Changes of biomarkers with oral exposure to benzo(a)pyrene, phenanthrene and pyrene in rats

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants present in air and food. Among PAHs, benzo(a)pyrene(BaP), phenanthrene (PH) and pyrene (PY) are considered to be important for their toxicity or abundance. To investigate the changes of biomarkers after PAH exposure, rats were treated with BaP (150 µg/kg) alone or with PH (4,300 µg/kg) and PY (2,700 µg/kg) (BPP group) by oral gavage once per day for 30 days. 7-ethoxyresorufin-O-deethylase activity in liver microsomal fraction was increased in only BaP groups. The highest concentration (34.5 ng/g) of BaP, was found in muscle of rats treated with BaP alone at 20 days of treatment; it was 23.6 ng/g in BPP treated rats at 30 days of treatment. The highest PH concentration was 47.1 ng/g in muscle and 118.8 ng/g in fat, and for PY it was 29.7 ng/g in muscle and 219.9 ng/g in fat, in BPP groups. In urine, 114-161 ng/ml 3-OH-PH was found, while PH was 41-69 ng/ml during treatment. 201-263 ng/ml 1-OH-PY was found, while PH was 9-17 ng/ml in urine. The level of PY, PH and their metabolites in urine was rapidly decreased after withdrawal of treatment. This study suggest that 1-OH-PY in urine is a sensitive biomarker for PAHs; it was the most highly detected marker among the three PAHs and their metabolites evaluated during the exposure period and for 14 days after exposure.

Key words: benzo(a)pyrene, biomarker, PAHs, phenanthrene, pyrene

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are by-products from incomplete combustion or pyrolysis of organic materials containing carbon and hydrogen and are present as mixtures in air and food [41]. Some of the PAHs are classified as possible human carcinogens [18]. The low molecular weight PAHs have been shown to induce immune suppression, phototoxicity, neurological, and reproductive dysfunction in experimental animals [2,44,39]. Most of the PAHs are metabolized to hydroxylated compounds by the liver microsomal NADPH-dependent cytochrome P450 monooxidase system, then they are excreted into the urine and feces [15,18].

The biotransformation and metabolic activation, of carcinogenic PAHs, take place primarily by the action of the CYP1 family of cytochrome P450 monooxygenase [42]. The cytochrome P450 enzyme CYP1A1 catalyzes the generation of the reactive metabolites from the PAHs, and the metabolites subsequently result in the formation of adducts with DNA and protein [26,33,37]. The induction of the CYP1A1 enzymes in the liver microsomal fraction has been used as a biomarker for toxicity screening for many chemicals in experimental animals [12,38,40] by measurements of 7-ethoxyresorufin-O-deethylase (EROD) activity [35]. Benzo(a)pyrene (BaP) is the most potent carcinogen; it is embryo toxic and teratogenic in animals [18]. The level of BaP may be a good marker for carcinogenic PAH contamination in an environmental sample [7,41]. BaP is metabolized by the liver microsomal mixed function oxidase system to highly reactive compounds that can bind to specific target sites of DNA, which is of critical importance in the initiation of BaP-induced carcinogenesis [3,14].

Phenanthrene (PH) and pyrene (PY) have been found at high levels in the air and in food [10,11,32,41]. The level of PY in the air was highly correlated with the total amount of airborne PAHs; it is composed of a large portion of high molecular weight PAHs in occupational and environmental samples [5,22]. The 1-OH-pyrene in urine is representative of pyrene and the total amount of PAHs in occupational and dermal exposure in humans; it is a good indicator of mutagenic activity in animal and human hepatic fractions [6,21]. The 3-OH-benzo(a)pyrene and 3-OH-phenanthrene compounds have been reported to be the most abundant metabolites after BaP and PH exposure, re-
spective, in human urine [16].

To investigate the changes of biomarkers after exposure to PAHs, we administered BaP, PH and PY, representative chemicals of the PAHs, to female rats. We determined the concentration of BaP, PH and PY in muscle and fat, and their metabolites in urine; in addition, we studied the EROD activity in the liver and the DNA adducts of BaP in blood lymphocytes as well as the serum biochemical parameters in a time-dependent manner.

**Materials and Methods**

The chemical BaP, PH, PY, β-glucuronidase, ethoxyresorufin, NADPH, dimethylsulfoxide, and corn oil were purchased from Sigma-Aldrich (USA), and the 3-OH-benzo(a)pyrene, 1-OH-pyrene, 3-OH-phenanthrene and Benzo(a)pyrene-r-7,t-8,t-9,c-10-tetradyrotetrol(+/−) were purchased from the Midwest Research Institute (USA).

