorinated B[a]Ps were generated in chlorine water. B[a]P-Cl procedes for B[a]P-Cl2 and B[a]P-Cl3 at room temperature. Haloganated B[a]Ps ocuur the specific gene suppression in the some period in the nerve

differential process in mouse ES cells without visualable morphological change. Especially, B[a]P-Cl and B[a]P-Br showed storonger suppression than B[a]P and B[a]P-Cl2 mix. In the present of bromate ion, B[a]P-Br might be generated similar to B[a]P-Cl. B[a]P-Cl, B[a]P-Br and B[a]P-Cl2 mix showed the mutagenecity under the condition with metabolic actibation. These results indicate the grasps and controls of the behaviour in the environment and various treatment processes of haloganated B[a]P, prior mono-haloganated B[a]P in the other haloganated B[a]P, is important.

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