**Animals and housing**

Eight-week-old female Slc : SD rats (SLC, Japan) were provided with tap water and commercial diet ad libitum. The animal room was maintained at a temperature of 22 ± 2°C, relative humidity 50 ± 10% and a 12-h light/dark cycle. All animals were cared for in accordance with the guidelines established by the National Veterinary Research and Quarantine Service, Korea.

**Experimental design**

BaP, PH and PY were dissolved in dimethylsulfoxide and further diluted with corn oil to the dose of BaP (150 µg/kg), PH (4,300 µg/kg) and PY (2,700 µg/kg). One group of rats was treated with BaP alone and another with BaP with PH and PY simultaneously given by oral gavage once per day for 30 days at a volume of 2 ml/kg and maintained for another three weeks after withdrawal. Muscle, fat, blood, liver and urine samples were collected every 10 days during treatment and every 7 days after withdrawal of the treatment. Three rats were housed in a cage specially designed to collect the overnight urine. The urine samples were kept at ~80°C until measurement.

**Determination of 7-ethoxyresorufin-O-deethylase (EROD) activity in liver**

Individual liver samples were homogenized in ice-cold solution (0.25 M sucrose, 0.1 M Tris-HCl, 1 mM EDTA, pH 7.4). The microsomal fraction was separated by ultracentrifugation and the collected pellet was further dissolved with 1 ml of 20% glycerol solution (pH 7.4) containing 0.1 M Tris-HCl, 1 mM EDTA and 0.25 M sucrose. Liver microsomal EROD activity was assayed according to the method of Pohl and Fouts [35].

**Determination of the biochemical parameters in serum**

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in serum were determined using a commercial kit (Bayer, Germany) and a blood chemical analyzer (Express 550 Ciba Corning, USA).

**Determination of benzo(a)pyrene-r-7,t-8,t-9,c-10-tetradyrotetrol (+/−) in blood lymphocytes**

Blood lymphocyte DNA was isolated with a DNA isolation kit (Roche Molecular Biochemicals, USA). Then 100 µg of DNA was dissolved in 0.1 N HCl solution and incubated at 97°C for 30 min to hydrolyze the BaP-DNA adduct. After cooling to room temperature, methanol was added to obtain a 10% methanol solution. Benzo(a)pyrene-r-7,t-8,t-9,c-10-tetradyrotetrol (+/−) was separated with a SeP-Pak C18 cartridge (Waters, USA). The purified samples were analyzed with liquid chromatography according to the method of Islam et al. [19].

**Determination of parent compounds in muscle and fat, and their metabolites in urine**

For the determination of BaP, PH and PY, 3 g of muscle (0.3 g of fat) was homogenized with liquid nitrogen and extracted with 50 ml of hexane by sonication for 30 min. Homogenates were filtered through anhydrous sodium sul-

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**Table 1.** Changes of relative liver weight by treatment with a vehicle, benzo(a)pyrene alone (BaP) or benzo(a)pyrene with pyrene and phenanthrene (BPP) (Liver weight / body weight, %)

| Groups | Treatment (day) | Withdrawal (day) |
|--------|----------------|------------------|
|        | 10             | 20               | 30               | 7     | 14     | 21     |
| Vehicle| 3.27 ± 0.14    | 2.96 ± 0.13      | 3.07 ± 0.10      | 3.27 ± 0.14 | 3.00 ± 0.29 | 2.95 ± 0.13 |
| BaP    | 3.50 ± 0.11    | 3.18 ± 0.24      | 3.03 ± 0.17      | 3.05 ± 0.30 | 3.10 ± 0.05 | 3.23 ± 0.15 |
| BPP    | 3.31 ± 0.06    | 3.13 ± 0.12      | 3.02 ± 0.04      | 2.96 ± 0.14 | 3.00 ± 0.14 | 3.14 ± 0.19 |

Chemicals were treated for 30 days via gavages to 9 week old female SD rats. Values are the mean ± SD (n = 3).
fate and concentrated to about 1 ml at 45°C with a rotary evaporator. The concentrated solution was further purified using an activated Florisil cartridge (Silica cartridge for fat). The PAHs were eluted with 18 ml of hexane and dichloromethane (3 : 1, v/v) solution, and the eluate dried at 45°C, then dissolved with 1 ml of acetonitrile by sonication. Next, 20 µl of filtered solution was analyzed using liquid chromatography with a fluorescence detector according to the method of Chen et al. [9]. Analysis of the parent compound, and their metabolites in urine, was conducted using the method of Jongeneelen et al. with modifications [21]. A 7 ml sample of 0.2 M sodium acetate buffer (pH 5.0) was added into 5 ml of urine for acidification; then β-glucuronidase (13,200 unit) and sulfatase (220 unit) solution was added. The mixture was incubated with a shaking incubator (37°C, 210 rpm) for 16 h. After centrifugation (3,000 × g for 10 min), the supernatant was loaded onto an activated Sep-Pak C18 cartridge, washed with 5 ml of 40% methanol, eluted with 8 ml of acetonitrile, and 10 ml of hexane and dichloromethane (3 : 1, v/v). The final eluate was evaporated at 45°C and dissolved with 1 ml of acetonitrile.

Statistical analysis
The body weight gain, organ weight, feed consumption, and blood chemistry parameters were analyzed by break down and one-way ANOVA, followed by the Duncan test as a post-hoc comparison using a statistics program (Ver. 5.5; StatSoft, USA).

Results

Body weight, organ weights and blood chemistry
The treatment groups showed no significant difference in body weight and relative liver weight compared to those in the vehicle control group (Fig. 1 & Table 2). Rats exposed to BaP or BPP did not show significant changes in the activity of ALT, AST and ALP. However, the ALP activity was increased by 21 days after the withdrawal of BPP compared to the ALP in the vehicle control group (Table 2).

Table 2. Serum biochemical values by treatment with a vehicle, benzo(a)pyrene alone (BaP) or benzo(a)pyrene with pyrene and phenanthrene (BPP)

| Groups | Treatment (day) | Withdrawal (day) |
|--------|----------------|------------------|
|        | 10             | 20               | 30               | 7    | 14  | 21  |
| ALT    | Vehicle        | 28.0 ± 5.9       | 23.4 ± 1.9       | 29.5 ± 2.4 | 25.8 ± 4.0 | 21.8 ± 2.1 | 25.7 ± 0.5 |
| (unit/l) | BaP            | 40.5 ± 15.0      | 30.5 ± 3.1       | 31.8 ± 1.8  | 30.9 ± 1.9  | 26.4 ± 1.1  | 30.2 ± 3.0  |
|        | BPP            | 51.5 ± 19.8      | 25.6 ± 1.7       | 27.7 ± 2.3  | 25.8 ± 2.1  | 26.1 ± 2.8  | 26.0 ± 0.5  |
| AST    | Vehicle        | 60.7 ± 9.1       | 67.6 ± 6.7       | 80.5 ± 6.3  | 64.0 ± 7.9  | 58.2 ± 1.5  | 68.1 ± 2.2  |
| (unit/l)| BaP            | 75.9 ± 10.5      | 83.2 ± 6.6       | 80.0 ± 5.5  | 72.9 ± 8.5  | 68.7 ± 1.7  | 69.7 ± 5.5  |
|        | BPP            | 80.5 ± 13.0      | 71.8 ± 3.9       | 78.7 ± 0.05 | 57.1 ± 0.4  | 62.5 ± 6.9  | 61.7 ± 0.12 |
| ALP    | Vehicle        | 158.7 ± 34.7     | 154.3 ± 26.2     | 135.3 ± 9.0 | 117.7 ± 7.8 | 122.0 ± 8.0 | 109.0 ± 5.0 |
| (unit/l)| BaP            | 185.3 ± 29.9     | 155.3 ± 9.8      | 182.0 ± 25.4| 131.7 ± 3.8 | 126.0 ± 8.1 | 132.0 ± 2.9 |
|        | BPP            | 150.0 ± 25.4     | 175.0 ± 31.9     | 143.5 ± 21.5| 125.0 ± 13.5| 107.0 ± 10.2| 152.0 ± 15.6*|

Chemicals were treated for 30 days via gavages to 9 week old female SD rats. Values are the mean ± SD (n = 3). ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase. *Significantly different from vehicle control at p < 0.05.
EROD activity in liver and BaP adduct in lymphocytes

EROD activity, a major enzyme of CYP1A1 in the liver microsomal fraction was significantly increased \( (p < 0.05) \) only in rats exposed to 30 days of BaP alone. There were no significant differences in the time course of EROD activity during BPP treatment and during the withdrawal periods of BaP and BPP (Fig. 2). Benzo(a)pyrene-r-7, t-8,t-9,c-10-tetradyrotetrol (+/-) was not detected in the blood lymphocytes of rats exposed to BaP alone or with PH and PY.

Time-course changes of BaP, PH and PY in muscle and fat

The highest mean amount of BaP in muscle was 34.4 ng/g in the BaP treated group; it was 23.6 ng/g in BPP treated rats, an amount achieved after 20 days of treatment in both groups. The BaP concentration in muscle showed no significant difference in comparisons between both treatment groups (BaP and BPP). In addition, the amount of BaP in muscle rapidly decreased after withdrawal of the treatment.

The highest mean amount of BaP in fat was 3.2 ng/g in the BaP treated group; this amount was obtained by 20 days of treatment. The highest mean amount of PH was 47.1 ng/g in muscle and 118.8 ng/g in fat; for PY it was 29.7 ng/g in muscle and 219.9 ng/g in fat, which was also achieved by 20 days of treatment. Both compounds were rapidly removed from the system after withdrawal of treatment (Figs. 3 & 4, Table 3)

Time-course changes of BaP, PH and PY and their metabolites in urine

The 3-OH-BaP compound, a major metabolite of BaP, was not detected in urine during treatment or after withdrawal of treatment in both the BaP and BPP exposed groups. The 3-OH-PH compound is a major metabolite of PH; it was found in the ranges of 114-161 ng/ml and the highest concentrations were reached by 30 days of treatment. The concentrations of the parent compound (PY) were 42-69 ng/ml with the highest concentration reached by 30 days of treatment. The 1-OH-PY compound, a major metabolite of PY, was found to be in the range of 201-263 ng/ml and the highest concentration was achieved by 10 days of treatment; the parent compound was in the range of
Fig. 4. Changes of PAHs in fat at different time points by treatment with benzo(a)pyrene 150 µg/kg alone (BaP) and benzo(a)pyrene with pyrene 1,700 µg/kg and phenanthrene 4,300 µg/kg (BPP). Values are mean ± SD.

Fig. 5. Changes of PAHs and their metabolites in urine at different time points by treatment with benzo(a)pyrene 150 µg/kg alone (BaP) and benzo(a)pyrene with pyrene 1,700 µg/kg and phenanthrene 4,300 µg/kg (BPP). Values are mean ± SD.

9-17 ng/ml with the highest concentration reached by 30 days of treatment. The amounts of PY, PH and their metabolites were rapidly eliminated from the system after the withdrawal of treatment (Fig. 5, Table 3).

Discussion

The PAHs are a group of several hundred compounds that are present as a mixture in air and food. BaP is the most toxic among the PAHs and well known as a carcinogen in animals. The level of BaP in environmental samples is a good marker for carcinogenic PAH contamination [7]. The correlation between BaP and the carcinogenic PAHs was reported to be 0.98 in food [24]. PH and PY are less toxic than BaP, but they are found in high levels in the air, animal feed and food compared to the other PAHs [9,30,32]. Therefore, in this study, BaP, PH and PY were selected as representative PAHs. We determined the treatment dose of BaP as 150 µg/kg/day based on the reference maximal exposure amount in humans, which was 0.055 µg/kg/day [41] and multiplying the maximal margin factor by 3,000. The dosages of PH and PY were determined to be 4,300 µg/kg/day and 2,700 µg/kg/day, respectively; these doses were chosen based on the mean contamination ratios of PH and PY to BaP in air, food and feed.

The metabolic activation of carcinogenic PAHs occurs primarily through the action of the CYP1A family P450 monooxygenase; CYP1A1 acts mainly by the hydroxylation of BaP [35]. The activity of CYP1A1 was measured using the EROD activity [8,35,40]. Although the EROD activity in rats exposed to BaP was increased about 44.7 fold in another reported experiment [28], in this study
the increase was three fold compared to the control with the treatment of BaP alone for 30 days. These differences may be caused by the treatment dose used. The dose of Moorothy et al. [28] was more 20 times higher than that used in the present study. This result is also supported by Nilsen et al. [29] who showed that mice exposed to 10 mg diesel exhausts for 14 days caused no increase of EROD activity. The threshold dose for the induction of hepatic EROD activity was reported to be about 300 µg/kg for 5 and 6 ring PAHs [37]. PH and PY did not change the EROD activity in H4IIE cells, while the cells were sensitive to BaP [4]. Willett et al. [42] reported that fluoranthene inhibited the BaP induced EROD activity in fish. This study, EROD activity in rats exposed to BaP with PH and PY was not changed. However, it was increased in rats exposed to BaP alone. PAHs bind to aryl hydrocarbon receptors (AhR) in the liver and are further metabolized to a hydroxylated compound. The high carcinogenic potency of certain PAHs is correlated with the Ah-receptor (AhR) affinity, and the induction of the CYP1A enzymes leading to increased rates of formation of reactive metabolites [40]. Therefore, PH and PY may interrupt BaP binding to AhR; however, scaled experiments with additional samples as well as in vitro studies are required for the characterization of the changes in CYP1A1 activity with BaP with other PAH chemicals.

DNA adducts of BaP in various organs have been studied as biomarkers for PAH exposure or toxicity. In addition, blood lymphocytes have been suggested to be useful biological targets for DNA adduct formation [19,34,36]. Benzo(a)pyrene-7,8,9-tetradyrotetrol (+/−), one of the main DNA adducts of benzo(a)pyrene, was not detected in the blood lymphocytes of rats exposed to BaP alone or to PH in our study. The detection limit for benzo(a)pyrene-7,8,9-tetradyrotetrol (+/−) was about 2.5 pg, which is comparable to that reported by Alexandrov et al.[1]. The same metabolite was found as an albumin adduct in rats exposed to 100 mg BaP/kg for 3 days orally [19] and 10 mg BaP/kg by intra-peritoneal injection [13]. Although the dose of BaP in this study was higher than the maximum exposure level in humans, the dose of BaP used in this study may not have been enough to induce EROD activity to form reactive metabolites to bind to DNA. Our data suggests that DNA adducts in blood lymphocytes may have been too low to be detected. Therefore, a more sensitive method is needed to analyze BaP-specific DNA adducts.

BaP is known to be readily absorbed from the gastrointestinal tract in animals. By contrast, the pretreatment of BaP, for 7 days, inhibited the accumulation of BaP in the body fat [41]. PY in an aqueous suspension was poorly absorbed from the GI tract; the amount observed was highest in the peritoneal fat compared to the liver, kidney, lung, heart, testes, spleen, and brain [27,43]. Data from the present study showed that the amount of PH and PY in fat also was higher than in muscle. However, BaP was higher in muscle than in fat. BaP, PH and PY were rapidly removed from muscle, consistent with other reports [27,43], showing that the rate of clearance of the PAHs form organs was very rapid.

Some of the PAHs are quickly metabolized by phase 1 enzymes in the liver to a hydroxylated form and they are then mainly excreted in the urine. Metabolites of PH and PY, or other PAHs in body fluid, may be useful biomarkers for PAH exposure in humans and animals [16,17,21,25]. Urine samples can be obtained quickly and easily in animals and humans, and may provide a useful biological sample for the study of exposure and toxicological assessment for environmental contaminants. 1-OH-PY, 3-OH-PH and 3-OH-BaP are the major hydroxylated metabolites for PY, PH and BaP, respectively, in humans and animals [16,20,25]. Pyrene is a relatively large portion of the high molecular weight PAHs, and it is found at high levels in the diet; its metabolite, 1-OH-PY, found in urine has been suggested to reflect the total PAH contamination and is an indicator of the mutagenic activity of the PAHs in animals [5,6,22]. In the human, the ratio of 1-OH-PY to 3-OH-BaP was reported to be 200 times higher [16]. The 1-OH-PY in urine reflects a recent exposure while the PAH-adduct reflects a more persistent and long-time exposure [23,31]. In the

Table 3. Maximum mean level of benzo(a)pyrene, pyrene and phenanthrene in muscle, fat and urine and their metabolites in the urine of rats orally exposed to BaP or BPP for 30 days

| Treatment group | Parent compounds | Urine | Muscle | Fat | Metabolites | Urine |
|-----------------|------------------|-------|-------|-----|-------------|-------|
| BaP             | BaP              | ND    | 23.6 (20)* | 3.2 (30) | 3-OH-BaP | ND |
| BPP             | BaP              | ND    | 34.5 (20) | 1.3 (30) | 3-OH-BaP | ND |
| PH              | 69.0 (30)        |       | 47.1 (20) | 118.8 (20) | 3-OH-PH | 161.6 (30) |
| PY              | 17.6 (30)        |       | 29.7 (20) | 219.9 (20) | 1-OH-PY | 263.0 (10) |

*Data are maximum mean level (ppb) at date (day) when maximum level was found. BaP: benzo(a)pyrene, BPP: benzo(a)pyrene + phenanthrene + pyrene.

ND: not detected.
present study, BaP and its metabolite, 3-OH-BaP, were not detected and the concentration of PH was five times higher than PY, while 1-OH-PY was detected at a two fold higher level than 3-OH-PH in urine. Our data show that the amount of parent compound in tissue and urine are proportional to the dose used for treatment. However, the level of the metabolites may not be directly related to the dose; that is, although the treatment dose of PH was 1.6 times higher than that of PY, the metabolites of PH were 0.61 times higher than that of PY. The 1-OH-PY and 3-OH-PH compounds were rapidly excreted into the urine after the withdrawal of treatment; they were not detected 14 days after withdrawal.

In conclusion, the results of this study suggest that 1-OH-PY in urine may be a candidate biomarker for exposure to PAHs.

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